# Plasma levels of marine n-3 fatty acids and cardiovascular risk markers in renal transplant recipients

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**Abbreviations:**

| | |
|---|---|
| Adj. $R^2$: | Adjusted explained variance |
| β-coeff.: | Regression coefficient |
| CV: | Cardiovascular |
| DBP: | Diastolic blood pressure |
| DHA: | Docosahexaenoic acid |
| EPA: | Eicosapentaenoic acid |
| ESRD: | End-stage renal disease |
| fPG: | Fasting plasma glucose levels |
| HDL cholesterol: | High-density lipoprotein cholesterol levels |
| JELIS: | Japan Eicosapentaenoic acid Lipid Intervention Study |
| LDL cholesterol: | Low-density lipoprotein cholesterol levels |
| PUFA: | Polyunsaturated fatty acid |
| PWV: | Pulse wave velocity |
| RCTs: | Randomized controlled trials |

| | |
|---|---|
| rHR: | Resting heart rate |
| RTRs: | Renal transplant recipients |
| SBP: | Systolic blood pressure |
| SCD: | Sudden cardiac death |


# Abstract

**Background/Objective:** Cardiovascular disease is the leading cause of death after renal transplantation. Marine n-3 polyunsaturated fatty acids (PUFAs) exert potential cardio-protective metabolic effects and might reduce cardiovascular morbidity and mortality in renal transplant recipients (RTRs).

**Subjects/Methods:** In this cross-sectional study of 1990 Norwegian RTRs, transplanted between 1999 and 2011, associations between plasma phospholipid marine n-3 PUFA levels and various cardiovascular risk markers at ten weeks post-transplant were evaluated.

**Results:** Higher plasma marine n-3 PUFA levels were associated with lower resting heart rate, lower fasting plasma glucose levels, lower plasma triglyceride levels and higher plasma high-density lipoprotein (HDL) cholesterol levels. Plasma levels of eicosapentaenoic acid, but not docosahexaenoic acid showed a positive association with plasma HDL cholesterol levels. Plasma marine n-3 PUFA levels were not associated with plasma low-density lipoprotein cholesterol levels, pulse wave velocity or systolic and diastolic blood pressure. A negative association between plasma marine n-3 PUFA levels and cardiovascular mortality was weakened by additional adjustment for plasma triglyceride levels and resting heart rate. The ratio of n-6 to n-3 PUFAs showed similar associations with cardiovascular risk markers as absolute plasma marine n-3 PUFA levels.

**Conclusions:** This is the first study in RTRs showing that marine n-3 PUFAs are negatively associated with resting heart rate and fasting plasma glucose in addition to beneficial effects on plasma HDL cholesterol and triglyceride levels. Especially effects on autonomic nervous function and triglyceride metabolism might contribute to explain the lower cardiovascular mortality risk with higher plasma marine n-3 PUFA levels previously shown in this cohort.


# Introduction

Cardiovascular (CV) disease is the leading cause of death after renal transplantation,[1] and the CV mortality rate is twice that of the general population.[2] Traditional CV risk factors, such as hypertension, hyperglycemia and dyslipidemia, are more prevalent in renal transplant recipients (RTRs) compared with the general population.[3] In addition, several transplant-

specific factors, e.g. side-effects of immunosuppressive drugs and renal allograft function may influence the risk of CV disease.[3] Marine n-3 polyunsaturated fatty acids (PUFAs) have been reported to exert cardio-protective metabolic effects,[4] including lipid modulation,[5] reduced blood pressure,[6] impairment of artery calcification,[7] anti-inflammatory,[8] anti-arrhythmic [9] and anti-thrombotic effects.[10] The effects of marine n-3 PUFA intake from fish consumption or supplements have been extensively studied in non-transplant populations.[11] In renal transplantation, however, no previous observational study and only small randomized controlled trials (RCTs) have studied the effects of marine n-3 PUFAs on lipids and blood pressure,[12-15] and no study has to our knowledge investigated associations between marine n-3 PUFAs and heart rate, plasma glucose levels or measures of arterial stiffness.

The aim of this cross-sectional study was to examine associations between plasma levels of marine n-3 PUFAs and CV risk markers in a large cohort of RTRs.

