EMERGING NON-TRADITIONAL BIOMARKERS FOR ASSESSING LONG TERM RISK IN RENAL TRANSPLANT RECIPIENTS

Hege K Pihlstrøm

Faculty of Medicine
University of Oslo

Section of Nephrology, Department of Transplant Medicine.
Oslo University Hospital Rikshospitalet

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List of Papers

I. Hege Pihlstrøm, Geir Mjøen, Winfried März, Dag Olav Dahle, Sadollah Abedini, Ingar Holme, Bengt Fellström, Alan Jardine, Stefan Pilz, Hallvard Holdaas

Neopterin is associated with cardiovascular events and all-cause mortality in renal transplant patients

II. Hege Pihlstrøm, Geir Mjøen, Dag Olav Dahle, Stefan Pilz, Karsten Midtvedt, Winfried März, Sadollah Abedini, Ingar Holme, Bengt Fellström, Alan Jardine, Hallvard Holdaas

Symmetric Dimethylarginine as Predictor of Graft loss and All-Cause Mortality in Renal Transplant Recipients.

III. Hege Pihlstrøm, Dag Olav Dahle, Geir Mjøen, Stefan Pilz, Winfried März, Sadollah Abedini, Ingar Holme, Bengt Fellström, Alan G Jardine, Hallvard Holdaas

Increased risk of all-cause mortality and renal graft loss in stable renal transplant recipients with hyperparathyroidism.
Transplantation 2015;99(2):351-9
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADMA</td>
<td>asymmetric dimethylarginine</td>
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<tr>
<td>ALERT</td>
<td>Assessment of LEscol in Renal Transplantation</td>
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<td>AZA</td>
<td>azathioprine</td>
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<td>CaR</td>
<td>calcium-sensing receptor</td>
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<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>CKD</td>
<td>chronic kidney disease</td>
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<tr>
<td>CHD</td>
<td>coronary heart disease</td>
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<td>CNI</td>
<td>calcineurin inhibitor</td>
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<td>CKD</td>
<td>chronic kidney disease</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>CV</td>
<td>cardiovascular</td>
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<td>CVD</td>
<td>cardiovascular disease</td>
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<td>DD</td>
<td>deceased donor</td>
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<td>ED</td>
<td>endothelial dysfunction</td>
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<td>ECD</td>
<td>expanded criteria donor</td>
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<td>ESRD</td>
<td>end stage renal disease</td>
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<td>FGF-23</td>
<td>fibroblast growth factor-23</td>
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<td>GFR</td>
<td>glomerular filtration rate</td>
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<td>HD</td>
<td>hemodialysis</td>
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<td>HLA</td>
<td>human leucocyte antigen</td>
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<td>HPT</td>
<td>hyperparathyroidism</td>
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<td>HR</td>
<td>hazard ratio</td>
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<td>IL-6</td>
<td>interleukine 6</td>
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<td>IFN-γ</td>
<td>interferon gamma</td>
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<td>LD</td>
<td>living donor</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>MACE</td>
<td>major cardiovascular events</td>
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<td>MI</td>
<td>myocardial infarction</td>
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<td>MMF</td>
<td>mycophenolate mofetil</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>NOS</td>
<td>nitric oxide synthase</td>
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<td>NRI</td>
<td>net reclassification improvement</td>
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<td>PD</td>
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<td>PTH</td>
<td>parathyroid hormone</td>
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<td>PTHr1</td>
<td>PTH-receptor 1</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>ROC</td>
<td>receiver operating curve</td>
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<td>RCT</td>
<td>randomized controlled trial</td>
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<td>RRT</td>
<td>renal replacement therapy</td>
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<td>RTRs</td>
<td>renal transplant recipients</td>
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<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SDMA</td>
<td>symmetric dimethylarginine</td>
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<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
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<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
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General introduction

Transplantation in Norway

The first kidney transplantation in the Nordic countries was performed in 1956 at the National Hospital by a team led by Leif Efskind (1). The patient survived one month after the transplantation, but died of a heart attack during another surgical procedure. The next four patients who underwent kidney transplantation in Norway (1960-1962) all died within 40 days after the procedure, due to either acute rejection or septicaemia. At Ullevål Hospital, in the year of 1963, the American surgeon R.E. Wilson performed Norway’s first transplantation resulting in a long-term functioning graft, introducing immunosuppressive treatment with Azathioprine (AZA) (2).

Scandiatransplant was established in 1969, an organisation for organ exchange between the Scandinavian countries. A Norwegian renal transplant program was launched, and its continuing success is dependent on co-operation between regional and central hospitals and The Transplant Centre at the National Hospital. The National Hospital (Rikshospitalet) has since 1983 been responsible for all solid organ transplantations in Norway (3).

Since 1980, The Norwegian Renal Association has collected national data on renal replacement therapy (RRT), and a formal registry (The Norwegian Renal Registry) was constituted in 1994. Annual reports are published, containing updated information on patients waitlisted for, or already receiving, RRT. Given that kidney transplantation has been shown to improve both survival and quality of life in patients with end-stage renal disease (ESRD) (4, 5), Norway is aiming towards offering the option of kidney transplantation to all eligible uremic patients within a reasonable time frame, and there is no upper age limit (6). In 2014, a total of 16% of the patients on the waiting list were older than 70 years.

A total of 274 renal transplants were performed at The Transplant Centre at Oslo University Hospital Rikshospitalet in 2014. In 68 (25%) of them the allograft came from a living donor (Figure 1). In Norway, like in most other western societies, the number of individuals in need of RRT is increasing steadily. The overall number of transplants performed each year is varying between 50 and 60 per million of population, which is among the highest frequencies in Europe (Figure 2).
By the end of 2014, 296 patients were on the active waiting list for a deceased donor (DD) renal graft, a number that represented an 11% increase since 2013. Since the end of 2012, the prevalent number of waitlisted patients has increased by more than 45%. Median time on the waiting list for patients who received a DD-graft in 2013 was 9 months, while for the 2014 - cohort of recipients, median time in waiting had increased to 12 months. A steady high rate of living donation over the last decades is one of the main reasons that DD-transplant recipients in Norway are spending relatively short time in waiting for a suitable renal graft. However, we have seen a slight tendency towards lower rates of living donations during the last couple of years. Hence, to meet the enhanced demand for kidney transplants, we have been encouraging the increased use of very old (>75 years) donors and expanded criteria donors (ECD). This policy is supported by the rather satisfying results of a retrospective quality control study evaluating the outcomes of single kidney transplantations from donors older than 75 years(7).

**Renal transplantations performed at Rikshospitalet 1969-2014**

![Renal transplantations performed in Norway since 1969. Stratification by type of kidney donor. LD= living donor, DD= deceased donor. Data from the Norwegian Renal Registry.](image)

*Figure 1. Renal transplantations performed in Norway since 1969. Stratification by type of kidney donor. LD= living donor, DD= deceased donor. Data from the Norwegian Renal Registry.*
Renal transplantation in 2013

*Graft sources and waiting lists (★) pr. mill. pop.*

**Figure 2. Renal transplantations performed in Norway in 2013 and patients on the kidney transplant waiting list (per million population). Comparison with other western countries. Data from the Norwegian Renal Registry.**

Out of the total prevalent patient population on RRT in Norway, more that 70% are transplant recipients. This is in contrast to other European countries like e.g. Italy, where the frequency of transplantation per million inhabitants is about half that of Norway(8). In our opinion, this large fraction of transplant recipients puts Norway in a position where we have a particular responsibility: Continuing research efforts should be made in this field, with the goal of optimizing patient- and graft survival.

Transplant medicine in Norway is centralized to the extent that only one hospital is performing the surgery and taking care of the recipients during the first three months of follow-up. Patients are then transferred back to their local nephrologists, but 12 months after transplantation they return to the Transplant Centre for a final routine evaluation including a clinical examination, a complete laboratory work-up as well as a protocol transplant biopsy. This centralized approach gives us unique opportunities
for standardizing the postoperative care of these patients, creating valuable databases for future research as well conducting representative intervention trials. Like in most other developed countries, the standard post transplant immunosuppressive regimen in Norway has for several decades consisted of AZA, one of the calcineurine inhibitors (CNIs), cyclosporine or tacrolimus, and low-doses of oral steroids. A marked decrease in the incidence of acute rejection was achieved world wide when mycophenolate mofetil (MMF) replaced AZA in the standard regimen in 2001(9). Treatment with CNIs is associated with potentially harmful side effects, including nephrotoxicity, but in lack of reliable evidence for the superiority of newer drugs, the vast majority of patients globally are still on an immunosuppressive regimen containing a CNI.

Comorbidities in renal transplant recipients; short- and long term prognosis

Though the main causes of renal failure are similar around the world, what is etiologically most important in each society will vary somewhat according to demographical factors like ethnicity/ genetic background of the population, age distribution, lifestyle/ health behaviour as well as the quality of the health care system. Type 2 diabetes is now the most prevalent cause of chronic kidney disease globally(10). In the USA, much due to the increasing challenge of obesity in the population, diabetic nephropathy is now responsible for 40.4% of newly developed ESRD(11). In Norway, the scenario is slightly different, with diabetes nephropathy being responsible for only 19% of patients starting RRT in 2013. The main change over time has been an increase in vascular/hypertensive nephropathy (34%) and a relative reduction of glomerulonephritis (16%) as primary cause of stage 5 CKD in Norway, but one cannot rule out that this is merely a reflection of changes in coding practice. Figure 3 depicts the main causes of ESRD for patients in Norway starting RRT, illustrating the pattern of change in the dominating diagnoses over time (Data from The Norwegian Renal Registry).
Primary cause of ESRD in Norway

Figure 3. Main causes of ESRD for patients embarking on RRT, by 5 year periods. Data from the Norwegian Renal Registry.

In their landmark report, Wolfe et al(4) showed that transplant recipients had a 68% decrease in mortality compared with those who remained on the wait list. This survival advantage was also seen in older individuals. Guidelines on transplant eligibility are now generally supportive of transplantation for older patients with ESRD. As the mean age of transplant recipients is increasing, renal transplant recipients (RTRs) today will naturally have more complex medical histories and larger burden of comorbidity than before(12). Cardiovascular disease is frequent at start of RRT. The Norwegian data report from 2013 indicates that coronary heart disease is present in about 29%, anamnestic heart failure in 16%, left-ventricular hypertrophy in 22%, cerebrovascular disease in 17%, peripheral atherosclerotic disease in 15% and chronic obstructive pulmonary disease in 10% of incident RTRs. Despite steady improvements in short-term results after kidney transplantation over the past decades, the global long-term prognosis for graft and patient survival has
improved minimally in comparison(13). Figure 4 presents Norwegian results for long-term first DD-graft survival stratified by era of transplantation. From this figure it becomes evident that our most recent results are in line with updated calculations for the rest of Europe showing a 5 year graft survival of 77% and a 10 year survival of 56% (14). Norwegian figures are clearly more favourable than in the USA where the 5- and 10 year graft survival is 67% and 43%, respectively(14). As a rule of thumb, half of the RTRs die with a functioning allograft, while the other half lose their graft due to various causes(15). The leading causes of death in RTRs are fatal cardiovascular events (30-48%), infections (17-30%) and malignancy (8-18%)(16).

Survival of first renal DD grafts

*By vintage. Norway 1969-2013*

Figure 4. Long term outcomes among recipients of a first-time deceased donor kidney transplant by graft vintage. Data from The Norwegian Renal Registry 2014.
Traditional / established risk factors

Type of kidney donor (LD, DD or ECD), donor and recipient age, number of human leukocyte antigen (HLA) mismatches, presence of donor-specific antibodies, cold ischemia time, ethnicity, and delayed graft function are the main pre- or perioperative factors determining long term graft survival. In addition, stable glomerular filtration rate (GFR) at 3-12 months after transplantation, degree of proteinuria, development of chronic allograft dysfunction and factors related to the immuno-suppressive regimen are important predictors(17). Chronic allograft dysfunction is presumably a result of many intricate deleterious factors working together. For the individual patient, there will be a combination of immune factors (clinical and subclinical rejection, reactivation of dormant viral infections, treatment adherence) and non-immune factors (hypertension, diabetes, anaemia, dyslipidaemia) potentially exhibiting harmful influences on the allograft. Nephrotoxic effects of immunosuppressive drugs have for the last 20-30 years been considered the major cause of late allograft loss(18), though several recent review articles have claimed that the role of CNIs in causing chronic graft dysfunction is exaggerated(19, 20).

Recurrence of native kidney disease, e.g. glomerulonephritis, will also pose a threat to the graft.

The reduced patient survival in RTRs compared with other populations, might be explained by interaction effects between a heavy burden of cardiovascular disease (CVD) and infectious and neoplastic processes(21). Important cardiovascular (CV) risk factors like diabetes, arterial hypertension and dyslipidemia are frequently present in RTRs. However, these traditional risk factors cannot fully explain the heightened CV mortality. Kasiske et al has shown that the Framingham risk score underestimated risk in the transplanted population(22). In fact, about 40% of transplanted patients experience one or more CV events during the first 10 years after TX (23). Decreased GFR and proteinuria have both been independently associated with coronary artery disease and all-cause mortality in community-based studies(24-26). Some reduction in kidney function is usually present in RTRs, even when the treatment course is judged otherwise satisfactory. Reduced GFR can probably account for some of the increased CV-risk, as shown in a post-hoc analysis from the Favorit Trial(27). Furthermore, immunosuppressive drugs are thought to
exaggerate the negative effects of CV risk factors(22). Using the Assessment of Lescol in Renal Transplantation (ALERT) trial population, a formula for 7-year CVD and mortality risk calculation for prevalent RTRs was developed(28). A seven-variable model including age, previous coronary heart disease, diabetes, low-density lipoprotein, creatinine, number of transplants, and smoking could predict the occurrence of major adverse cardiac events. Total mortality could be predicted by the six variables: age, coronary heart disease, diabetes, creatinine, total time on renal replacement therapy, and smoking.

Figure 5 gives a summary of the main predictors of transplant and graft survival.

**Figure 5. Early predictors of long-term transplant and patient survival.**
*Variables are listed in order of descending association with long-term survival.*
*Adaption from Bottomley M J, and Harden P N Br Med Bull 2013;106:117-134*

The transplant community is continuously striving to further improve long term graft- and patient survival in RTRs. The appropriate selection and matching of organ recipients and donors, the optimization of peri- and postoperative care, the
individualization of the immunosuppressive regimens, as well as the attentive management of the relatively large burden of comorbidity are amongst possible strategies to achieve better long-term results. Worldwide, use of living kidney donors varies widely, from less than 10% to more than 75%(29). Most developed countries encourage the use of living donors, as it is associated with a considerably better short- and long time prognosis for the recipient(30). Even so, a decline in living kidney donation has been observed recently, at least in the USA(31). One of the strategies to combat organ shortage, is the increased use of more marginal organ donors (ECD)(32). This shift might have an undesirable impact on outcome, but there is reason to believe that if given to the right recipients, kidneys from older donors or from donors suffering a cardiac death could serve as a means for expanding the donor pool without affecting clinical outcomes significantly(33). Furthermore, it has been shown that recipients of kidney allografts from elderly deceased donors (>65 years) have better survival than their paired counterparts remaining on dialysis(34).

The pursuit of efficient and tolerable CNI-free immunosuppressive regimens continues to be a stimulus in drug development programs by the pharmaceutical industry, much due to their presumed side effect of chronic nephrotoxicity, but also due to their tendency to induce or aggravate diabetes and arterial hypertension.