## Materials and methods

### Study Design and Population

This single center study was performed at Oslo University Hospital, Rikshospitalet, which serves the Norwegian population of 5.2 million inhabitants (October 2015). Between the 30$^{th}$ of September 1999 and the 13$^{th}$ of October 2011, 2978 renal transplantations were performed in 2837 patients with end-stage renal disease (ESRD). At our center, we performed an in-depth clinical investigation and biobanking of plasma samples of all RTRs at ten weeks after transplantation. Patients not eligible for inclusion in the present study were either below the age of 16 years (n=78), transferred to local hospitals before ten weeks after transplantation (n=335), suffered graft loss (n=58) or death (n=21) within the first ten weeks after transplantation (Figure 1). Of the eligible patients, 344 patients were not offered a clinical visit ten weeks post-transplant due to understaffing at the laboratory. Furthermore, the amount of plasma collected was inadequate for individual fatty acid determination in another 11 patients, which leaves 1990 patients included in the study. Informed consent was obtained from all patients.

### Data Collection and Registry

Blood was sampled after a minimum of eight hours overnight fast. Routine blood samples were analyzed at a central biochemical department and laboratory test results were entered into a database, including plasma levels of triglycerides, plasma high-density lipoprotein (HDL) cholesterol levels, plasma low-density lipoprotein (LDL) cholesterol levels and fasting plasma glucose (fPG). Other blood samples were immediately frozen and stored at -80º C and were later on sent to The Lipid Research Center, Aalborg University Hospital for analysis of plasma phospholipid fatty acid composition by gas chromatography, as previously described.[16] In short, individual fatty acids were identified and quantified as weight

percentage of total plasma phospholipid fatty acids. Marine n-3 PUFA levels were defined as the sum of plasma phospholipid levels of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid.

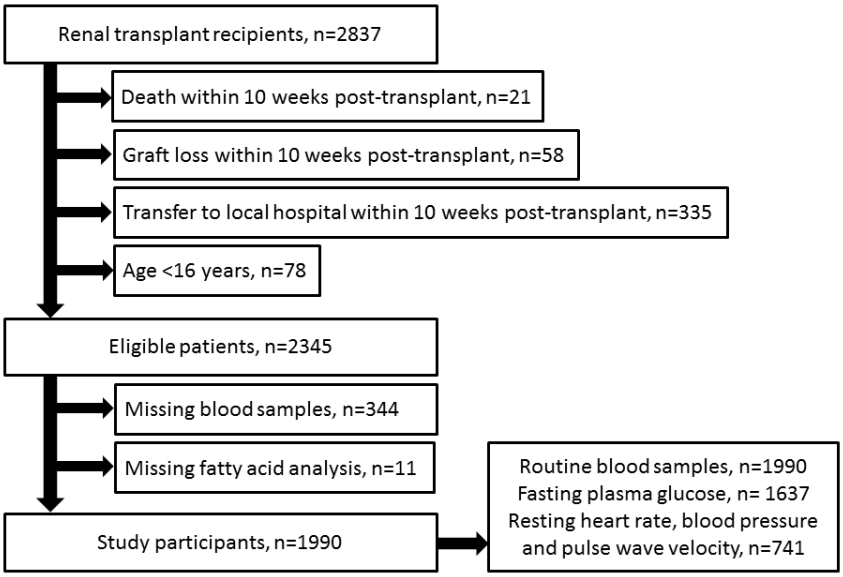


**Figure 1.** Inclusion of patients.

Registration of pulse wave velocity (PWV), resting heart rate (rHR), systolic blood pressure (SBP) and diastolic blood pressure (DBP) measurements started in 2007. From three automated oscillometric upper arm blood pressure measurements (CAS Medical Systems, Branford, CT, US) with the patients resting in a sitting position, the average of the two last measurements was recorded. PWV and rHR were measured with the patient in the supine position using SphygmoCor® version 8.0 (AtCor Medical, West Ryde, NSW, AUS) as previously described.[17] The measurements were performed at the morning of the clinical visit ten weeks post-transplant immediately after blood sampling. The patients had been instructed not to take any medication or drink coffee or tea prior to the measurements, as this might influence the results. We used estimated glomerular filtration rate (eGFR) according to the Modification of Diet in Renal Disease formula [18] as a measure of renal graft function and fPG levels were assessed in whole blood samples using a plasma calibrated HemoCue AB™ B-glucose Analyzer® (HemoCue, Ängelholm, Skåne, SWE). Clinical data were retrieved from medical records and from The Norwegian Renal Registry as previously described.[16]

During most of the study period, the immunosuppressive regimen consisted of methylprednisolone and basiliximab induction, followed by maintenance therapy with

mycophenolate, prednisolone and a calcineurin inhibitor (either cyclosporine A or tacrolimus).[16] Before 2007, nearly all patients received cyclosporine A. The choice of calcineurin inhibitor in recent years was mainly based on recipient age and whether the patient had diabetes mellitus or not. Statins were discontinued during the first three months after transplantation.[16]

**Statistical Analysis**

Demographical and clinical data at ten weeks post-transplant have previously been described in detail in this cohort.[16] Patient characteristics for selected variables across quartiles of marine n-3 PUFA levels are given in Table 1. Differences between groups were evaluated using logistic regression for binary variables, Mantel-Haenszel test of linear trend for other categorical data, Kruskal-Wallis test for time in dialysis therapy and linear regression for other continuous variables.

We evaluated unadjusted, recipient age- and gender adjusted and multivariable adjusted associations between marine n-3 PUFA levels and CV risk markers in linear regression analysis (Table 2). The table display unstandardized regression coefficients (β-coeff.) with corresponding 95% confidence interval, standardized regression coefficients, p-values and adjusted explained variance. We used $p<0.10$ as main inclusion criteria of variables in the final multivariable adjusted model. Variables included in the fully adjusted models are listed in the table legend. Candidate variables included: 1) Recipient related variables recorded at the time of transplantation: Recipient age and gender, atherosclerotic disease (a history of coronary artery, cerebrovascular and / or peripheral artery disease), diabetes mellitus, smoking status (current smoker, former smoker or life-long non-smoker), first or previous renal transplantation, preemptive transplantation (no previous renal transplantation or dialysis therapy) and time in dialysis therapy. In addition, donor age and type (living or deceased) as well as human leukocyte antigen DR mismatches between donor and recipient were evaluated. 2) Variables recorded at ten weeks post-transplant: Number of anti-hypertensive drugs, calcineurin inhibitor used (tacrolimus or cyclosporine A), body mass index, albumin, eGFR and marine n-3 PUFA levels.

Time in dialysis therapy was logarithmically transformed to obtain normal distribution. There was complete data on all candidate variables in 99.3% of patients.

We evaluated associations between EPA and DHA levels and CV risk markers using multivariable linear regression and univariate linear regression. EPA levels were logarithmically transformed to obtain normal distribution. Since EPA and DHA consumption are derived from the same sources, associations between EPA and DHA levels and CV risk markers were analyzed separately.

In addition, we evaluated associations between n-6 PUFA levels and the ratio of n-6 PUFA to n-3 PUFA levels and CV risk markers (Supplementary Appendix).

Finally, we used Cox proportional hazard regression to estimate age- and gender adjusted CV mortality hazard ratios for CV risk markers and evaluate the impact of these markers on multivariable adjusted associations between marine n-3 PUFA levels and CV mortality, as described in the Supplementary Appendix. PASW Statistics® version 17.0 (IBM, New York, NY, US) and STATA® version 13.0 (Stata Corp, College Station, TX, US) were used for the statistical analysis. The study was approved by the Regional Committees for Medical and Health Research Ethics in Norway and was performed in accordance with the Declaration of Helsinki (ClinicalTrials.gov number NCT02017990).

**Table 1. Patient characteristics across quartiles of marine n-3 polyunsaturated fatty acids for selected variables in renal transplant recipients**