Although the most important donor and recipient risk factors as well as peri- and postoperative prognostic factors have been established, a full mapping of causes for graft failure, CVD and premature death in RTRs remains to be completed. There is an ongoing search for new and possibly modifiable risk factors in the hope of further improving long term prognosis in these patients. The research process often begins with the investigation of novel biomarkers for possible association with long term outcomes, then the application of predictive statistical models, and consequently the clinical intervention in causal factors. Important areas of focus include systemic inflammation, endothelial dysfunction, oxidative stress, bone and mineral disease as well as hormonal balance.
Emerging risk factors

Risk markers, risk factors and the concept of causality.

A risk marker refers to a measurable variable which shows statistically significant associations with the subsequent clinical outcome of interest. An independent risk marker should retain its statistical association with the outcome despite controlling for other risk factors in a multivariate statistical model. Strictly speaking, a risk marker cannot be considered a risk factor unless intervention to modulate this factor results in parallel modulation of risk, given that possible confounding factors are taken into account in the analysis.

Causality is (in science) defined as a relationship between one phenomenon or event (A) and another (B) in which A precedes and causes B. The direction of influence and the nature of the effect are predictable and reproducible and may be empirically observed. Causality is difficult to prove, maybe even impossible, as seems to be the opinion of some social scientists. Whether a variable could be considered a causal risk factor, is not really a statistical question, but rather a matter of clinical and biological knowledge acquired experimentally. A statistical association is necessary, but not sufficient.

A well designed randomized controlled trial (RCT) is the optimal way to test causality (e.g. intervention with a drug that is known to reduce the risk marker), but this approach is not always feasible. Novel statistical methods for causal inference are being developed for observational data, and a common denominator for such methods is that datasets are transformed and weighted in order to mimic the interventional trial setting, where there is exchangeability between treatment groups. Such strategies include controlling for selection bias using propensity scores(35) and inverse probability weighting(36), and dealing with multiple treatment times using structural marginal models(37).

However, the establishment of an independent risk association from observational data is often necessary before a successful RCT can be designed and conducted. This thesis presents three such investigations of risk associations, the strengths of which include a well defined-patient cohort, an extensive time of follow-up as well as validated clinical end-points. Before summarizing and discussing the results of our
papers, I would like to give an overview of three important research areas, all particularly relevant for RTRs, and show how the biomarkers we are studying place themselves in this wider context.

**Systemic inflammation and the macrophage activation marker neopterin**

Chronic systemic inflammation is the result of release of pro-inflammatory molecules (cytokines) from immune-related cells and the chronic activation of the innate immune system. C-reactive protein (CRP), an established marker of systemic inflammation, has been identified as an independent risk factor for CV disease and CV death in several populations(38). At an early stage in chronic kidney disease (CKD), a link between CRP and renal function is established(39), and in ESRD, the association between CRP and risk of death and CV events is shown to be particularly strong(40). The pro-inflammatory milieu in ESRD is probably due to increased oxidative stress and chronic activation of various cell subsets belonging to the immune system, and these phenomena are strongly inter-related(41). Renal transplantation will ameliorate some of these imbalances, but some changes in immune function are likely to persist. There is, in fact, evidence for enhanced systemic inflammation in stable RTRs, even among those with low Framingham risk scores(42).

There is evidence to suggest that systemic inflammation favours vascular calcification(43, 44). The presence of calcification in the aorta has been associated with coronary artery disease, stroke and heart failure in RTRs(45). Osteoprotegrin, a glycoprotein involved in the regulation of vascular calcification processes, is associated with mortality as well as renal and CV events in RTRs(46), and our group has recently shown serum calcification propensity to be a strong and independent determinant of cardiac and all-cause mortality in kidney transplant recipients (Dahle et al, manuscript in press)

Inflammation is also a strong promoter in the atherosclerotic process. A central inflammatory molecule, interleukine 6 (IL-6), triggers the synthesis of fibrinogen and stimulates a pathway leading to thrombosis. In a stable renal transplant population high sensitive CRP, as well as IL-6, was shown to be associated with major
cardiovascular events (MACE) and all-cause mortality(47) independent of traditional risk factors, including measures of kidney function. An inflammatory glycoprotein, TKL-40, which is expressed by macrophages in the earliest atherosclerotic lesions, was shown to be elevated in RTRs(48). Another study has investigated the impact of inflammatory cytokine polymorphisms on posttransplant CV-disease(49), concluding that tumor necrosis factor-α (TNF-α) and interleukine -10 genotypes might represent cardiovascular risk markers.

Neopterin (D-erythro-1-2-3-trihydroxypropylpterin), a rather specific marker of macrophage activation, was hot in transplant research in the 1980s as a possible predictor of acute rejections and viral infections in the postoperative setting, but has since been somewhat “forgotten”(50). Neopterin is produced from guanosine triphosphate (51) by activated human monocytes, monocyte-derived dendritic cells and macrophages. During the cellular immune response, activated Th1-lymphocytes release interferon-γ (IFN-γ), which again is the main stimulus for neopterin production and release(52). IFN-γ will quickly bind to target structures or become neutralized by soluble receptors, but neopterin is biochemical inert, and its serum concentration is closely linked to the activity of the cellular immune system(53). Neopterin is shown to be a marker of disease in a variety of conditions like viral infections, inflammatory diseases, autoimmune diseases, neurodegenerative diseases and certain forms of cancer (54), and elevated serum neopterin has previously been associated with CV events and mortality in non-transplant populations (50, 55). As a marker of cellular immune response activation depending on IFN-γ release, neopterin may better reflect uremia-associated changes in the immune system prevailing in kidney transplant recipients, than less specific markers of inflammation. Neopterin level corresponds to the degree of cellular immune activation, but also to the extent of oxidative stress involving production of reactive oxygen species (ROS)(56). Neopterin may exert pro-oxidant properties itself, e.g. by being able to intensify the effects of ROS (57). Though neopterin measured early post transplant in kidney allograft recipients has been associated with subsequent adverse events, in particular acute immunological and infectious complications(57, 58), the predictive value of neopterin for clinical outcomes in stable RTRs has not been previously investigated.
**Endothelial dysfunction and the dimethylarginines, ADMA and SDMA**

The vascular endothelium carries far more biological functions than simply serving as a barrier between the blood constituents and the tissues. Important functions include modifying the coagulation process, assisting the immune response in the face of antigen, controlling the exchange of fluid and molecules between the blood stream and the tissues, as well as regulating vasoconstriction and vasodilatation. If one or more of these tasks cannot be performed adequately, endothelial dysfunction (ED) is present. However, when this concept is used in the literature, it almost invariably refers to the establishment of an imbalance between dilating and constricting factors produced in the endothelium(59, 60). Among these factors nitric oxide (NO), one of the most potent dilators in the vasculature, is considered the most clinically important. ED is believed to be the first initiating step in the process of atherosclerosis(61, 62) and has been called the “risk of risk factors”, reflecting that it may be seen as a common pathway through which much of the CV risk associated with other risk factors is mediated(63). Conditions associated with an increased level of oxidative stress(64) such as the traditional CV-risk mediators: diabetes, hypertension, dyslipidemia, smoking and high age are all predisposing factors for ED. Furthermore, a close relationship between inflammation and endothelial dysfunction has been demonstrated in healthy volunteers, patients at risk, and patients with established CV disease, thus providing a link between these two concepts(62).

When ED is established, the vasculature is predisposed to suffering repeated lesions followed by remodelling, continuing inflammation, vasoconstriction, thrombotic events as well as plaque rupture and erosion(65) (Figure 6). The presence of ED is associated with a less favourable prognosis in patients with established CV disease(66-68), and in the renal transplant setting, associations between clinical measures of ED and long term outcomes have recently been demonstrated(69, 70). The gold standard for measuring ED is angiographic estimation of the coronary artery response to acetylcholine, given as the change in vessel diameter(71), though less invasive methods are gaining acceptance(60). However, if endothelial function could be easily assessed via blood tests, the value of ED as a risk stratification tool would increase markedly. As a consequence, among the new molecules currently under
investigation as possible serum biomarkers for CV disease, we find several markers potentially reflecting endothelial function(72).

Figure 6. Endothelial dysfunction as the “risk of the risk factors.” The endothelium represents a mechanical and biological barrier between the blood and the vascular wall. ‘Endothelial Dysfunction- A Marker of Atherosclerotic Risk’ Bonetti et.al., Arterioscler Thromb Vasc Biol.2003, 23(2):168-75.

The dimethylarginine siblings, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA), are products of the process of protein turnover in the cells, formed when constituent methylated arginine residues are released from intracellular proteins. There is increasing evidence for a role of these molecules in ED as they may interfere with NO-production. Asymmetric dimethylarginine (ADMA) has the potential to reduce the levels of NO by inhibiting the enzyme nitric oxide synthase (NOS) and limiting the uptake of L-arginine, the substrate for NOS, into endothelial cells(73). ADMA has become an established risk factor for CV events and mortality in different populations(74). The structural isomer symmetric dimethylarginine (SDMA) has largely been overlooked in research, as it was believed to be a by-product of
metabolism without any biological activity. It has now become clear that though SDMA does not directly inhibit NOS, it might indirectly reduce the activity of the enzyme by limiting L-arginine supply (75, 76). In a very recent study of 64 CKD-patients and 52 RTRs, a strong and predictable association was demonstrated between hsCRP, SDMA and endothelial dysfunction (77). Figure 7 shows a simplified overview of the pathways in which the dimethylarginines seem to be involved. Interestingly, in addition to being involved in pathways central to endothelial function, SDMA seems also to partake in inflammatory pathways. Mechanisms include activation of nuclear factor kappa B (NF-κB) and increased expression of IL-6 and TNF-α (78).

Consequently, interest in this novel biomarker is on the rise, and SDMA has recently been associated with mortality in several populations, including patients referred for angiography (44, 80), patients having suffered an ischaemic stroke (81) and stable
patients with coronary heart disease(82). SDMA was found to independently predict mortality in a large (n=3523) multi-ethnic cohort representative of the general population(83).

Abedini et al conducted a post-hoc analysis from the ALERT trial(80) showing that ADMA is associated with renal and cardiovascular outcomes as well as all-cause mortality in renal transplant recipients. However, up until now the potential association between SDMA level and long term outcomes in RTRs has not been investigated separately. Busch et al studied a heterogenous population of CKD patients, including 37 renal transplant recipients(84) for associations between the dimethylarginines and long term outcomes. Their results pointed in the direction of an association between elevated SDMA and progression to ESRD.

**Hyperparathyroidism and bone- and mineral balance**

Chronic kidney disease has a strong impact on the mineral and bone metabolism. In ESRD, long lasting stimulation of the parathyroid gland by low serum calcium, low levels of active vitamin D and high serum levels of phosphate will initially lead to polyclonal hyperplasia(85). If uremia persists, these generalized diffuse changes will frequently be followed by monoclonal nodular hyperplasia resulting in autonomous hormone production (86). If such a state of autonomy is established, the condition is less likely to resolve after renal transplantation(87). Typically, by 6 months after transplantation, the parathyroid functional mass will be markedly reduced, and parathyroid hormone (PTH) levels will have dropped by approximately 50%(88). A more gradual decline is to be expected thereafter, but complete normalization cannot be assured. In fact, hyperparathyroidism (HPT) beyond 1 year after transplantation has been reported in up to 50% of RTRs (89, 90) The main predisposing factors for such persistent HPT are high levels of serum calcium, alkaline phosphatases and PTH at the time of transplantation, as well as poor graft function and a prolonged time course of dialysis treatment before transplantation(87, 91). Vitamin D-deficiency is very frequent in the post-transplant setting, and this is also thought to contribute to the persistence of HPT (90, 92). Recently, a central role for fibroblast growth factor 23 (FGF-23) in the regulation of phosphate-vitamin D-PTH homeostasis has also been confirmed (93).
In addition to being acknowledged for its involvement in bone disease in different populations, PTH has recently been launched as a novel CV risk marker (94). There are receptors for PTH present in cardiomyocytes and the vessel walls (95), and this might implicate direct effects of the hormone outside bone and kidney (96, 97). Elevated PTH-levels are associated with reduced survival and increased CV morbidity in patients with mild primary HPT (98), in the general population (99, 100), and in patients referred for cardiac angiography (101). Furthermore, there is evidence that both HPT and an altered vitamin D status contribute to the pattern of CV pathology and excess mortality from CV causes seen in CKD-patients (102-104).

When it comes to RTRs, data on the possible effect of HPT on clinical outcomes are limited. Associations between high levels of PTH and decreased bone mineral density (105), as well as increased rate of fractures (106) have recently been demonstrated in the transplant setting. Severe persistent HPT is frequently associated with a chronically elevated level of serum calcium (107). There is evidence for a detrimental effect of hypercalcemia, not only on bone metabolism, but also on other tissues and organs, including the allograft (108, 109).

Potential effects of chronically elevated PTH in renal transplantation have been suggested in a recent review by Evenepoel (110), from which Figure 8 is borrowed. However, independent relationships between serum PTH and long term renal and cardiovascular outcomes in renal transplantation have not been described previously.
Figure 8. In persistent HPT, inappropriately high PTH levels, along with high FGF23 levels, may cause allograft dysfunction and contribute to the high burden of fractures and cardiovascular disease in renal transplant recipients. NODAT, new-onset diabetes after transplantation; pHPT, persistent hyperparathyroidism.
The Alert Trial

The ALERT trial (Assessment of LEscol in Renal Transplantation)(111) was a double-blind randomized placebo-controlled study designed to evaluate the effect of treatment with fluvastatin on long term CV outcomes in renal allograft recipients. Prior to this study, the role of dyslipidemia and the effect of lipid lowering drugs had not been fully established in RTRs(22, 112). Fluvastatin was the drug of choice in order to minimize interactions with immunosuppressive therapy(113). The ALERT trial was a multicenter study initiated and coordinated by The Section of Nephrology at Oslo University Hospital, Rikshospitalet. 2102 clinically stable RTRs between the age of 30 and 75 were randomized to daily treatment with either fluvastatin or placebo, and participants were followed for 5-6 years. All events (primary or secondary endpoints) were registered and validated by an independent critical events committee consisting of two nephrologists and two cardiologists blinded to treatment assignment. The primary endpoint of the study was MACE (major cardiovascular events), and the conclusions from the core trial was a 17% reduction in these events in the treatment arm, though the result was statistically non-significant. Low density lipoprotein (LDL)-cholesterol was significantly reduced, and the treatment was found to be safe and well tolerated by the majority of participants. Secondary endpoints included individual cardiac events, combined cardiac death or non-fatal myocardial infarction (MI), combined cerebrovascular events, non-cardiovascular death; all-cause mortality, and the composite renal endpoint of graft loss or doubling of serum creatinine. There was a significant 35% reduction in combined cardiac deaths and non-fatal MI’s, an effect size comparable to the beneficial effects of lipid lowering treatment in other populations, while for the other secondary endpoints no significant treatment effect was detected. Of course, in lack of a statistically significant primary efficacy analysis, effect estimates for secondary endpoints will have to be interpreted with caution.

Available survey data from the year 1994 indicated a yearly 5% rate of MACE in RTRs, and the power calculation for the ALERT trial was based on this assumption and the suggestion of a 25% size effect of fluvastatin. However, the rate of events turned out to be much lower than expected, probably due to the fact that the recruited study population turned out to be stable RTRs with a rather moderate CV risk profile. Most patients with pre-existing coronary heart disease were excluded, as the majority
of them were already using statins, and patients with a large burden of comorbidity were probably not included due to complex therapeutic regimens. As the investigators realized this early in the course of the trial, the number of patients enrolled was increased. In order to maximize the potential value of the trial, the dosage of fluvastatin was doubled about two years into the study period, mainly because of emerging safety data and results from large clinical outcome trials, which were not available at the time point when the ALERT was planned and designed. These changes to the protocol was recommended by the Data Safety and Monitoring board and implemented by the Steering Committee.

The lack of significant treatment effect in the primary efficacy analysis, despite the above mentioned efforts, was most likely due to a combination of a low event rate in the selected study population and the fact that as many as 32% of the patients in the placebo group were actually using statins in the last year of the core trial follow-up. This figure includes patients who at scheduled study visits presented with lipid levels above protocol thresholds, as well as patients who were prescribed statins outside the study by non-participating physicians.

In 2002, based on the findings of two large RCTs in the general population demonstrating effective long-term prevention of CV events with lipid lowering treatment, the Steering Committee decided to extend the ALERT trial another two years, now offering treatment with fluvastatin 80 mg to all participants. Ninety-two percent of the original study participants took part in the extension trial, and the conclusion was a significant 21% reduction of MACE and a 29% reduction in the composite endpoint “cardiac death or non-fatal myocardial infarction”(114). Hence, the extension study established a cardioprotective effect of statin therapy in RTRs. Soon after publication of these results, recommendations for statin therapy was implemented into international guidelines and has now become mainstay as part of the standard treatment protocol for this patient group(115, 116).

Several examples exist of larger interventional trials having provided extensive datasets for further epidemiological research on risk factors and clinical outcomes in the field of CKD and renal transplantation. The Aurora Study(117), the Favorit Trial(118), and the 4D study (119) are amongst them. The LURIC trial, a prospective
cohort study of individuals with and without CV disease at baseline is an example of a well designed trial aiming to provide a thoroughly characterized cohort for the study of environmental and genetic risk factors, functional genomics and pharmacogenomics(120). Likewise, in addition to seeking answers to the main research question concerning the effectiveness of statins, the ALERT trial established a valuable database for post-hoc research. Blood and urine was frozen at time of inclusion, opening up the possibility of studying associations between baseline levels of potential risk factors and the occurrence of validated cardiac and renal end points up to 8 years later. Over the last decade, the ALERT cohort has been the source of numerous investigations concerning statin safety and effectiveness(121-126), risk prediction and stratification in RTRs (127-134), as well as the identification of novel risk factors associated with long term outcomes(46, 47). The papers presented in this thesis are, for now, the latest additions to this substantial list of research.
Aims / Research questions

Considering that long term patient and graft survival in RTRs has shown only very modest improvements over the last decades, there is an unmet need to identify new, and possibly modifiable, risk factors for adverse outcomes. The general aim of this thesis was to explore the possible value of a few such novel biomarkers in transplant recipients. Causal inference is seldom possible based on observational studies like these, but establishing associations between the level of a biomarker and future outcomes in a population at risk, is often a necessary prerequisite for designing a successful interventional trial where concepts of causality can be tested.

This thesis addresses the following questions, all of which have not been sufficiently answered by previous research in the field:

I. Is neopterin, an inflammatory marker released from activated macrophages and monocytes, an independent predictor of long term survival or cardiovascular / renal outcomes in clinically stable RTRs?

II. Is SDMA, a novel marker of endothelial function and possibly a partaker in inflammatory pathways, associated with long term outcomes (mortality or cardiovascular / renal adverse events) in clinically stable RTRs?

III. Is persistent hyperparathyroidism after kidney transplantation associated with poorer long term outcomes in RTRs?
Patients and methods

Laboratory measurements and study population.

For all three studies we used the ALERT database, and all baseline information was available for each patient (135). The original participants were 2102 male and female RTRs aged 30–75 years, included from June 1996 to October 1997. They had received a renal transplant more than six months previously, had a stable graft function and a total serum cholesterol concentration between 4.0–9.0 mmol/L (155–348 mg/dL). Patients with familial hypercholesterolemia, a recent acute rejection episode, or a predicted life expectancy of less than one year were excluded. Patients already receiving statin therapy were not eligible for randomization.

For the ALERT trial, lipids, serum creatinine, creatine kinase and hepatic enzymes were measured at a central laboratory (Eurofins Medinet) which has a solid reputation and many years of experience in analyzing samples for clinical trials. Several national and international agencies have previously evaluated their performance, and so the ALERT Steering Committee did not independently supervise the analytic process or validate the data. At baseline, samples of serum, plasma and full blood were obtained from the majority of participants and frozen at minus 70° C. Several pre-specified biomarkers were selected to be measured at a later time point, and neopterin, SDMA and PTH were amongst these. At the end of the study, the frozen samples were analysed at a central laboratory in Germany (Synlab laboratories, Heidelberg) under the direction of Prof. Winfried März. Synlab specializes in measuring a wide range of biomarkers in frozen samples, and again the ALERT steering committee did not perform independent validation of measurements. The methodology for measurement of neopterin, SDMA and PTH is described in the individual papers.

For the studies described in paper I and III we used the whole ALERT cohort for statistical analysis, and events registered in the extension trial period were also included in the follow-up data. This choice of study population seemed justified by the fact that initial statistical analyses revealed no heterogeneity between treatment arms regarding the associations between the biomarkers and risk of outcomes.
However, for the study on SDMA we used the placebo group alone, analysing the core trial follow-up data only. We made this choice because of evidence in the literature supporting a possible effect of statins on endothelial function\(^{(136, 137)}\), and we wanted a `clean approach`. Moreover, initial statistical analyses revealed that the association between high SDMA and adverse outcomes were markedly stronger in the placebo arm than in the treatment arm, thus suggesting the presence of an interaction between dimethylarginines and lipid lowering treatment\(^{(138)}\).

Common for all three studies is that measurements of the serum levels of the biomarkers, as well as the registration of relevant confounding variables, were conducted at baseline only. Ideally one would like to have repeated measurements throughout the follow up-period, but for such a large study cohort it was not possible due to financial restrictions. We have to accept that the baseline value of a risk marker might not reflect the mean exposure during follow up! There is also a possibility of bias when risk estimation is confined to “single measurements”, this being due to the possibility of measurement errors as well as the within-person variability in the serum levels of the biomarkers. Regression dilution bias is a statistical phenomenon which has been discussed in several methodological publications\(^{(139, 140)}\).

We are, however, somewhat reassured by the fact that mean levels of SDMA and neopterin in the ALERT cohort seem in line with levels reported by other publications in the field of renal transplantation\(^{(141-143)}\). We acknowledge that PTH-values in our cohort were substantially lower than what is reported in most observational studies of RTRs\(^{(144, 145)}\). Patients with the highest burden of comorbidity were not included the ALERT, which might explain some of the discrepancy in PTH levels. Also, mean time since last transplantation was longer than in most studies (>5 years), allowing for parathyroid hyperplasia regression to have occurred.
Statistical analysis

The three studies presented in this thesis are methodologically fairly similar. After having assessed baseline demographics and identified possible confounding covariates, survival analyses were performed using MACE, all-cause mortality and renal graft loss as the major clinical outcomes of interest.

Trends or variations in baseline demographics, risk factors and standard laboratory measurements across categories of the exposure variables (quartiles/quintiles of neopterin-to-creatinine ratio, SDMA and PTH) were analysed by ANOVA and $\chi^2$ statistics. Results of unadjusted survival analyses were presented as Kaplan-Meyer curves or as Nelson-Aalen cumulative hazard plots, and Log Rank test was used to compare survival distributions of groups.

In all of the three studies presented we performed Cox proportional hazard regression; univariate calculations followed by multivariate analyses. The choice of covariates to enter into the multivariate regression models was based on available literature and clinical knowledge, but also the presence of significant associations with the clinical outcomes in initial statistical analyses or significantly skewed distributions across levels of the biomarker at interest. We chose not to apply automatic model building procedures such as forward- or backward stepwise inclusion of covariates, a decision made on the basis that they can be misleading, as they do not consider the real-world importance of each predictor.

Correlations between biomarkers and other variables were calculated using Spearman’s correlation test. This non-parametric correlation test was chosen since biomarker levels did not show normal distributions in our population. Levels of different biomarkers may to a varying extent reflect the same biological pathways. Furthermore, if the molecules of interest are not metabolized in the body, as is the case for SDMA and neopterin, serum levels may be strongly dependant on the efficacy of renal elimination. In situations like these, correlation analyses will allow for the evaluation of potential collinearity issues. Collinearity (sometimes termed multicollinearity) is a statistical phenomenon in which two or more predictor variables in a multiple regression model are highly correlated,
meaning that one can be linearly predicted from the others with a fair degree of precision. In a Cox regression model this means that the hazard ratio (HR) estimates may become unstable and change erratically in response to small changes in the model or the data. If collinearity is present in a model, the affected coefficients will tend to have large standard errors, and a type 2 error is more likely to be made. We therefore made careful considerations before entering variables that showed a high degree of statistical correlation together into our final regression models.

In all three studies, there were no obvious violations of the proportional hazard assumption, judged by testing of Schoenenfeld residuals. Hazard ratios (HR) were calculated with a 95% confidence interval (CI), both when comparing groups and when using continuous exposure variables. Increasing bias and variability, unreliable confidence interval coverage, and problems with model convergency become the case if too many variables are included in the regression model. Also the problem with missing data generally increases with the number of covariates analysed. For the current studies, in the few cases where the "rule" of at least ten events per adjusting variable(146) was relaxed, we chose to seek expert statistical advice concerning the validity of the calculated HRs.

For the purpose of this thesis, we examined correlations between neopterin, SDMA and PTH. Also, a multivariable regression model was constructed using all the three biomarkers in focus, as well as the main traditional CV risk factors.

In the study of PTH, to guide our subsequent choice of statistical model, we applied a restricted cubic spline method(147) for assessing if there was significant non-linearity in the relationship between the exposure variable and clinical outcomes.

In the study of SDMA as a risk marker, possible competing risks between MACE (as study endpoint) and all-cause mortality (as competing risk endpoint) were examined by sensitivity analysis(148), including the sub-distribution hazards method described by Fine and Gray(149).

Statistical analyses were performed mostly using SPSS version 18.0 (IBM, New York,USA), but also STATA version 11 (StataCorp, College Station,Texas,USA) and R Project for Statistical Computing (Free Software Foundation.Inc, Boston, MA).
Results

Paper 1: Neopterin is associated with cardiovascular events and all-cause mortality in renal transplant patients.

This study used the ALERT cohort to investigate the association between serum level of neopterin at time of inclusion and subsequent MACE, all-cause mortality and death censored renal graft loss.

Neopterin values were available for 629 patients at baseline. Initial statistical analysis revealed no relevant difference between trial participants with- or without available neopterin measurements. The correlation coefficient between neopterin and creatinine was 0.61, between neopterin and IL-6 was 0.26, while for neopterin and CRP it was 0.14. All correlations were highly significant.

As has been done in the majority of previous studies on neopterin in populations with compromised renal function, we calculated the neopterin-to-creatinine ratios for all patients. Levels ranged from 33 to 325 μmol/mol, mean 96 (SD 42) μmol/mol. The incidence of MACE and all-cause death was significantly higher in the fourth neopterin/creatinine quartile compared with the first. For renal graft loss the event rate was higher in quartile 4 than in quartile 1, but the difference was not statistically significant.

Neopterin-to-creatinine ratio as a continuous variable was highly significantly associated with all endpoints in the univariate Cox regression analysis. After multivariate adjustments for established and potentially important risk factors, a positive associated was found with all-cause of death (HR 1.06 per 10 units, p=0.002,95% CI 1.02-1.09) and MACE (HR 1.06 per 10 units, p=0.009,95% CI 1.01-1.10). We found no independent predictive value for graft loss, the association between neopterin-to-creatinine ratio and outcome becoming markedly attenuated by the inclusion of level of proteinuria in the multivariate model.

In this sub-cohort of ALERT, while neopterin was significantly associated with outcomes, results were weaker for other markers of inflammation. Neither high
sensitive CRP nor IL-6 was independently associated with any of the study endpoints in the multivariable analyses.

To our knowledge, this is the first study conducted in clinically stable RTRs demonstrating an independent association between serum neopterin concentration and the long-term clinical outcomes of MACE and all-cause mortality.
Again the ALERT cohort provided a valuable database. We investigated the association between serum level of SDMA at time of inclusion and subsequent long term outcomes: MACE, renal graft loss and all-cause death. Sub-analyses also explored the effect of having a high SDMA level on the three most important causes of mortality. The study population consisted of ALERT participants in the placebo group, and we used core trial data with a mean follow up of 5.1 years. 925 of the 1052 participants had available SDMA measurements at baseline (mean 1.20 (SD 0.48) μmol/L; range 0.46-4.41 μmol/L).

Correlations with SDMA had a coefficient of 0.77 for creatinine and -0.72 for estimated GFR. Significant correlations were also found between SDMA and ADMA (r=0.40). Correlations between SDMA and inflammatory markers were weak.

Due to apparent non-linear associations between the exposure variable and risk of outcome, we split patients into quartiles based on their SDMA-values. SDMA showed a positive association with all study outcomes in univariate analysis. After adjustment for important possible confounders there was significantly increased risk of death (HR 4.56, 95% CI 2.15-9.71, p<0.001) and graft loss (HR 5.51, 95% CI 1.95-15.57, p=0.001) in the highest SDMA quartile compared with the first. There was a non-significant trend indicating increased risk of MACE with higher SDMA-values.

A surprisingly strong association was seen between SDMA and non-cardiovascular death (HR 7.54, 95% CI 2.54-22.40, p<0.001). Compared with the first quartile there was a more than nine-fold increase in the risk of dying from cancer and a more than seven-fold increased risk of death by infection in the 4th SDMA quartile. Both associations were highly statistically significant.

This study is the first to report that increased serum levels of SDMA, adjusted for traditional and nontraditional risk factors, are associated with reduced long-term graft and patient survival in RTRs; the reduced survival driven mainly by non-cardiovascular causes.
Paper 3: Increased risk of all-cause mortality and renal graft loss in stable renal transplant recipients with hyperparathyroidism.

The majority of patients in the ALERT cohort had available PTH-measurements at time of inclusion, and in this study we explored the possible association between persistent HPT and long term graft and patient survival, as well as MACE. Mean follow up was 6.7 years. Mean serum PTH was 51.9 (SD 67.6) pg/mL, ranging as wide as from 1.20 to 1007 pg/mL. All patients with HPT, as defined by the upper reference level (65 pg/mL), were located in the fifth quintile. There were no clinically significant differences in baseline variables between patients with available PTH-level (n=1840) and the rest of the ALERT participants (n=262).

The correlation coefficient between PTH and creatinine was 0.28 (p<0.001), and correspondingly for estimated GFR it was -0.28 (p<0.001). CRP was not significantly correlated with PTH. Furthermore, there was no overall correlation between PTH and serum calcium or serum phosphate.

Taking into account the possible non-linearity in associations between PTH and risk of outcomes, we created univariate restricted cubic splines for serum PTH as a risk factor for all-cause mortality and graft loss. While univariate analyses might indicate non-linearity, in the multivariate analyses the tests for non-linear trend were non-significant. Based on these initial statistical analyses we chose to evaluate serum PTH both as a continuous variable and a categorical variable in the final Cox Proportional Hazard Regression models.