| | All patients | Quartile 1 | Quartile 2 | Quartile 3 | Quartile 4 | Trend (p) | |
|---|---|---|---|---|---|---|---|
| Marine n-3 PUFA level, *wt%* | 1.35 – 23.87 | ≤ 6.20 | 6.21 – 7.94 | 7.95 – 10.02 | ≥ 10.03 | Unadj | Adj* |
| Number of patients | 1990 | 499 | 499 | 495 | 497 | | |
| EPA level, *wt%* | 2.23 (1.57) | 0.94 (0.33) | 1.46 (0.45) | 2.30 (0.66) | 4.23 (1.67) | <0.001 | <0.001 |
| DHA level, *wt%* | 5.11 (1.54) | 3.24 (0.74) | 4.64 (0.56) | 5.59 (0.63) | 6.94 (0.94) | <0.001 | <0.001 |
| n-6 PUFA level, *wt%* | 35.44 (3.32) | 38.40 (2.38) | 36.61 (2.10) | 34.96 (1.87) | 31.79 (2.63) | <0.001 | <0.001 |
| Recipient age, *years* | 51.6 (14.6) | 43.3 (14.0) | 51.6 (14.1) | 54.2 (13.3) | 57.3 (13.1) | <0.001 | N/A |
| Donor age, *years* | 47.2 (16.1) | 44.2 (15.7) | 46.1 (16.7) | 49.3 (15.3) | 49.5 (16.1) | <0.001 | 0.09 |
| Gender (Male), *%* | 66.9 | 69.0 | 64.8 | 65.6 | 68.3 | 0.90 | 0.48 |
| Diabetes mellitus, *%* | 18.2 | 23.5 | 21.1 | 15.3 | 13.0 | <0.001 | N/A |
| Current smoker, *%* | 16.0 | 23.5 | 18.0 | 12.9 | 9.4 | <0.001 | <0.001 |
| eGFR, *ml/min x 1.73m²* | 56.9 (18.8) | 64.5 (20.4) | 56.5 (18.3) | 54.2 (17.3) | 52.6 (16.9) | <0.001 | 0.05 |
| Tacrolimus, *%* | 23.1 | 35.5 | 22.2 | 19.4 | 15.3 | <0.001 | 0.56 |
| Cyclosporine A, *%* | 74.2 | 60.5 | 74.9 | 78.8 | 82.7 | <0.001 | 0.29 |

Categorical data are given as proportions and continuous data as mean and standard deviations. Differences in patient characteristics were evaluated using logistic regression for categorical data and linear regression for continuous data. Marine n-3 polyunsaturated fatty acid (*PUFA*) level was defined as the sum of plasma phospholipid levels of eicosapentaenoic acid (*EPA*), docosahexaenoic acid (*DHA*) and docosapentaenoic acid in weight percentage (*wt%*) of total plasma phospholipids. Abbreviations: *eGFR*: Estimated glomerular filtration rate. *N/A*: Not applicable. * After adjusting for recipient age and diabetes mellitus.

**Table 2. Associations between marine n-3 polyunsaturated fatty acid levels and cardiovascular risk markers in renal transplant recipients**