In univariate analyses we found significant associations between continuous PTH and all study outcomes. In multivariate analyses, there were still significant associations with all-cause mortality (HR 1.042 (1.014-1.071), p=0.004) and graft loss (HR 1.055 (1.030-1.081), p<0.001), though the risk was somewhat attenuated. For MACE, the association was weak and far from significant (HR 1.014 (0.982-1.047) p=0.393), but associations between PTH and fatal cardiovascular, cerebrovascular or vascular events remained of comparable magnitude as for total mortality (HR 1.039 (1.000-1.079), p=0.050). We then compared patients with serum PTH > 65pg/ml to those with normal/low values, finding a HR of 1.85 (1.41-2.42; p<0.001) for graft loss,
and a HR of 1.46 (1.12-1.92; p=0.006) for all-cause death. Similar results were seen in quintile analyses.

In contrast to PTH, serum calcium and serum phosphate were not significant predictors of mortality in the fully adjusted model. Higher phosphate levels and lower calcium levels were associated with higher risk of graft loss.

Supplementary Risk marker comparison

Pathways of inflammation, endothelial function and bone-mineral disturbances are intertwined. We were tempted, for the sake of this thesis, to assess the three biomarkers relationship with each other. We found a strong correlation of 0.65 (p<0.001) between neopterin and SDMA, and correlation of 0.27 (p<0.001) between neopterin and PTH. Between SDMA and PTH, the correlation was 0.31 (p<0.001). Now using both treatment arms, analysing patients with available measurements of all three markers (n= 595), we constructed a multivariate model which included the most important cardiovascular risk factors: age, gender, diabetes mellitus, coronary artery disease, systolic blood pressure (BP), estimated GFR and LDL. ADMA and high sensitive CRP was also included for comparison (Tables 1 and 2). For all-cause death we found that one standard deviation (SD) increase in neopterin was associated with 30% increased risk, while one SD increase in SDMA increased the risk by 35%. Both results were statistically significant.

For graft loss there were significant independent associations for SDMA (40% increased risk per SD) and PTH (14% increased risk per SD).
### 1 All-cause death

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<th>p-value</th>
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### 2 Renal graft loss

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<td>LDL (mmol/L)</td>
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<td>8% incr risk per SD</td>
<td>.896</td>
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<td>GFR (ml/min)</td>
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<td>.000</td>
<td>83% red risk per SD</td>
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<td>Neopterin (nmol/L)</td>
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<td>SDMA (μmol/L)</td>
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<tr>
<td>hsCRP (mg/L)</td>
<td>1.001</td>
<td>.956</td>
<td>1% incr risk per SD</td>
<td>.977</td>
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*Tables 1 and 2. Risk marker comparison; associations with patient and graft survival expressed as HR per unit and per SD. HR; hazard ratio, SD; standard deviation, CI; confidence interval.*
Discussion

The current view on emerging cardiovascular risk markers seems to be that in the low-risk general population, the contribution of novel markers to the total CV risk estimation is quite modest. The “old” traditional risk factors are still the ones that explain most of the variation in prognosis between individuals\(^{(150)}\).

Even so, the scenario might be rather different in intermediate- to high risk populations. The transplant recipient is frequently in a state of low-grade systemic inflammation, will quite often have some degree of endothelial dysfunction and may experience persistent derangements in parameters associated with bone- mineral balance. Consequently, the renal transplant population is a patient group in which there is frequent elevation of a number of emerging risk factors at the same time. Based on this, there is a larger potential for new biomarkers to become of clinical value.

In this thesis we have explored three rather promising emerging risk factors that might eventually prove of clinical value in RTRs for risk estimation, and possibly risk reduction. Most of the novel biomarkers being examined in CKD populations are evaluated in a thorough and comprehensive review by Fassett et al\(^{(151)}\). The most promising novel risk markers in CKD seem to be the inflammation markers CRP and IL-6, pro brain natriuretic peptide (proBNP) and troponin as markers of cardiac dysfunction, ADMA as a marker of endothelial function, transforming growth factor - \(\beta1\) (TGF-\(\beta1\)) as a mediator of fibrosis and FGF-23 as mediator of fibrosis as well as of cardiovascular disease\(^{(152)}\) (Table 3). Additionally, vitamin D seems to be of importance in various functions including immune modulation, cardiac contractility and bone health. Of these markers, reliable assays are available in clinical practice for proBNP, CRP and troponin only.
<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Native kidney cohorts</th>
<th>Transplant kidney cohorts</th>
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<tr>
<td>IL-6</td>
<td>All-cause and CV-mortality</td>
<td>MACE, all-cause mortality, doubling of serum creatinine and graft loss</td>
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<td>CRP</td>
<td>All-cause mortality and vascular stiffness</td>
<td>MACE and all-cause mortality</td>
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<td>ProBNP</td>
<td>All-cause mortality, progression of ESRD</td>
<td>Mortality</td>
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<td>Troponin</td>
<td>All-cause mortality, progression to ESRD</td>
<td>MACE and mortality</td>
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<td>ADMA</td>
<td>Endothelial dysfunction, vascular stiffness, progression of CKD</td>
<td>MACE and all-cause mortality</td>
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<td>TGF-β</td>
<td>Left ventricular hypertrophy in CKD, development of CKD.</td>
<td>Studied in CNI-toxicity and fibrosis.</td>
</tr>
<tr>
<td>FGF-23</td>
<td>Vascular calcification, CV-outcomes, progression to ESRD, mortality</td>
<td>Mortality, graft loss</td>
</tr>
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<td>1,25-OH Vit D</td>
<td>Excess: arterial stiffness and vascular damage in CKD.</td>
<td>Deficiency: Post-tx malignancy.</td>
</tr>
<tr>
<td>25-OH Vit D</td>
<td>Deficiency: hypertension/cardiac contractility, immune dysfunction</td>
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IL-6, interleukine-6; MACE, major cardiovascular events; BNP, brain natriuretic peptide; CKD, chronic kidney disease; CRP, C-reactive protein; CNI, calcineurin inhibitor; ESRD, end stage renal disease; FGF, fibroblast growth factor; TGF; transforming growth factor.

Table 3. Promising novel biomarkers in CKD and their association with clinical endpoints. (Adaption from DeSerres et al.(143)).

When it comes to renal transplant patients, so far most research efforts in biomarkers have focused on the identification of rejection among recipients with graft dysfunction. Though a substantial number of publications have described significant associations of these markers with outcome, descriptions of formal evaluations of the diagnostic performance of the proposed markers are rare.

Studies evaluating emerging biomarkers of CV-events and mortality in the transplant setting are scarce. To summarize a few of the investigations, Abedini et al demonstrated that ADMA was independently associated with long term outcomes(80). IL-6 and CRP were also associated with major cardiovascular events.
and all-cause mortality in the ALERT cohort (47), but the associations were markedly weaker than for ADMA. Recently, FGF-23 was shown to be significantly and independently associated with death and graft loss in RTRs(144). The work presented in this thesis adds the investigations of three novel biomarkers to the relatively short list of association studies conducted in this field. I will continue this discussion by focusing on each paper individually.

Paper 1

In this study, we have shown that the inflammatory marker neopterin is significantly associated with cardiovascular events and all-cause mortality in a large cohort of RTRs from the ALERT trial. The increment in risk associated with higher neopterin-to-creatinine ratio remained statistically significant after adjustment for conventional and new risk factors.

Inflammation represents a non-traditional risk factor which is potentially important and modifiable(153). In CKD-populations, chronically elevated levels of inflammatory markers like IL-6 and CRP are predictive of increased CV risk (32, 154). Much of the attention in the research area of systemic inflammation has been focused on these two biomarkers. In a comprehensive review(152), DeSerres et al argue that it is unlikely that the combined analyses of a broad spectre of inflammatory markers (including a large range of cytokines) for estimating CV risk will prove more useful that a selected, limited number of markers, due to the issues of collinearity and the fact that they represent multiple facets of the same biological process. However, in the uremic state, reduction in the number and function of lymphoid cells, combined with normal or increased activity in the myeloid cell lines, is a frequent feature. It has been shown that the cellular composition of the immune system does not normalize after kidney transplantation, even if the level of systemic inflammation and oxidative stress is markedly reduced(155). Levels of pro-inflammatory CD16+ monocytes are shown to remain high in RTRs independent of the type of immunosuppression used or how long time has passed since the transplantation(156). They preferentially produce proinflammatory cytokines, including IFN-γ, and have been associated with subclinical atherosclerosis in RTRs(157). This evidence suggests that persistent low-grade macrophage activation
and cytokine release might be a specific feature of the immune system in renal transplant recipients, and that the degree of such activation is related to long term CV- prognosis. The fact that baseline neopterin values were substantially higher in our cohort compared to the general population(158) as well as patients with known CV disease(159), is in harmony with this theory. Levels in our cohort are in line with findings in clinically stable RTRs in other studies (160, 161).

We find it interesting that neopterin, a marker of macrophage activation, performs this well in our multivariate analysis despite adjustments for both IL-6 and CRP, and that the correlations between neopterin and the other markers of inflammation were relatively weak. In the field of inflammation research, the utility of different biomarker panels is under active investigation. Our results suggest that neopterin might be evaluated for inclusion in such panels, especially if developed for the transplant setting. We nevertheless acknowledge the challenge in establishing reference levels and specific thresholds for these emerging inflammatory markers, including neopterin, in the CKD/transplant setting.

According to our findings, neopterin does not seem independently associated with renal graft loss. This might appear as contradictory to the findings of several groups who have demonstrated significant associations between early post transplant neopterin and subsequent adverse renal outcome(57, 162, 163). However, there are differences in the design and conduction of these studies that could explain the variation in conclusions. Our study population consisted of clinically stable recipients with a reasonably good renal function, while the above mentioned publications investigated neopterin measured in the immediate posttransplant period. Furthermore, we chose to include level of proteinuria, an established risk marker for poor patient and graft survival, as a plausible confounder in our final multivariate regression model. This means that if some of the negative effect of having high serum neopterin is mediated through pathways which increase urinary excretion of protein, we will not be able to account for this in our risk estimates. Weimer et al (142) reported significant associations between neopterin concentration one year after transplantation and the subsequent development of chronic rejection and chronic allograft dysfunction, but proteinuria was not included in their multivariable analysis. Interestingly, if proteinuria is removed from our regression model, there is a
significant association between neopterin and graft loss after all with a 5% increase in risk per 10 units increase in neopterin-to-creatinine ratio (HR 1.05, p=0.013). Whether proteinuria is a confounder or a mediator in this clinical context is not fully known. Our results should thus be interpreted as a suggestion that in RTRs neopterin does not add significantly to the prognostic information given by the degree of proteinuria.

When investigating biomarkers whose elimination is highly dependant on GFR, how to correctly adjust for the confounding effect of renal function, will necessarily be an important issue in the statistical analysis. As neopterin is chemically inert and its elimination is solely through the kidneys, compromised renal function leads to a rise in serum neopterin that is not caused by increased inflammatory activity (164). As our main interest lay in the fraction of the serum neopterin concentration determined by degree of macrophage activation, we chose to express neopterin levels relative to the level of serum creatinine. This is an approach used by most authors investigating this biomarker in populations with compromised kidney function. Additionally, we included serum creatinine as a separate covariate in the regression model to allow for estimation of the independent association between renal function and endpoints. Whether this represents a statistical over-correction could be discussed, but collinearity in the model did not appear to be a problem, judging by the size of the standard errors.

Diminished monocyte activity has been demonstrated in long-term stable kidney transplant recipients (25). Measuring neopterin in a stable clinical post transplant setting might actually be helpful in determining the intensity of immunosuppression, as a low level of neopterin might be a reflection of sufficient suppression of the immune response directed against the allograft. Several groups have proposed the usage of neopterin measurements in clinical practice to guide dosage decisions. This is an interesting aspect when discussing the possible practical value of neopterin as a risk marker. We, however, did not have the necessary data to examine the possible correlation between neopterin level and intensity of immunosuppressive treatment in the ALERT cohort.
This study is the first in renal transplant recipients documenting an association between increased serum levels of SDMA and reduced long-term graft and patient survival after adjusting for traditional and novel cardiovascular risk factors. The association with all-cause death seemed primarily driven by non-cardiovascular causes.

SDMA has been shown to accumulate in the serum of patients with CKD(79). This is mainly a result of impaired renal clearance, as the molecule is almost exclusively eliminated by renal filtration. Claes et al (141) showed recently that patients with ESRD generally have high serum SDMA, and after renal transplantation, the level falls quickly until it is stabilized at levels similar to non-transplanted CKD patients (matched for age, gender, estimated GFR and diabetic status). ADMA displayed a more biphasic pattern in this study, and the metabolism of ADMA is different from SDMA with only 20% being excreted through the kidney.

Before implying that a high SDMA level is in fact negatively associated with clinical outcomes, one has to consider the possible confounding role of kidney function, as was the case for neopterin. Our analyses is in line with the available literature, reporting correlation coefficients ranging from - 0.70 to - 0.80 between SDMA and various GFR estimates(165). Even though estimated GFR was included in our multivariate regression model, we cannot claim to have made an optimal adjustment for renal function. This is due to the fact that estimated GFR itself is less than perfect as a measure of the true glomerular filtration rate. There is an ongoing attempt to identify new and better markers of organ function for laboratory diagnostics. Some authors have actually launched SDMA as a potentially better marker of renal function than serum creatinine(166, 167). Hence residual confounding may be one of the reasons for the associations we have demonstrated between SDMA and outcomes, particularly when it comes to graft loss. We believe, however, that the degree of association and the level of significance makes it unlikely that all statistical significant results in our analyses should be explained by variations in unmeasured confounders. Moreover, even if SDMA elevation in RTRs is mainly a reflection of
decreased renal elimination, the molecule may still play a part in biochemical pathways or exert biological actions.

Novel biomarkers reflecting endothelial function are under investigation in many patient populations, including patients with reduced kidney function. Given the general interest in ADMA as a novel risk marker, and the previous findings of associations between ADMA and long term outcomes in the ALERT cohort(80), results of the current study are intriguing: When entering SDMA into our regression model together with ADMA, the association of ADMA with graft loss and mortality became markedly attenuated, even non-significant. SDMA thus seems to be an even stronger predictor of adverse events than ADMA in our cohort. In the study of a heterogenous population of CKD-patients(84), an increasing SDMA level could be identified as a predictor for renal outcome whereas ADMA level could not, a conclusion consistent with our findings. SDMA is still characterized by some authors as a biochemically inactive substance, though there is increasing evidence pointing towards the existence of biological effects. Suggested mechanisms include an indirect influence on NO-synthesis, as well as a possible participation in inflammatory pathways, as mentioned previously(79).

We have demonstrated an association between SDMA and mortality in RTRs, This is in line with findings in several other populations (44, 80-82). While SDMA was found to independently predict mortality in a large (n=3523) multiethnic cohort representative of the general population; the associations for ADMA were less robust in this material (83). However, contrary to our expectations, SDMA seemed to be more convincingly associated with death from cancer and infections, than from CV causes. Furthermore, we were not able to demonstrate a significant association between SDMA and MACE, a composite CV endpoint which included the “softer” events: myocardial infarction and performance of a coronary artery revascularization procedure. One interpretation of these findings is that the involvement of SDMA in alternative biochemical pathways (e.g. pathways of inflammation or cell turnover) is more important for long term outcomes than the proposed effect on NO availability and endothelial function, which has invariably been linked with cardiovascular health.
Consistent with this theory, in a study of the role of dimethylarginines in patients with stable coronary artery disease(82), the authors demonstrated significant associations between SDMA and secondary CV events as well as mortality, but they could not find clear association to outcomes for L-arginine (the substrate for NO-production) or L-arginine/SDMA ratio. Furthermore, associations between ADMA and study outcomes were generally weaker than for SDMA. The authors concluded that mechanisms besides those involved in the synthesis of NO should be considered for both dimethylarginines.