| **Univariate linear regression analysis** | | | | | |
|---|---|---|---|---|---|
| **Cardiovascular risk markers** | **Unstd. β-coeff. (95% CI)** | | **Std. β-coeff.** | **p** | **Adj. $R^2$** |
| Triglycerides, *mg/dL* | -5.563 | (-7.196, -3.930) | -0.149 | <0.001 | 0.02 |
| HDL cholesterol, *mg/dL* | 0.503 | (0.223, 0.782) | 0.079 | <0.001 | 0.01 |
| LDL cholesterol, *mg/dL* | 1.042 | (0.014, 2.069) | 0.045 | 0.05 | 0.002 |
| Fasting plasma glucose, *mg/dL* | -0.941 | (-1.546, -0.336) | -0.076 | 0.002 | 0.01 |
| Resting heart rate, *bpm* | -0.642 | (-0.945, -0.338) | -0.151 | <0.001 | 0.02 |
| Systolic blood pressure, *mmHg* | 0.561 | (0.119, 1.003) | 0.091 | 0.01 | 0.01 |
| Diastolic blood pressure, *mmHg* | -0.116 | (-0.394, 0.162) | -0.030 | 0.41 | 0.001 |
| Pulse wave velocity, *m/sec* | 0.117 | (0.035, 0.198) | 0.101 | 0.01 | 0.01 |
| **Age- and gender adjusted linear regression analysis** | | | | | |
| **Cardiovascular risk markers** | **Unstd. β-coeff. (95% CI)** | | **Std. β-coeff.** | **p** | **Adj. $R^2$** |
| Triglycerides, *mg/dL* | -6.335 | (-8.052, -4.618) | -0.169 | <0.001 | 0.03 |
| HDL cholesterol, *mg/dL* | 0.387 | (0.102, 0.673) | 0.061 | 0.01 | 0.07 |
| LDL cholesterol, *mg/dL* | -0.057 | (-1.128, 1.014) | -0.002 | 0.92 | 0.02 |
| Fasting plasma glucose, *mg/dL* | -0.971 | (-1.605, -0.336) | -0.078 | 0.003 | 0.01 |
| Resting heart rate, *bpm* | -0.594 | (-0.911, -0.277) | -0.140 | <0.001 | 0.04 |
| Systolic blood pressure, *mmHg* | -0.063 | (-0.497, 0.371) | -0.010 | 0.78 | 0.13 |
| Diastolic blood pressure, *mmHg* | 0.085 | (-0.202, 0.371) | 0.022 | 0.59 | 0.04 |
| Pulse wave velocity, *m/sec* | -0.070 | (-0.141, 0.002) | -0.061 | 0.06 | 0.31 |
| **Multivariable linear regression analysis** | | | | | |
| **Cardiovascular risk markers** | **Unstd. β-coeff. (95% CI)** | | **Std. β-coeff.** | **p** | **Adj. $R^2$** |
| Triglycerides, *mg/dL* [a] | -6.549 | (-8.228, -4.870) | -0.175 | <0.001 | 0.09 |
| HDL cholesterol, *mg/dL* [b] | 0.295 | (0.017, 0.574) | 0.046 | 0.05 | 0.14 |
| LDL cholesterol, *mg/dL* [c] | -0.581 | (-1.646, 0.484) | -0.025 | 0.29 | 0.06 |
| Fasting plasma glucose, *mg/dL* [d] | -1.042 | (-1.617, -0.412) | -0.083 | 0.001 | 0.04 |
| Resting heart rate, *bpm* [e] | -0.563 | (-0.864, -0.263) | -0.133 | <0.001 | 0.09 |
| Systolic blood pressure, *mmHg* [f] | -0.129 | (-0.555, 0.298) | -0.021 | 0.55 | 0.21 |
| Diastolic blood pressure, *mmHg* [g] | -0.035 | (-0.319, 0.248) | -0.009 | 0.81 | 0.11 |
| Pulse wave velocity, *m/sec* [h] | -0.033 | (-0.101, 0.036) | -0.028 | 0.35 | 0.40 |

Abbreviations: *HDL*: High-density lipoprotein. *LDL*: Low-density lipoprotein.

Marine n-3 polyunsaturated fatty acid level was defined as the sum of plasma phospholipid levels of eicosapentaenoic acid, docosahexaenoic acid and docosapentaenoic acid in weight percentage of total plasma phospholipids.

Associations between marine n-3 polyunsaturated fatty acid level and cardiovascular risk markers were estimated using univariate, recipient age- and gender adjusted and multivariable linear regression analysis, estimating unadjusted and adjusted associations between marine n-3 polyunsaturated fatty acid levels and cardiovascular risk markers. In addition to adjusted explained variance ($R^2$) for the final model, unstandardized regression coefficients (*Unstd. β-coeff.*) with corresponding 95% confidence intervals (*CI*), standardized regression coefficients (*Std. β-coeff.*) and p-values are shown.

In addition to marine n-3 polyunsaturated fatty acid levels, the following variables were included in the fully adjusted multivariable models (p<0.10 for inclusion): Recipient age (a-h), gender (a,b,e,h), donor age (d,g,h), atherosclerotic disease (a,b,c,d,f,h), diabetes mellitus (c,f,g,h), smoking status (f), body mass index (a,b,c,d,e,g,h), number of anti-hypertensive drugs (a,b,c,e,f,g), first renal transplant (a,c,e,g,h), time in dialysis therapy (a,b,g,h), living donor (b,e,g), number of human leukocyte antigen DR mismatches (e), use of tacrolimus (a,b,c,d,h), use of cyclosporine A (e,g,h), albumin (b,c,d,e,f) and estimated glomerular filtration rate (a,c).

## Results

Patient characteristics are presented in Table 1. Patients with high marine n-3 PUFA levels were older, had lower n-6 PUFA levels and lower prevalence of smoking and diabetes mellitus. Adjusted for recipient age and diabetes mellitus, other associations with marine n-3 PUFA levels were non-significant.

Table 2 shows unadjusted and adjusted associations between marine n-3 PUFA levels and CV risk markers. Marine n-3 PUFA levels were negatively associated with rHR, fPG and triglycerides and positively associated with HDL cholesterol in unadjusted, age- and gender adjusted and multivariable linear regression analyses (Table 2).