Though not fully elucidated at present, the molecular processes that regulate ADMA and SDMA, and the pathways that transduce their biological function, might become targets for intervention in renal disease. We agree with Paroni et al(168) who already 10 years ago suggested the determination of both dimethylarginines in the design of future clinical trials in this field.

**Paper 3**

In the last paper we show persistent HPT after renal transplantation to be significantly associated with all-cause mortality and adverse renal outcome. When looking at cardiovascular, cerebrovascular, and vascular deaths combined, there was also a borderline significant association to increased PTH, but there was no evidence for an association between PTH and non-fatal manifestations of coronary heart disease.

When held up against the few other studies looking at PTH and hard endpoints in RTRs, results are somewhat conflicting. In the study of 984 stable RTRs(144), whose main focus was on the value of FGF-23 in predicting clinical outcomes, an association between PTH and a composite endpoint of death and graft loss was significant only in univariate analyses, disappearing after adjustment for GFR.

Features of their study population, which might explain some of this discrepancy, include a cohort half the size of ours and a median serum-PTH almost twice the value of that in our cohort.

In the study of Bleskestad et al(169), looking at RTRs with GFR >60ml/min 10 weeks after transplantation, there was a significant association between PTH-level and a composite clinical endpoint consisting of CV events, graft loss, and all-cause death.
While a J-shaped risk pattern for PTH was seen in this population, like in studies of ESKD-patients(103), we, in our study, could not find significant increase in mortality associated with PTH-values below reference. In evaluating these differences it is of note that PTH levels in the cohort of Bleskestad to a large extent reflected pre-transplantation values, PTH being measured rather early post transplant. Further regression of parathyroid hyperplasia and reduction of tissue PTH resistance would be expected in the majority of patients in the months to follow. In dialysis patients, PTH-levels are almost invariably elevated, and modest increases in PTH (150-300 pg/ml) seem not associated with increased mortality(103, 170). In contrast, in RTRs we find an increased risk of mortality already at the upper reference level of PTH, 65pg/mL. We believe our findings suggest that stable RTRs are more in line with the general population(171, 172) as in contrast to dialysis populations, when it comes to the risk profile for PTH and the level at which risk increases.

There is, as previously mentioned, evidence for an association between PTH-level and mortality in several populations(98, 101, 170, 171). We consider our results to be in concert with these studies. More surprising is the weaker association between PTH and MACE in our data. High levels of PTH have been associated with hypertension(173), left ventricular hypertrophy(174), clinical heart failure(175), as well as coronary heart disease(100), and much of the negative effect of high PTH on survival in different patient groups have been thought to involve the heart muscle and the vessel walls via changes in the expression and function of both the PTH/ PTHrP-receptor (PTHR1) and the calcium-sensing receptor (CaR)(176-179).

We postulate in our paper that in the transplant population persistent PTH elevation might be less associated with the early traditional manifestations of coronary artery disease than with arterial calcification, key processes of cardiac remodelling and the development of cardiomyopathy. There are animal studies to support the involvement of PTH in these pathways(180, 181), and if this theory holds true, it provides an explanation for the stronger association of PTH to fatal than to non-fatal cardiovascular events. Acute coronary syndrome is thought to account for approximately half of the CV-deaths in RTRs(182), but in this patient group as well as in dialysis patients(183, 184), increased incidence of cardiac dysfunction such as heart failure and arrhythmias are important causes of heightened cardiovascular
mortality compared with the general population. Intriguingly, a recent study has shown associations between elevated PTH and sudden cardiac death in older adults without established CV disease(185).

Through the propensity for persistent HPT to induce chronic hypercalcemia(186) and nephrocalcinosis(187, 188), an association between disturbances in bone- and mineral balance and unfavourable graft outcome has been established previously. An elevated calcium phosphate product six months after transplantation has been associated with subsequent renal graft loss(108).

We are, however, the first group to show significant associations between PTH and long term renal outcome independent of levels of serum minerals. Whether there is, in fact, a causal pathway by which PTH may cause damage to the allograft is unknown, but we allow ourselves to speculate that effects of PTH on the vasculature supplying the graft may be involved(189, 190).

In the context of the general aim of this thesis, which is to address the value of different novel biomarkers associated with clinical outcomes in RTRs, it is of interest also to report the independent value of serum minerals as potential risk markers. Previous studies have provided inconsistent results as to whether higher serum phosphate and calcium levels are associated with increased all-cause mortality in RTRs(191, 192). Our results are in concert with the study of Schaeffner et al, finding no significant associations.

However, increasing levels of phosphate were independently associated with increased risk of graft loss in our analyses (J-shaped risk curve), whilst a higher serum calcium seemed protective of graft loss. Others have also encountered this rather contradict association for calcium(192). Since we cannot claim to have the optimal adjustment for kidney function in our multivariate analyses, the mechanism for such a finding might be that the vitamin D deficiency accompanying loss of GFR most often leads to reductions in serum calcium.

In conclusion, we dare to suggest that PTH is a novel risk marker for adverse patient and graft survival in the renal transplant population. PTH levels might be of value for risk stratification in this patient group.
Supplementary analyses

When examining possible novel risk markers, one has to take into account that there is most likely a complex interplay between pathways involving inflammation, endothelial dysfunction and mineral balance. Knowledge about the relative contribution of these pathways to the total burden of risk is limited. Our supplementary analyses revealed a strong correlation between SDMA and neopterin, a finding which is rather expected, since both of these parameters are closely dependent on GFR. More interestingly, in a multivariate Cox regression analysis with GFR and other important traditional risk factors included as covariates, the hazard ratios for each of the three biomarkers examined was to some extent affected by including one or both of the other markers in the model.

This supplemental analysis is not meant to be prognostic and must be interpreted with caution. Nevertheless, in this smaller sub-cohort of ALERT participants, it seems like all three biomarkers retain independent associations with outcomes, neopterin possibly showing the strongest association with all-cause mortality and SDMA seeming the most important for graft loss. PTH seems convincingly associated with renal graft loss, but no independent association was seen for mortality when the other novel risk markers were included in the model.

However, the following aspect is important: Available treatment options for HPT exists, both in the form of surgery and calcimimetic drugs. Furthermore, techniques for measurement are validated and reference values are somewhat established. Also, when evaluating the clinical effect of PTH-lowering treatments, it is the total biological effect mediated through PTH, not just the possible independent pathways that will be of interest.

In any case, while making comparisons between multiple markers is tempting, results should be interpreted with caution, as conclusions will be of a speculative nature! Causality in risk factor comparison should be addressed properly in a prognostic paper using appropriate models for prediction.
Limitations

The limitations which have to be considered are basically the same for all three papers in this thesis. Despite efforts to include an extensive number of potential confounding variables in the multivariate analyses, we are not able to rule out residual confounding. In practice, unmeasured confounders will always be an issue in observational studies. In paper 3, for example, one would have liked to have available measurements of other relevant biomarkers, such as FGF-23 and vitamin D. Biomarkers like neopterin and SDMA, which are mainly eliminated from the body by renal excretion, will naturally be strongly correlated with serum creatinine and estimated GFR. Attempts to adjust for glomerular filtration rate in our multivariate analyses will not be perfect, though, since estimated GFR and creatinine themselves are not ideal markers of kidney function. Also, missing data for one or more important covariates led to some patients having to be excluded from multivariate analyses in all three studies.

Statistical associations between biomarkers and clinical outcomes cannot be interpreted as representing causal relationships. The conduction of properly designed interventional trials is necessary before one can arrive at conclusions about which risk markers are truly causal risk factors.

Furthermore, the study population, a cohort from an RCT investigating the effect of statin treatment in RTRs, is not necessarily representative of the general transplant population. In particular, patients with the highest CV risk might have been excluded due to knowledge emerging in the late 1990’s concerning benefits of lipid lowering treatment in populations at risk. As mentioned before, the biomarker levels were only measured once, at baseline. This prevents us from knowing, e.g. if high biomarker levels in a patient were a constant feature during follow up, corresponding to the mean “exposure”, or simply representative of the levels at that particular moment.

Due to the above mentioned concerns, and to the retrospective origin of the studies presented, one should be careful before translating our findings into recommendations for daily clinical practice.
Clinical implications and future perspectives

In this thesis I have analysed three different novel, or non-traditional, biomarkers for a possible relationship with long term outcomes in renal transplant recipients. The main goal of association-studies like these is to get closer to establishing whether the biomarker of interest could be a useful target for intervention in order to modify risk in the patient population. The next step after showing associations between the biomarkers and outcomes would be writing a prognostic paper, along the lines of the paper by Holme et al(121) who used the ALERT cohort to evaluate the predictive ability of lipoprotein components to that of traditional risk factors. Analytical strategies like receiver operating statistics (ROC) and net reclassification improvement (NRI) could determine how well our biomarkers perform in the prediction of clinical events.

Paper 1

The findings in paper 1 led us to believe that neopterin could be an important marker for risk stratification in RTRs. Others have suggested the use of neopterin as a possible marker to guide immunosuppressive treatment intensity. No drug currently on the market will reduce neopterin levels specifically, though a general increase in the level of immunosuppression is associated with lower serum neopterin.

As previously mentioned, there are indications of an effector role for neopterin in amplifying the effects of reactive oxygen species. It is unknown, however, whether a therapeutic strategy of increasing the elimination of neopterin or inhibiting neopterin production and release would prove fruitful in clinical practice. Clinical trials are needed in order to determine if this biomarker should be considered a true causal factor and not merely a risk marker.

Eventually, more research would also be needed in order to establish a target level for neopterin in interventional settings. If calculating the neopterin-to-creatinine ratio is the way to go in adjusting for compromised renal function, will also have to be evaluated further.
In paper 2 we found a strong link between SDMA, a marker of endothelial function and patient and graft survival in RTRs. This new risk marker may prove useful for risk stratification in this high-risk population. As for most novel biomarkers, there is a demand for both basic research and clinical trials before one can conclude that knowing a patient’s SDMA level would be of practical value in the clinical management of the individual.

The somewhat surprising finding that SDMA was less associated with CV events and more so with non-CV fatal events, has led us to believe that this molecule is indeed more than an indirect inhibitor of NO-production. Inflammatory pathways and possibly viral replication activity could be involved. However, whether the relationship between elevated SDMA and unfavourable clinical outcomes is a causal one, has yet to be elucidated.

Hopefully, future research will establish if an association with cancer and severe infections holds true also in other patient groups. What are the levels of SDMA in active malignancy or ongoing systemic infections, and how does it vary with the intensity of the disease? SDMA might turn out to be an interesting marker for malignancy or even for low-grade infectious activity in transplanted patients, and possibly also other patient groups at risk.

In conclusion, RCTs are required in order to find out whether SDMA-lowering therapeutic strategies actually may reduce the risk of graft loss and mortality in RTRs. As previously proposed, both dimethylarginines should be measured and evaluated in the course of such studies, in order to determine which marker might be most useful in the different clinical settings.
In paper 3 we have investigated the value of PTH as a risk marker for long term events in RTRs. Of the tree papers in the thesis, this is the one that in our opinion may carry the most important clinical implications at present. Intervention trials with PTH-lowering drugs are being conducted in the transplant population(193). The goal of these trials has been mainly to establish the efficacy and safety of drugs like cinacalcet for lowering of PTH.

Hopefully, RCTs designed to investigate long-term outcomes after PTH-lowering treatment will follow the initial trials mentioned above. Judging from the study by Evenepoel et al., the effects of calcimimetica on serum calcium and PTH seem temporary, as values quickly reverted back to pre-treatment level after the treatment was stopped. Economical issues might thus limit the possibilities of studying the effect of drugs like cinacalcet on long term clinical outcomes in RTRs.

As for now, PTH has yet to be established as a definite causal risk factor for reduced graft- and patient survival. This is reflected in the fact that despite an increased interest in PTH as a potential multi-faceted risk factor, we still lack guidelines for the prevention and treatment of persistent HPT in RTRs. Likewise, there is no consensus on how to handle severe secondary HPT in ESRD patients being considered for kidney transplantation.

We humbly suggest that our research adds to the notion that persistent HPT should be actively treated, not only due to the risk of persistent hypercalcemia and bone/mineral disturbances, but also due to the proposed negative effect of HPT on long term graft and patient survival. Persistent hyperparathyroidism should be recognized in this patient group, and the decision on whether or not to embark on PTH-lowering treatment should probably be based on more than the levels of serum minerals.
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Appendix

Paper 1-3
Neopterin is associated with cardiovascular events and all-cause mortality in renal transplant patients


Abstract: Background: Inflammatory markers show significant associations with cardiovascular events and all-cause mortality after kidney transplantation. Neopterin, reflecting interferon-γ-release, may better reflect the proinflammatory state of recipients than less specific markers.

Methods: Kidney transplant recipients in the Assessment of LEScol in Renal Transplant (ALERT) trial were examined and investigated for an association between serum neopterin and subsequent clinical events: graft loss, major cardiovascular events (MACE) and all-cause mortality.

Results: After adjustment for established and emerging risk factors neopterin expressed as neopterin-to-creatinine ratio was significantly associated with MACE (p = 0.009) and all-cause mortality (p = 0.002). Endpoints were more frequent with increasing quartiles of neopterin-to-creatinine ratio. The incidence rates of MACE and all-cause mortality were significantly increased in the upper quartiles compared with the first.

Conclusions: This long-term prospective analysis in stable kidney allograft recipients suggests that neopterin is associated with long-term risk of cardiovascular events and all-cause mortality, but not renal outcomes.

Hege Pihlstrøm, Geir Mjøen, Winfried März, Dag Olav Dahle, Sadollah Abedini, Ingar Holme, Bengt Fellström, Alan Jardine, Stefan Pilz and Hallvard Holdaas

*Department of Organ Transplantation, Oslo University Hospital Rikshospitalet, Oslo, Norway, 1Division of Nephrology, Department of Medicine, Oslo University Hospital Ullevål, Oslo, Norway, 2Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Graz, Austria, 3Synlab Center of Laboratory Diagnostics, Heidelberg, Germany, 4Medical Faculty Mannheim, Mannheim Institute of Public Health, Social and Preventive Medicine, University of Heidelberg, Mannheim, Germany, 5Division of Nephrology, Department of Medicine, Sykehuset i Vestfold, Tønsberg, Norway, 6Department of Research and Development, Oslo University Hospital Ullevål, Oslo, Norway, 7Division of Nephrology, Department of Internal Medicine, Uppsala University Hospital, Uppsala, Sweden, 8British Heart Foundation, Glasgow Cardiovascular Research Centre, Glasgow, UK, 9Division of Endocrinology and Metabolism, Department of Internal Medicine, Medical University of Graz, Graz, Austria and 10Department of Epidemiology and Biostatistics, EMGO Institute for Health and Care Research, VU University Medical Centre, Amsterdam, the Netherlands

Key words: inflammatory marker – kidney transplantation – long-term – neopterin – outcomes

Corresponding author: Hege Pihlstrøm, Department of Organ Transplantation, Division of Nephrology, Oslo University Hospital Rikshospitalet, P.B. 4960 Nydalen, 0424 Oslo, Norway. Tel.: +47 92 214585; fax: +47 23 073928; e-mail: hegphi@ous-hf.no

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Cardiovascular (CV) events and premature deaths are significantly more frequent in kidney transplant recipients (KTR) compared with the general population, even when adjusting for the higher prevalence of traditional risk factors such as diabetes mellitus, hypercholesterolemia, and hypertension (1). Although long-term statin therapy reduces the incidence of major cardiovascular events (MACE) in this population, there is significant residual risk for both cardiac events and all-cause mortality (2). Several non-traditional risk factors, both modifiable and non-modifiable, have been proposed to contribute to this excessive risk (3). We have previously demonstrated in KTR with stable graft function that the inflammatory markers interleukin 6 (IL-6) and C-reactive protein (CRP) show significant associations with CV events and all-cause mortality (4). In this study, we explored the possibility that neopterin may be a more appropriate inflammatory marker for patients undergoing renal transplantation (5).