**Table 3. Associations between eicosapentaenoic acid and docosahexaenoic acid levels and cardiovascular risk markers in renal transplant recipients**

| | Eicosapentaenoic acid | | | | |
|---|---|---|---|---|---|
| **Cardiovascular risk markers** | **Unstd. β-coeff. (95% CI)** | | **Std. β-coeff.** | **p** | **Adj. $R^2$** |
| Triglycerides, *mg/dL* [a] | -83.362 | (-100.589, -66.135) | -0.213 | <0.001 | 0.10 |
| HDL cholesterol, *mg/dL* [b] | 10.658 | (7.806, 13.511) | 0.161 | <0.001 | 0.15 |
| LDL cholesterol, *mg/dL* [c] | -5.698 | (-16.738, 5.343) | -0.023 | 0.31 | 0.06 |
| Fasting plasma glucose, *mg/dL* [d] | -10.417 | (-17.006, -3.829) | -0.079 | 0.002 | 0.04 |
| Resting heart rate, *bpm* [e] | -6.933 | (-9.948, -3.918) | -0.161 | <0.001 | 0.10 |
| Systolic blood pressure, *mmHg* [f] | -0.934 | (-5.202, 3.337) | -0.015 | 0.67 | 0.21 |
| Diastolic blood pressure, *mmHg* [g] | 0.488 | (-2.326, 3.301) | 0.013 | 0.73 | 0.11 |
| Pulse wave velocity, *m/sec* [h] | -0.362 | (-1.046, 0.321) | -0.031 | 0.30 | 0.40 |
| | **Docosahexaenoic acid** | | | | |
| **Cardiovascular risk markers** | **Unstd. β-coeff. (95% CI)** | | **Std. β-coeff.** | **p** | **Adj. $R^2$** |
| Triglycerides, *mg/dL* [i] | -6.528 | (-9.835, -3.220) | -0.091 | <0.001 | 0.07 |
| HDL cholesterol, *mg/dL* [j] | -0.921 | (-1.460, -0.383) | -0.076 | 0.001 | 0.14 |
| LDL cholesterol, *mg/dL* [c] | -0.233 | (-2.315, 1.848) | -0.005 | 0.83 | 0.06 |
| Fasting plasma glucose, *mg/dL* [k] | -1.857 | (-3.080, -0.634) | -0.078 | 0.003 | 0.04 |
| Resting heart rate, *bpm* [l] | -0.624 | (-1.172, -0.072) | -0.081 | 0.03 | 0.08 |
| Systolic blood pressure, *mmHg* [f] | -0.344 | (-1.125, 0.437) | -0.031 | 0.39 | 0.21 |
| Diastolic blood pressure, *mmHg* [g] | -0.125 | (-0.645, 0.394) | -0.018 | 0.64 | 0.11 |
| Pulse wave velocity, *m/sec* [h] | -0.049 | (-0.175, 0.049) | -0.024 | 0.44 | 0.40 |

Abbreviations: *HDL*: High-density lipoprotein. *LDL*: Low-density lipoprotein.
Associations between plasma phospholipid levels of eicosapentaenoic and docosahexaenoic acids in weight percentage of total plasma phospholipids and cardiovascular risk markers were evaluated by multivariable linear regression analysis. Eicosapentaenoic levels were logarithmically transformed to obtain normal distribution. In addition to adjusted explained variance ($R^2$) for the final model, unstandardized regression coefficients (*Unstd. β-coeff.*), 95% confidence intervals (*CI*), standardized regression coefficients (*Std. β-coeff.*) and p-values are shown. In addition to either eicosapentaenoic or docosahexaenoic acid levels, the following variables were included in the fully adjusted multivariable models (p<0.10 for inclusion): Recipient age (a-l), gender (a,b,e,g,h,i,j), donor age (d,g,h,k), atherosclerotic disease (a,b,c,d,f,h,i,j,k), diabetes mellitus (c,f,g,h), smoking status (f), body mass index (a,b,c,d,e,g,h,i,j,k,l), number of anti-hypertensive drugs (a,b,c,e,f,g,i,j,l), first renal transplant (a,c,g,h,i,l), time in dialysis therapy (a,b,g,h,i,j), living donor (b,e,g,j,l), number of human leukocyte antigen DR mismatches (e,l), use of tacrolimus (a,b,c,d,h,i,j,k), use of cyclosporine A (e,g,h,l), albumin (b,c,d,e,f,j,k,l) and estimated glomerular filtration rate (a,c,i).