Neopterin (D-erythro-1-2-3-trihydroxypropyl-pterin) is produced from guanosine triphosphate (6) by activated human monocytes, monocyte-derived dendritic cells, and macrophages. Release and production of neopterin is stimulated mainly by interferon-\( \gamma \) (IFN-\( \gamma \)) released by activated Th1-lymphocytes during the cellular immune response (7). In contrast to IFN-\( \gamma \), which quickly binds to target structures or is neutralized by soluble receptors, neopterin is biochemical inert, and its serum concentrations were closely linked to the activity of the cellular immune system (8). Neopterin is shown to be a marker of disease in a variety of conditions (9) and has previously been associated with CV events and mortality in non-transplant populations (10, 11). As a marker of cellular immune response activation depending on IFN-\( \gamma \) release, neopterin may better reflect the proinflammatory state of KTR than less specific markers of inflammation, but the predictive value of neopterin for clinical outcomes in stable KTR is unknown.

In the current analysis, long-term data from the randomized Assessment of LESool in Renal Transplant (ALERT) trial (1) were examined to investigate the association between serum neopterin level and subsequent adverse clinical outcomes in a population of KTR.

**Patients and methods**

**Study design**

The study design and baseline data of the ALERT trial have been described previously (12). In brief, ALERT was a randomized, double-blind, placebo-controlled study of the effect of fluvastatin (40–80 mg/d vs. placebo) on cardiac and renal outcomes in 2102 male and female KTR aged 30–75 yr, included from June 1996 to October 1997. Patients had received a renal transplant more than six months previously, had a stable graft function and a total serum cholesterol between 4.0 and 9.0 mM (155–348 mg/dL). Exclusion criteria were familial hypercholesterolemia, recent acute rejection episodes, predicted life expectancy of less than one yr or ongoing statin therapy. Follow-up was 5–6 yr in the core study, after which trial participants were offered open-label fluvastatin 80 mg/d in a two-yr extension trial. Mean total follow-up time for the extension study was 6.7 yr. Prior to unblinding the ALERT study, neopterin was chosen as one of the pre-specified cardiovascular risk factors to be analyzed. Serum neopterin concentration was measured in 30% of patients (randomly chosen) by radioimmunoassay (IBL Diagnostics, Hamburg, Germany) in samples taken at the time of study entry (baseline), a mean of 5.4 yr after transplantation.

The study adhered to the International Conference on Harmonization guidelines for Good Clinical Practice and was conducted in accordance with the Declaration of Helsinki Principles. All participants provided written informed consent, and the ethics committee at each participating center approved the trial.

**Outcome definitions**

Renal endpoint was the time to graft loss (RGL), defined as return to dialysis or retransplantation. Cardiac endpoint was the occurrence of a MACE,
defined as cardiac death, non-fatal myocardial infarction verified by hospital records, or coronary revascularization procedure, including coronary artery bypass graft or percutaneous coronary intervention. Death by any cause was also chosen as study outcome. All endpoints were validated by an independent clinical end point committee blinded to study randomization.

Statistical analysis
Since treatment and placebo arms of the original study showed no significant heterogeneity in relation to demographics, known cardiovascular or renal risk factors or levels of inflammatory markers (12), the current analysis was based on the pooled patient population.

In comparing baseline characteristics between groups, independent samples t-test and Mann–Whitney U-test for continuous variables and chi-square test for associations between categorical variables were used. Spearman’s rank correlation was used in checking for statistical associations between neopterin and creatinine, as well as the inflammatory markers IL-6 and CRP.

Univariable and multivariable Cox proportional hazard models were used to evaluate the influence of possible prognostic variables, including conventional cardiovascular risk factors, other inflammatory markers and factors associated with graft survival. These models were used to examine the association between neopterin and MACE, all-cause mortality and graft loss. Since high sensitivity CRP (hsCRP) and IL-6 are closely linked etiologically and considered to be mutual confounders, they were included as covariates in separate multivariable regression models, the other variables remaining the same. Covariates were examined using Schoenfeld residuals and found to fulfill the assumptions of proportionality. Hazard ratios (HR) were estimated with 95% confidence intervals.

Neopterin estimates
As neopterin is chemically inert and its elimination is solely through the kidneys, compromised renal function leads to a rise in serum neopterin that is not caused by increased inflammatory activity (13). We found a high degree of correlation between neopterin and creatinine. Therefore, as done in the majority of previous studies on neopterin in populations with compromised renal function, neopterin levels were calculated relative to the serum concentration of creatinine, thus adjusting for kidney function. Values are expressed in μmol/mol of creatinine.

SPSS version 18 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses except for generation of Figs. 1 and 2 where we used Stata version 11 (StataCorp, College Station, TX, USA).

Results
Baseline characteristics
Table 1 lists baseline characteristics for the ALERT participants, comparing those with and without available baseline neopterin data. Demographics, risk factors, and neopterin levels were similar in the fluvastatin and placebo arms (not shown). Since there were no clinically important differences, neither between the two treatment arms nor between the overall study population and those for whom neopterin levels were available – subsequent analysis was performed on the pooled population with available neopterin measurements (4, 14).

![Fig. 1. Cumulative all-cause mortality according to quartiles of neopterin-to-creatinine ratio.](image1)

![Fig. 2. Cumulative major cardiovascular events (MACE) according to quartiles of neopterin-to-creatinine ratio.](image2)
The correlation coefficient between neopterin and creatinine was 0.61. For purposes of survival analysis, we categorized patients into quartiles according to their neopterin-to-creatinine ratios at baseline. Levels ranged from 33 to 325 μmol/mol. In Table 2, demographic data and background risk factors are presented for each quartile of neopterin/creatinine. There was a tendency towards higher proportions of patients with hypertension, chronic heart disease, panel reactive antibodies, delayed graft function (DGF), longer time on dialysis prior to transplantation and treatment for CMV-infection/reactivation in the highest or the two highest quartiles. IL-6 and hsCRP increased progressively across the quartiles, as did age.

**Outcomes**

The proportions of patients reaching the renal, cardiac and mortality endpoints in each quartile for neopterin-to-creatinine ratio are listed in Table 3. Log-rank tests were used to check the significance of differences between each of the three upper quartiles compared with the lowest.

The rate of death from all causes increased in higher neopterin/creatinine quartiles, and the differences were statistically significant between all three upper quartiles and the first quartile. The number of events more than trebled from the first to the fourth quartile (9.5–33.6%). For MACE, the incidence rate was more than twice as high (11.1–25.0%) in the fourth neopterin/creatinine quartile compared with the first one, and the difference was statistically significant. For renal graft loss the incidence rates was slightly higher in the fourth quartile, but no statistical significance was reached.

Figs. 1 and 2 presents the Kaplan–Meier failure estimates plots for all-cause mortality and MACE showing time to event, or end of follow-up, by neopterin/creatinine quartile.

### Multiple risk factor analysis

Table 4 shows risk factor evaluation using univariable and multivariable models of Cox regression analyses. For all study outcomes we present HR with 95% confidence intervals (95% CI) and their respective p-Values. In the univariable model, baseline neopterin expressed as neopterin-to-creatinine ratio, as well as potential risk factors and baseline demographics, is examined separately against the study outcomes. The multivariable model is adjusted for the following baseline covariates: age, gender, smoking habit, diagnosis of coronary heart disease, LDL-cholesterol, systolic blood pressure, diabetes mellitus, serum creatinine, and proteinuria. IL-6 and hsCRP were included in separate models, as they are part of the same etiological inflammatory pathway and thus have a high collinearity. Results are shown only for the analysis including hsCRP, as the hazard ratio for neopterin was virtually identical in the two models.

In the univariable model, neopterin was highly significantly associated with all endpoints in our study. After adjustment for other established and potentially important risk factors, we found neopterin (in μmol/mol creatinine) to have a significant positive association with all-cause of death (HR 1.06 per 10 units, p = 0.002, 95% CI 1.02–1.09) and MACE (HR 1.06 per 10 units, p = 0.009, 95% CI 1.01–1.10) but no independent predictive value for graft loss. HR and p-Values for neopterin remained the same for all endpoints when randomization group was included in the multivariate model (not shown). Though reaching significance for MACE in the univariable analyses, HsCRP was not independently associated with any of the
study endpoints in the multivariable analyses, nor was IL-6. Using Spearman’s rank correlation, we found the correlation coefficient between neopterin and IL-6 to be 0.26, p < 0.001, while for neopterin and CRP it was 0.14, p < 0.001. Among the remaining risk factors entered into the model, diabetes mellitus was strongly associated with all outcomes, as was current smoking for all outcomes but MACE. As might have been expected, coronary heart disease was predictive of future MACE and all-cause mortality, while serum creatinine and level of proteinuria was significantly associated with the renal endpoint. Also, LDL-cholesterol was significantly associated with MACE.

Neopterin was also included in a multivariate model with osteoprotegrin, asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) to assess its independency of other markers related to inflammation and endothelial function. No relevant change in HR was seen, and the association with MACE and all-cause death remained highly significant (data not shown).

<table>
<thead>
<tr>
<th>Neopterin/creatinine quartile µmol/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Age at baseline, yr</td>
</tr>
<tr>
<td>Male gender</td>
</tr>
<tr>
<td>Current smoker</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Hypertension</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
</tr>
<tr>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>Serum creatinine, µM</td>
</tr>
<tr>
<td>Proteinuria, g/24 h</td>
</tr>
<tr>
<td>HDL cholesterol, mM</td>
</tr>
<tr>
<td>LDL cholesterol, mM</td>
</tr>
<tr>
<td>Triglycerides, mM</td>
</tr>
<tr>
<td>hsCRP, mg/L</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
</tr>
<tr>
<td>Time since last transplant, yr</td>
</tr>
<tr>
<td>Time on dialysis, yr</td>
</tr>
<tr>
<td>Cold ischemia time, hours</td>
</tr>
<tr>
<td>Panel reactive antibodies</td>
</tr>
<tr>
<td>Delayed graft function</td>
</tr>
<tr>
<td>Treatment for cytomegalovirus</td>
</tr>
<tr>
<td>Neopterin, nM</td>
</tr>
<tr>
<td>Randomized to fluvastatin</td>
</tr>
<tr>
<td>Graft loss, n (%)</td>
</tr>
<tr>
<td>p-Value</td>
</tr>
<tr>
<td>MACE, n (%)</td>
</tr>
<tr>
<td>p-Value</td>
</tr>
<tr>
<td>All-cause mortality, n (%)</td>
</tr>
<tr>
<td>p-Value</td>
</tr>
</tbody>
</table>

Data expressed as number of patients with the event in each quartile (%). MACE, major adverse cardiac event.
Table 4. Hazard ratios for neopterin-to-creatinine ratio (per 10 units in μmol/mol) with covariates in relation to outcomes in 628 stable renal transplant patients by univariable and multivariable Cox regression analysis

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>MACE 106/628</th>
<th>All-cause mortality 122/628</th>
<th>Graft loss (death-censored) 124/628</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p-Value</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td><strong>Univariable analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.03 (1.01–1.05)</td>
<td>0.002</td>
<td>1.07 (1.05–1.09)</td>
</tr>
<tr>
<td>Male gender</td>
<td>1.51 (0.98–2.34)</td>
<td>0.064</td>
<td>1.05 (0.72–1.54)</td>
</tr>
<tr>
<td>Current smoking</td>
<td>1.03 (0.64–1.64)</td>
<td>0.917</td>
<td>1.55 (1.04–2.30)</td>
</tr>
<tr>
<td>Coronary heart disease</td>
<td>4.00 (2.55–6.27)</td>
<td>&lt;0.001</td>
<td>3.22 (2.09–4.99)</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>1.42 (1.18–1.69)</td>
<td>&lt;0.001</td>
<td>1.06 (0.88–1.26)</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>1.01 (1.00–1.02)</td>
<td>0.002</td>
<td>1.01 (1.01–1.02)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1.99 (1.32–2.99)</td>
<td>&lt;0.001</td>
<td>2.17 (1.49–3.16)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>1.01 (1.00–1.01)</td>
<td>0.002</td>
<td>1.01 (1.00–1.01)</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>1.22 (1.02–1.45)</td>
<td>0.030</td>
<td>1.23 (1.04–1.45)</td>
</tr>
<tr>
<td>hsCRP</td>
<td>1.03 (1.01–1.05)</td>
<td>0.004</td>
<td>1.02 (1.00–1.04)</td>
</tr>
<tr>
<td>Neopterin/creatinine</td>
<td>1.08 (1.04–1.12)</td>
<td>&lt;0.001</td>
<td>1.11 (1.07–1.14)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>MACE 100/585</th>
<th>All-cause mortality 117/583</th>
<th>Graft loss (death-censored) 114/583</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p-Value</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td><strong>Multivariable analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.02 (1.00–1.04)</td>
<td>0.057</td>
<td>1.07 (1.05–1.09)</td>
</tr>
<tr>
<td>Male gender</td>
<td>1.54 (0.96–2.45)</td>
<td>0.073</td>
<td>0.90 (0.59–1.35)</td>
</tr>
<tr>
<td>Current smoking</td>
<td>1.00 (0.60–1.65)</td>
<td>0.990</td>
<td>1.75 (1.16–2.65)</td>
</tr>
<tr>
<td>Coronary heart disease</td>
<td>3.35 (2.06–5.46)</td>
<td>&lt;0.001</td>
<td>2.02 (1.27–3.19)</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>1.47 (1.22–1.77)</td>
<td>&lt;0.001</td>
<td>0.99 (0.83–1.19)</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>1.00 (0.99–1.01)</td>
<td>0.503</td>
<td>1.00 (0.99–1.01)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2.01 (1.31–3.10)</td>
<td>0.002</td>
<td>2.38 (1.60–3.55)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>1.00 (1.00–1.01)</td>
<td>0.056</td>
<td>1.01 (1.00–1.01)</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>1.08 (0.87–1.34)</td>
<td>0.508</td>
<td>1.09 (0.87–1.35)</td>
</tr>
<tr>
<td>hsCRP</td>
<td>1.02 (1.00–1.04)</td>
<td>0.127</td>
<td>1.01 (0.99–1.03)</td>
</tr>
<tr>
<td>Neopterin/creatinine</td>
<td>1.06 (1.01–1.10)</td>
<td>0.009</td>
<td>1.06 (1.02–1.09)</td>
</tr>
</tbody>
</table>

The multivariable analysis adjusts for relevant demographic covariates (age, gender), known renal/cardiovascular risk factors (smoking, coronary heart disease, LDL-cholesterol, systolic blood pressure, diabetes mellitus, creatinine, level of proteinuria), and the inflammation marker hsCRP. CI, confidence interval; HDL, high-density lipoprotein; HR, hazard ratio; hsCRP, high sensitivity CRP; LDL, low-density lipoprotein; MACE, major adverse cardiac event.

Discussion

In this analysis of a large cohort of KTR from the ALERT study, we have shown that the inflammatory marker neopterin is significantly associated with cardiovascular events and all cause mortality in KTR, even after adjustment for conventional and new risk factors.

In patients with pre-dialysis chronic kidney disease (CKD), serum neopterin is elevated and significantly correlated with markers of inflammation including hsCRP, IL-6, and IFN-γ (15). We have shown previously that levels of IL-6 and hsCRP are associated with cardiovascular endpoints and all-cause mortality following kidney transplantation (4, 16). However, and of central importance, this study shows that in KTR the predictive power of neopterin was maintained after adjustment for hsCRP and IL-6. In the multivariable analyses including neopterin, CRP and IL-6 failed to reach significance as independent predictors of long-term outcomes. In addition, though statistically significant, the correlations between neopterin and these two inflammation markers were weak ones. Our epidemiological data on the predictive value of neopterin are in line with previous literature showing that neopterin rises quickly after macrophage activation (17), is an excellent marker for longer term activation of cellular immunity during the maintenance phase, and appear to remain relatively stable over time (18).

Inflammation is a key element of the development of atherosclerosis, with T-lymphocytes and monocye-derived macrophages being detected in atherosclerotic lesions. In accordance with our results, studies have highlighted neopterin as a useful marker for long-term risk of all-cause and cardiovascular death in patients from diverse populations, including individuals undergoing coronary angiography (19), patients with stable
coronary artery disease (20), newly diagnosed diabetics (21), and dialysis-dependent CKD patients (22). Furthermore, it has recently been shown that elevated neopterin, but not CRP level, predicts left ventricular dysfunction in patients with chronic stable angina pectoris (23). A recent report from the Hordaland study demonstrated that in elderly patients, without pre-existing coronary heart disease, higher levels of neopterin are associated with an increased risk of subsequent coronary events (24). Chronic low-grade inflammation is one of the main conditions associated with increased cardiovascular morbidity and mortality in patients with CKD, especially those on dialysis (25). Thus, it is not surprising that persistent inflammation, endothelial dysfunction, and associated oxidative stress in KTR (26) is reflected in progression of atherosclerosis (27) and adversely affects cardiovascular outcomes (4). The significant association between neopterin and outcomes was maintained even after adjustment for markers of endothelial function (SDMA, ADMA).

Immunologic responses to allografts involve humoral and cellular components of both the adaptive and innate immune system, the T-cell playing a pivotal role in the initial recognition of anti-self (28). The stronger association that we found between neopterin and the clinical endpoints than for other inflammatory markers may possibly reflect the dominance of T-cell and macrophage activation in the ongoing inflammatory status of allograft recipients. Consistent with this, baseline neopterin values were substantially higher in our cohort compared with the general population (29) and patients with known CV disease (18). This is in harmony with earlier findings on clinically stable KTR (30, 31).

In one of the earliest publications to assess levels of neopterin in KTR (32), Margreiter et al. measured urinary neopterin daily during the early post-operative period and found that acute rejection and early viral infection were preceded by a rising or high level of urinary neopterin in 95% and 100% of cases, respectively. They later extended their data to include urinary neopterin measurements in 294 kidney allograft recipients (33). Subsequent studies by others have confirmed that elevation of serum or urinary neopterin precedes the rise in creatinine by up to several days in patients with acute early complications (34, 35), and routine daily post-operative neopterin measurements have been suggested for the early detection of immunologic complications in kidney allograft recipients (36).

In the trial (33) conducted by Reibnegger and colleagues, a high neopterin level in the early post-transplant period was associated with a higher risk of graft failure in the long term. In a smaller cohort of patients, Grebe et al. (37) observed that elevated neopterin levels following transplantation were associated with inferior graft and patient survival, while a recent prospective study in 216 KTR showed an association between higher levels of neopterin and acute rejection in the first year of follow-up (38). Our population was recruited five yr after transplantation and was clinically stable with a reasonably good renal function. Elevated neopterin was not significantly associated with renal graft loss after adjusting for level of proteinuria. While Grebe et al. (37) were primarily interested in early post-transplant macrophage activation associated with elevated neopterin, our study suggests that in clinically stable allograft recipients, serum neopterin is not independently associated with renal graft failure and does not add significantly to the prognostic information given by the degree of proteinuria. Weimer et al. (39, 40) reported significant associations between neopterin concentration at one yr post-transplant and the development of chronic rejection and chronic allograft dysfunction within two yr, but proteinuria was not included in their multivariable analysis, possibly explaining this discrepancy.

A small study on children with primary nephrotic syndrome (41) showed a positive correlation between serum neopterin levels and proteinuria in patients with active disease. A link between neopterin levels and the progression rate in proteinuric diabetic nephropathy has been demonstrated (42), and one group (43) observed marked differences in serum and urine neopterin levels among diabetic patients with and without microalbuminuria. We are not proposing that enhanced macrophage activity is not an important factor in the development of a dysfunctional renal allograft, but the potential clinical value of neopterin with respect to graft function may be limited as long as proteinuria is considered a well established risk factor for poorer long-term renal outcomes in kidney allograft recipients (44, 45).

Reference ranges for neopterin are higher in the healthy elderly population (>75 yr). Several studies report rising neopterin levels from the age of 60–70 (46) or even earlier (47), probably reflecting the involvement of cellular immunity in the aging process, as well as the presence of low-grade inflammatory processes such as atherosclerosis, neurodegeneration, or unrecognized evolving disease (autoimmunity or malignancy) (29). However, we did not find significant differences in baseline neopterin between different age groups, perhaps because kidney transplants are not performed for
the oldest CKD patients (recipients in our cohort were aged 23.6–74.8 yr), and because comorbidity, the immunological consequences of long-standing uremia and the anti-allograft immune response may overshadow the component of neopterin production related to aging.

The strengths of this analysis are the prospective controlled design, the long follow-up time, the large patient cohort and the independent adjudication of all clinical endpoints. Potential limitations of our study, however, merit consideration. Although the results show a strong association between neopterin and clinical outcomes, the data do not prove a causal relationship. Residual confounding cannot be ruled out, though we have carefully adjusted for a wide range of covariates in our statistical models. Although neopterin may, at present, not be a clinically useful single parameter for risk prediction in KTR, it is conceivable that neopterin could be valuable for multi-marker risk prediction or for the evaluation of the clinical efficacy of established treatments in this patient group.

As in most large prospective trials, serum samples were obtained at inclusion and not followed consecutively. The study population, a cohort selected for entry into a clinical trial, is not necessarily fully representative of the general renal transplant population, although the qualitative relationships between neopterin and the specified clinical outcomes are likely to apply, at least for Caucasians. Mean neopterin values were not different for the two randomization groups, and there was no significant skewing in the proportion of patients receiving fluvastatin in each quartile of neopterin-to-creatinine ratio. Entering treatment group as a covariate in the multiple regression analysis did not change the hazard ratio for neopterin.

In conclusion, in clinically stable renal transplant recipients there appears to be an independent association between serum neopterin concentration and long-term clinical outcomes of MACE and all-cause mortality.

Acknowledgements
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Authors’ contributions
Hege Pihlstrøm: Data collection, data analysis/interpretation, statistics, drafting the article. Hallvard Holdaas: Concept/design, study protocol, data collection, drafting and critical revision of the article. Bengt Fellstrom and Alan Jardine: Concept/design, study protocol, data collection, critical revision of the article. Geir Mjøen, Ingar Holme, Sadollah Abedini, and Dag Olav Dahlé: Data analysis/interpretation, statistics, critical revision of the article. Winfried Mähr and Stephan Pflz: Data analysis/interpretation, drafting and critical revision of the article.

References
Neopterin in renal transplant recipients


Symmetric Dimethylarginine as Predictor of Graft loss and All-Cause Mortality in Renal Transplant Recipients

Hege Pihlstrøm, Geir Mjøen, Dag Olav Dahle, Stefan Pilz, Karsten Midtvedt, Winfried März, Sadollah Abedini, Ingar Holme, Bengt Fellström, Alan Jardine, and Hallvard Holdaas

Background. Elevated symmetric dimethylarginine (SDMA) has been shown to predict cardiovascular events and all-cause mortality in diverse populations. The potential role of SDMA as a risk marker in renal transplant recipients (RTR) has not been investigated.

Methods. We analyzed SDMA in the placebo arm of the Assessment of Lescol in Renal Transplantation study, a randomized controlled trial of fluvastatin in RTR. Mean follow-up was 5.1 years. Patients were grouped into quartiles based on SDMA levels at study inclusion. Relationships between SDMA and traditional risk factors for graft function and all-cause mortality were analyzed in 925 RTR using univariate and multivariate survival analyses.

Results. In univariate analysis, SDMA was significantly associated with renal graft loss, all-cause death, and major cardiovascular events. After adjustment for established risk factors including estimated glomerular filtration rate, an elevated SDMA-level (4th quartile, >1.38 μmol/L) was associated with renal graft loss; hazard ratio (HR), 5.51; 95% confidence interval (CI), 1.95–15.57; P=0.001, compared to the 1st quartile. Similarly, SDMA in the 4th quartile was independently associated with all-cause mortality (HR, 4.56; 95% CI, 2.15–9.71; P=0.001), and there was a strong borderline significant trend for an association with cardiovascular mortality (HR, 2.86; 95% CI, 0.99–8.21; P=0.051).

Conclusion. In stable RTR, an elevated SDMA level is independently associated with increased risk of all-cause mortality and renal graft loss.

Keywords: Symmetric dimethylarginine, Renal transplantation, Survival, Graft loss.
results

Baseline Characteristics

Participants in the Assessment of Lescol in Renal Transplantation (ALERT) study (19) were kidney allograft recipients with a stable graft function. Mean time from transplantation to randomization was 5.1 years. Baseline patient data including patient demographics, risk factors, and comorbidity have previously been presented (20).

Table 1 shows baseline characteristics for patients according to quartiles of SDMA. The groups were comparable in

TABLE 1. Demographic and baseline data according to quartiles of SDMA

<table>
<thead>
<tr>
<th>Variables</th>
<th>Q1 (n=248)</th>
<th>Q2 (n=216)</th>
<th>Q3 (n=232)</th>
<th>Q4 (n=229)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDMA quartiles µmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at baseline, yr</td>
<td>51.7 (10.4)</td>
<td>51.1 (11.2)</td>
<td>49.0 (10.7)</td>
<td>47.7 (11.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male sex</td>
<td>138 (55.6)</td>
<td>138 (63.9)</td>
<td>171 (73.7)</td>
<td>161 (70.3)</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td>Current smoker</td>
<td>36 (14.5)</td>
<td>29 (13.4)</td>
<td>48 (20.1)</td>
<td>53 (23.2)</td>
<td>0.003</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.9 (4.2)</td>
<td>26.2 (4.3)</td>
<td>25.5 (4.7)</td>
<td>25.1 (4.5)</td>
<td>0.015</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>60 (24.2)</td>
<td>32 (14.8)</td>
<td>39 (16.8)</td>
<td>46 (20.1)</td>
<td>0.316</td>
</tr>
<tr>
<td>Hypertension</td>
<td>170 (68.5)</td>
<td>150 (69.4)</td>
<td>181 (78.0)</td>
<td>173 (75.5)</td>
<td>0.023</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>143.0 (18.8)</td>
<td>143.0 (18.0)</td>
<td>145.2 (18.0)</td>
<td>147.0 (21.2)</td>
<td>0.012</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>85.7 (9.3)</td>
<td>85.6 (8.8)</td>
<td>86.5 (9.5)</td>
<td>85.9 (10.8)</td>
<td>0.548</td>
</tr>
<tr>
<td>Coronary heart disease</td>
<td>19 (7.7)</td>
<td>16 (7.4)</td>
<td>22 (9.4)</td>
<td>30 (13.1)</td>
<td>0.033</td>
</tr>
<tr>
<td>ADMA, µmol/L</td>
<td>0.72 (0.10)</td>
<td>0.76 (0.10)</td>
<td>0.80 (0.12)</td>
<td>0.86 (0.16)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum creatinine, µmol/L</td>
<td>104.1 (17.8)</td>
<td>124.3 (24.2)</td>
<td>146.6 (29.0)</td>
<td>196.2 (60.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>eGFR, ml/min</td>
<td>63.2 (12.5)</td>
<td>53.3 (12.0)</td>
<td>45.9 (10.4)</td>
<td>34.6 (11.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Proteinuria, g/24 hr</td>
<td>0.22 (0.37)</td>
<td>0.27 (0.57)</td>
<td>0.42 (0.79)</td>
<td>0.59 (0.99)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.42 (0.45)</td>
<td>1.39 (0.46)</td>
<td>1.35 (0.44)</td>
<td>1.20 (0.39)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>4.17 (0.98)</td>
<td>4.24 (1.10)</td>
<td>4.15 (0.96)</td>
<td>4.12 (1.02)</td>
<td>0.441</td>
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<tr>
<td>Triglycerides, mmol/L</td>
<td>2.19 (2.13)</td>
<td>2.14 (1.13)</td>
<td>2.22 (1.39)</td>
<td>2.37 (1.28)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hsCRP, mg/L</td>
<td>2.80 (4.27)</td>
<td>3.64 (5.75)</td>
<td>3.46 (6.46)</td>
<td>4.23 (8.14)</td>
<td>0.646</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>2.60 (1.52)</td>
<td>2.88 (1.89)</td>
<td>2.83 (1.80)</td>
<td>3.24 (2.02)</td>
<td>0.006</td>
</tr>
<tr>
<td>Time since last transplantation, yr</td>
<td>4.9 (3.3)</td>
<td>5.1 (3.3)</td>
<td>4.9 (3.4)</td>
<td>5.6 (3.6)</td>
<td>0.186</td>
</tr>
<tr>
<td>Time on dialysis, yr</td>
<td>2.0 (3.6)</td>
<td>2.1 (3.6)</td>
<td>2.5 (3.6)</td>
<td>2.8 (4.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cold ischemia time, hr</td>
<td>20.0 (7.8)</td>
<td>18.5 (7.8)</td>
<td>20.2 (7.1)</td>
<td>19.9 (7.9)</td>
<td>0.335</td>
</tr>
<tr>
<td>Panel reactive antibodies</td>
<td>43 (18.5)</td>
<td>39 (19.5)</td>
<td>30 (14.5)</td>
<td>30 (16.1)</td>
<td>0.305</td>
</tr>
<tr>
<td>Delayed graft function</td>
<td>31 (12.6)</td>
<td>31 (14.6)</td>
<td>47 (20.4)</td>
<td>54 (24.3)</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td>Treatment of cytomegalovirus</td>
<td>23 (9.4)</td>
<td>30 (14.4)</td>
<td>34 (15.5)</td>
<td>30 (13.6)</td>
<td>0.149</td>
</tr>
</tbody>
</table>

Total n=925.

Continuous variables are shown as mean (SD); categorical variables as n (% of total).

ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; HDL, high-density lipoprotein; LDL, low-density lipoprotein; hsCRP, high sensitivity CRP; IL-6, interleukin-6. P-values for linear trends (ANOVA/χ² test) are presented in the rightmost column, SD, standard deviation.
relation to time since transplantation, cold ischemia time, presence of panel reactive antibodies, and treatment of cytomegalovirus. There were, however, more patients with delayed graft function in the higher SDMA quartiles, and they experienced generally longer time on dialysis before transplantation. Symmetric dimethylarginine was positively associated with proteinuria and inversely correlated with estimated glomerular filtration rate (eGFR). Also, patients with elevated SDMA at baseline were slightly younger, more likely to be men and smokers, and showed a higher burden of coronary heart disease, hypertension, and dyslipidemia. Also, IL-6 values were significantly higher in the 4th SDMA quartile, whereas high sensitivity C-reactive protein (hsCRP) showed no such association with SDMA. P values for linear trends (analysis of variance and chi-square test) are presented in the rightmost column of Table 1.