We also assessed associations with CV risk markers for EPA and DHA levels in separate multivariable linear regression models. Both were negatively associated with rHR, fPG and plasma triglyceride levels (Table 3). In contrast, EPA levels were positively associated and DHA levels negatively associated with HDL cholesterol, after adjustment for multiple covariates (Table 3). In comparison, univariate linear regression analysis showed a significant positive association with HDL cholesterol with EPA levels, while DHA levels were not associated with HDL cholesterol levels (Unstandardized β -0.226, Standardized β -0.019, p=0.41).

Using age- and gender adjusted Cox proportional hazard regression, we found a tendency towards higher CV mortality risk with plasma triglyceride levels (per 50 mg/dL increase; adjusted hazard ratio [HR] 1.07, 95% confidence interval [CI] 0.98 to 1.16) and rHR (per 5 bpm increase; adjusted HR 1.09, 95% CI 0.99 to 1.20), but not for fPG or HDL cholesterol. In multivariable Cox proportional hazard regression analysis, adjusting for variables likely to confound associations between marine n-3 PUFA levels and CV risk, there was a 10% lower risk of death due to CV disease per 1.0 wt% increase in marine n-3 PUFA levels (multivariable adjusted HR 0.90, 95% CI 0.82 to 0.98). The association was weakened by 1% after additional adjustment for triglycerides (multivariable adjusted HR 0.91) and 2% when adjusting for the impact of rHR.

# Discussion

The major finding in this study was that marine n-3 PUFA levels were negatively associated with rHR, fPG and plasma triglycerides, but positively associated with plasma HDL cholesterol. EPA levels were positively associated, while in contrast, levels of DHA were negatively associated with HDL cholesterol in multivariable linear regression analysis.

### Plasma lipids

A triglyceride-lowering effect from marine n-3 PUFA intake has been repeatedly shown in diverse patient populations,[4] including patients with ESRD [19] and RTRs.[12-14] Consistent with these reports, we found negative association with triglycerides for EPA, DHA and marine n-3 PUFA levels. In the Japan Eicosapentaenoic acid Lipid Intervention Study (JELIS), addition of 1.8 g of purified EPA to statin therapy, significantly lowered triglycerides and reduced the incidence of major CV events in dyslipidemic patients.[20] This is of particular interest in RTRs, who frequently have dyslipidemia and suffer high CV morbidity and mortality rates.[2]

Uremic dyslipidemia is characterized by high triglycerides and low HDL cholesterol.[21] Consistent with the findings in the present study, a statistically significant small increase in HDL cholesterol of 2.2 mg/dL was found in a meta-analysis of interventional studies on the effects of marine n-3 PUFA supplementation for a minimum of three months following renal transplantation.[12]

In multivariable linear regression, levels of EPA were positively and DHA negatively associated with plasma HDL cholesterol levels. A similar finding has previously been shown in an epidemiological study in a Norwegian cohort.[22] However, this finding is not consistent with reports from head-to-head comparative studies, where DHA treatment more efficiently increased HDL cholesterol than EPA treatment.[23] In univariate linear regression analysis, DHA levels were not significantly associated with HDL cholesterol, suggesting a neutral rather than a negative effect. We have no good explanation to the unexpected divergent associations with HDL cholesterol between EPA and DHA levels in the present study.

We found no association between marine n-3 PUFA, EPA or DHA levels and plasma LDL cholesterol levels, consistent with previous reports in RTRs.[12-14]

**Plasma glucose**

Despite the higher age group, patients with high marine n-3 PUFA levels had less often diabetes mellitus in our cohort. Epidemiological studies from different regions with diverse dietary habits report equivocal and inconsistent results, with both positive, neutral and negative associations between fish consumption and the risk of developing type 2 diabetes mellitus.[24] Reports from European studies indicate that consumption of lean fish does not lower the risk of developing type 2 diabetes mellitus, as opposed to consumption of fatty fish.[25] A large American study reported a higher risk of developing type 2 diabetes mellitus with higher consumption of fish and seafood, while opposite associations have been found in Chinese and Japanese populations.[26-28] Consumption of marine n-3 PUFAs in our cohort was about five times higher than in the American cohort [26,29] and other dietary habits, like sugar consumption, also likely differ between the cohorts and could influence the results.[30] Meta-analysis of prospective studies that used fatty acid analysis for determination of fish consumption showed a non-significant negative tendency towards lower risk of developing type 2 diabetes mellitus with higher levels of marine n-3 PUFAs.[24] Also, this meta-analysis did not include a recent large Nordic study, which found a significant negative association between plasma marine n-3 PUFA levels and the risk of incident type 2 diabetes, even after adjustment for diet and life-style factors.[31]