Correlation Analysis

Correlation analyses were performed between SDMA and measures of kidney function as well as ADMA and selected parameters of inflammation (calculations not shown). The correlation coefficient was 0.77 for creatinine and 0.72 for eGFR. Symmetric dimethylarginine was significantly correlated with ADMA (r=0.40) and IL-6 (r=0.09), but not hsCRP (r=0.02, nonsignificant).

Survival Analysis

Results from univariate and multivariate Cox regression analyses are presented in Table 2. Fifty-two participants had missing values for one or more of the covariates and were excluded from the multivariate model. Hazard ratios (HRs) with corresponding 95% confidence intervals (95% CI) are shown for all study outcomes. The multivariate model is adjusted for baseline characteristics and potentially important risk factors including age, sex, smoking habit, established coronary heart disease, systolic blood pressure, low-density lipoprotein cholesterol, diabetes mellitus, hsCRP, ADMA, and eGFR split into quartiles.

Symmetric dimethylarginine showed a positive association with all study outcomes in univariate analysis, whereas after multivariate adjustment, there was a significant increased risk of death (HR, 4.56; 95% CI, 2.15–9.71; P=0.001) and graft loss (HR, 5.51; 95% CI, 1.95–15.57; P=0.001) in the highest SDMA quartile. When the mortality variable was further subdivided by cause of death, there was a strong association between SDMA and non-CV death (HR, 7.54; 95% CI, 2.54–22.40; P<0.001). For CV death, the increased risk associated with SDMA in quartile 4 was borderline significant (HR, 2.86; 95% CI, 0.99–8.21; P=0.051). A higher frequency of major adverse CV events (MACE) was seen in the 4th SDMA quartile, although this trend was not statistically

| TABLE 2. | Cox Regression Analysis for study outcomes using SDMA quartile 1 (Q1) as reference |
|-----------------|-------------------------------|-------------------------------|-------------------------------|-----------------|
| Outcome         | SDMA quartiles                |                               |                               |                 |
|                 | Q1 0.46–0.88 μmol/L          | Q2 0.88–1.08 μmol/L          | Q3 1.08–1.38 μmol/L          | Q4 1.38–4.41 μmol/L |
|                 | n=248                        | n=216                        | n=232                        | n=229           |
| MACE            |                               |                               |                               |                 |
| Number of events| 26 (10.5%)                   | 24 (11.1%)                   | 30 (12.9%)                   | 40 (17.5%)      |
| Univariate HR (95% CI) | 1 (0.39–1.80)               | 1.24 (0.73–2.09)             | 1.88 (1.15–3.09)             |                 |
| Multivariate HR (95% CI) | 0.99 (0.54–1.83)             | 1.10 (0.56–2.14)             | 1.64 (0.75–3.58)             |                 |
| All-cause mortality |                               |                               |                               |                 |
| Nr of events    | 21 (8.5%)                    | 20 (9.3%)                    | 23 (9.9%)                    | 61 (26.6%)      |
| Univariate HR (95% CI) | 1.04 (0.56–1.91)             | 1.15 (0.64–2.08)             | 3.53 (2.15–5.79)             |                 |
| Multivariate HR (95% CI) | 1.40 (0.72–2.71)             | 1.70 (0.83–3.47)             | 4.56 (2.15–9.71)             |                 |
| Cardiovascular death |                               |                               |                               |                 |
| Number of events| 12 (4.8%)                    | 11 (5.1%)                    | 13 (5.6%)                    | 29 (12.7%)      |
| Univariate HR (95% CI) | 1.06 (0.48–2.37)             | 1.12 (0.51–2.46)             | 2.91 (1.48–5.70)             |                 |
| Multivariate HR (95% CI) | 1.34 (0.56–3.22)             | 1.47 (0.56–3.87)             | 2.86 (0.99–8.21)             |                 |
| Noncardiovascular death |                               |                               |                               |                 |
| Number of events| 9 (3.6%)                     | 9 (4.2%)                     | 10 (4.3%)                    | 32 (14.0%)      |
| Univariate HR (95% CI) | 0.99 (0.38–2.57)             | 1.19 (0.48–2.93)             | 4.36 (2.08–9.14)             |                 |
| Multivariate HR (95% CI) | 1.41 (0.51–3.92)             | 1.97 (0.68–5.74)             | 7.54 (2.54–22.40)             |                 |
| RGL             |                               |                               |                               |                 |
| Number of events| 6 (2.4%)                     | 14 (6.5%)                    | 24 (10.3%)                   | 80 (34.9%)      |
| Univariate HR (95% CI) | 2.65 (1.02–6.88)             | 4.44 (1.82–10.87)            | 19.70 (8.59–45.16)           |                 |
| Multivariate HR (95% CI) | 1.62 (0.59–4.48)             | 1.86 (0.68–5.11)             | 5.51 (1.95–15.57)            |                 |

*Number of events (in percentage of total) registered in each SDMA quartile during a mean of 5.1 years of follow-up. Univariate and multivariate hazard ratios with 95% confidence intervals (HR, 95% CI) for study outcomes for each SDMA quartile compared with the first quartile. In the multivariate model adjustments were made for: age, sex, diabetes mellitus, smoking status, systolic blood pressure, LDL cholesterol, coronary artery disease, ADMA, hsCRP and eGFR in quartiles.

MACE, major adverse cardiovascular events; RGL, renal graft loss; cardiovascular death, cardiac, vascular, and cerebrovascular deaths; HR, hazard ratio; 95% CI, 95% confidence interval; hsCRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein.

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significant in multivariate analysis (HR, 1.64; 95% CI, 0.75–3.58; P=0.212). We were not able to reveal any significant competing risks between nonfatal MACE and all-cause mortality (data not shown).

Figure 1 presents adjusted HRs with corresponding P values for the three most important causes of death: CV events, infection, and cancer. Compared with the first quartile, there was a more than ninefold increase in the risk of dying from cancer and a more than sevenfold increased risk of death by infection in the fourth SDMA quartile. The occurrence of death from cancer or infection according to SDMA quartiles are shown in Table S1 (SDC, http://links.lww.com/TP/A994).

Figures 2 to 3 show adjusted Cox hazard functions for all-cause death and renal graft loss according to SDMA quartiles, illustrating the relationship between SDMA level and the risk of adverse events as a function of time.

In an initial analysis, IL-6 replaced CRP without causing noticeable changes in HR for any of the study outcomes (data not shown). Furthermore, we constructed an extended model for the prediction of graft loss, adding to the multivariate analysis the following plausible risk factors for adverse renal outcome: time since last transplantation, total time on renal replacement therapy, baseline level of proteinuria, delayed graft function, and treatment of rejection (before randomization). Importantly, this extensive multivariate adjustment rendered the HR for SDMA quartile 4 essentially unchanged (HR, 4.02; CI, 1.37–11.80; P=0.011).

**DISCUSSION**

This study is the first to report that increased serum levels of SDMA, adjusted for traditional and nontraditional risk factors, are associated with reduced long-term graft and patient survival in kidney transplant recipients. The association between SDMA and clinical outcomes does not appear fully linear, the risk increasing substantially from the third to the fourth quartile. The increased HR for all-cause mortality in the fourth quartile was mainly driven by non-CV causes, but there was still borderline significance for an association between SDMA and CV death.

An association between SDMA and mortality has previously been reported in several nontransplanted populations: in patients referred for angiography (21, 22), in patients after an ischemic stroke (23) and in stable coronary heart disease patients(24). Recently, SDMA was found to independently predict mortality in a large (n=3523) multiethnic cohort representative of the general population (25). One study indicates that in an older population, plasma levels of SDMA seem predictive of CV events (26). Significant relationships between SDMA and development of major CV events have been found in patients undergoing elective diagnostic cardiac catheterization (21, 22), patients with stable coronary heart disease (24)
and patients with non-ST-elevation myocardial infarction. In patients with CKD and in primary care patients with and without peripheral arterial disease, increased CV risk was not related to SDMA.

The relationship between high SDMA and increased risk of all-cause death seems robust in our cohort of RTR. We identified a trend, but no significant association between SDMA levels and the composite endpoint of major CV events. For CV death, there was an almost threefold higher risk associated with the highest SDMA-quartile, although just borderline significant. In conclusion, our findings extend and corroborate that SDMA may be a marker for CV events and all-cause death.

We also showed that a high SDMA level in clinically stable RTR is independently predictive of renal graft loss. The possibility of SDMA being a separate risk factor for adverse renal outcomes in RTR has not previously been studied. Such et al. (29) looked for the prognostic role of both ADMA and SDMA in a heterogeneous population of 200 CKD-patients including 37 renal transplant patients. Their results indicate that an increased serum level of SDMA (but not ADMA) might be a predictor for the progression to end-stage renal disease.

Compared to the healthy general population, mean SDMA levels were elevated in our RTR cohort (31, 32). Our SDMA values were in concert with a review by Fleck et al. (33) demonstrating high levels of dimethylarginines in end-stage renal disease patients, SMDA levels decreasing after renal transplantation, though not reaching reference values for healthy subjects.

Possible mechanisms for the relationship between high SDMA and poor long-term outcomes are not well defined. Symmetric dimethylarginine is believed to be biochemically inert and eliminated solely by renal filtration. Hence, most of the difference in SDMA concentrations between various populations could be explained by its strong covariance with kidney function; an association first shown in a study on numerous populations could be explained by its strong covariance with high serum levels of SDMA and the development of infection or malignancy in RTR. A high proportion of cancers in RTR are lymphoproliferative and related to infections with Epstein-Barr virus, human herpesvirus 8, human papilloma virus or the hepatitis B and C viruses (41). Symmetric dimethylarginine residuals are shown to be important constituents of the Epstein-Barr virus–encoded nuclear antigen 2 which is responsible for growth transformations in B lymphocytes (42, 43), but whether virus-related malignancy is associated with increased plasma levels of SDMA is not known. Further studies are needed to elaborate on this as well as to investigate whether associations between SDMA and specific non-CV causes of death can be found in other populations.

The main analysis is based on the placebo arm of the study. This cohort was selected because we wished to avoid the risk of interactions with the active intervention (statin therapy), as it is possible that statin use could modify SDMA levels or the biologic actions of SDMA. Degree of endothelium-dependent vasodilatation achieved by simvastatin treatment was indeed shown to vary across levels of ADMA (44), pointing in the direction of a possible interaction between statins and dimethylarginines. Statin treatment might improve endothelial function both in RTR (45) and in the general population (46). Endothelial NO synthase is upregulated by statins (47). Possibly, some of the beneficial effects of statins are mediated through pathways involving dimethylarginines. In our study, SDMA was measured at baseline only, before the initiation of statin therapy. Hence, speculations on mechanisms involved in the suspected interaction between SDMA and statin treatment was beyond the scope of this article.

The prospective controlled design, the long time of follow-up, the large patient cohort, and the independent adjudication of all clinical endpoints are major strengths of our study. However, there are potential limitations which merit consideration. Although the statistics show significant associations between SDMA and mortality as well as the renal endpoint, the data do not prove a casual relationship. Furthermore, the study population, a cohort selected for entry into a clinical trial, is not necessarily fully representative of the general renal transplant population.

In conclusion, this is the first study to report that increased plasma levels of SDMA in stable RTR are significantly associated with future graft loss and all-cause mortality.

**MATERIALS AND METHODS**

**Study Design**

A post hoc analysis was performed using the data from RTR included in the ALERT trial. Study design with baseline data has previously been described (20). In short, this randomized, double-blind, placebo-controlled study examined the effect of fluvastatin (40–80 mg daily) on cardiac and renal outcomes in 2102 RTR. Inclusion criteria were stable RTRs aged 30–75 years having received a renal transplant more than 6 months before study start and having serum cholesterol in the range of 4.0–9.0 mmol/L (155–348 mg/dL). Exclusion criteria were ongoing statin therapy, familial hypercholesterolemia, an acute rejection episode in the last 3 months before inclusion or predicted life expectancy of less than 1 year. The original trial had a follow-up mean time of 5.1 years.
The ALERT study adhered to the International Conference on Harmonization guidelines for Good Clinical Practice and was conducted in accordance with the Declaration of Helsinki Principles. Written informed consent was obtained from all patients included, and the trial was approved by the local ethics committee at each participating center.

**Outcome Definitions**

Cardiac study outcome was the original primary endpoint in the ALERT trial—a composite endpoint of MACE, defined as time to cardiac death, non-fatal myocardial infarction or performed coronary revascularization procedure. Renal outcome was time to first renal graft loss (death-censored). General survival outcome was time to death from all causes. An independent clinical endpoint committee blinded to study drug allocation validated all endpoints (19, 20).

**Laboratory**

Baseline laboratory values of the ALERT trial have been reported previously (20). Symmetric dimethylarginine level was measured at baseline to be assessed as a risk factor at inclusion, a mean 5.1 years after transplantation. Reversed-phased high-performance liquid chromatography was used to measure SDMA level in frozen serum (−80°C) obtained from 925 of the 1,052 participants in the placebo arm, the last 127 samples missing at random. Estimated GFR (mL/min per 1.73 m²) was calculated by the formula from the Modification of Diet in Renal Disease study (48).

**Statistical Analysis**

In reviewing the literature before conducting our analyses, we found evidence of a possible effect of statins on endothelial function. Initial statistical analyses indicated a significant interaction between SDMA and ranitidine on markers of endothelial function. Subsequent analyses indicated a possible effect of statins on endothelial function. Initial analyses indicated a significant interaction between SDMA and randomized group. Consequently, for a clean approach, we used the placebo arm only in subsequent analyses.

Study participants were stratified into quartiles according to SDMA levels. For comparison of demographics and known risk factors across quartiles, P values for continuous variables were calculated using analysis of variance with linear trend, whereas for categorical variables, we used the chi-square linear test for proportions. The variables IL-6, hsCRP, triglycerides, ADMA, creatinine, proteinuria, time since last transplant, and time on dialysis were logarithmically transformed to correct for skewness.

Survival analyses were performed by Cox proportional hazard models. We calculated HRs with 95% CI by comparing the upper three quartiles to the first. We did not calculate HRs for SDMA as a continuous variable because the association between SDMA and outcomes appeared nonlinear when using categorical approach. Crude and multivariate adjusted HRs are presented. The multivariate model was adjusted for plausible confounders based on clinical knowledge and published literature. Collinearity between eGFR and SDMA was not a problem because standard errors remained of acceptable size when including both parameters in the statistics. Because eGFR as a continuous variable did not fully meet the proportional hazards assumption, we split this variable into quartiles for inclusion in subsequent statistical analyses. Remaining covariates fulfilled the proportionality assumptions according to the Schoenfeld residuals test. Possible competing risks between MACE (as study endpoint) and all-cause mortality (as competing risk endpoint) were examined by sensitivity analysis (49), including the subdistribution hazards method described by Fine and Gray (50).

All analyses were performed using SPSS version 18.0 (IBM, New York) and STATA version 11 (StataCorp, College Station, TX).

**Acknowledgments**

The authors thank all patients who participated in the ALERT study as well as all investigators, study nurses, and collaborators involved in the trial.

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**References**