We found a negative association between marine n-3 PUFA levels and fPG in non-diabetic RTRs. Hepatic glycogenolysis and as well as hepatic and renal gluconeogenesis are key determinants of fPG in the morning. These processes are regulated by substrate availability (gluconeogenesis) and hormonal effects (insulin and glucagon).[32,33] One could speculate that marine n-3 PUFAs in some way affect one or more of these pathways. Mechanistically, reports from animal models and humans suggest that marine n-3 PUFAs might improve glucose homeostasis through maintenance of insulin growth factor 1 secretion, improving peripheral insulin responses, increased glucagon-like peptide 1 release, increasing insulin secretion from pancreatic beta-cells and increased adiponectin levels, preventing insulin resistance linked to obesity.[34]

There is no evidence for improved glycemic control or reduced CV morbidity or mortality after marine n-3 PUFA supplementation in non-transplant patients with type 2 diabetes mellitus or impaired glucose tolerance.[34,35] However, the effects of marine n-3 PUFAs have not been studied in patients with posttransplantation diabetes mellitus.

In non-diabetic populations, the effects of marine n-3 PUFA supplementation on insulin secretion and sensitivity differ between patient populations.[36-38] Interventional studies with adequate sample size and follow-up time are needed to clarify whether high intake of marine n-3 PUFAs might improve glucose homeostasis in organ transplant recipients.

**Resting heart rate, blood pressure and pulse wave velocity**

Marine n-3 PUFA intake is found to reduce resting heart rate and increase heart rate variability in several patient populations,[9] including cardiac transplant recipients.[39] We have previously reported a negative association between marine n-3 PUFA levels and CV mortality risk in this cohort.[16] The risk of sudden cardiac death was particularly low in patients with high compared with low marine n-3 PUFA levels,[16] and a similar association has also been shown in patients with ESRD.[40] Consistently, we found a negative association between marine n-3 PUFA levels and rHR, which might infer a reduced risk of CV disease. Uremic autonomic neuropathy is very frequent in ESRD patients, which partly may help explain the high incidence of SCD.[40] Normalization of renal function by renal transplantation ameliorates or reverses autonomic dysfunction within the first three to six months after transplantation.[41] In the present study, measurements of rHR were performed at ten weeks post-transplant, by which time autonomic dysfunction still might inhibit beneficial effects of marine n-3 PUFAs on autonomic function. Both EPA and DHA make cardiomyocytes less excitable through different effects on cardiac ion channels,[39] which also contribute to lower rHR and increase heart rate variability.[9] In this cohort, levels of EPA were more strongly associated with rHR than DHA. Purified EPA supplementation in the JELIS trial did not significantly lower the incidence of SCD,[20] but this might be due to the high background consumption of marine fatty acids in a Japanese population.[42] In contrast, low-dose combined EPA and DHA supplementation in a large Italian RCT significantly lowered the risk of SCD.[43]

Marine n-3 PUFA intake has also been reported to lower both SBP and DBP.[6,44] A meta-analysis of RCTs in RTRs reported a minor, but statistically significant effect on DBP and a trend towards lower SBP after marine n-3 PUFA supplementation.[12] We failed to find any significant associations between marine n-3 PUFA levels and blood pressure in the present study. However, nearly all patients received anti-hypertensive therapy and we have only information about the number, but not the type and dose of anti-hypertensive drugs used, which obviously might be a confounding factor. Moreover, reduced blood pressure after consumption of marine n-3 PUFAs has only been clearly demonstrated in interventional studies with supplemental doses > 2 g/day,[44] which is considerably higher than the amount of marine n-3 PUFAs normally obtained through the diet, even in an Norwegian cohort.[22] Despite the high background consumption of fish, a marked reduction in SBP and DBP has

previously been shown with high-dose marine n-3 PUFA supplementation in a Norwegian cohort.[6]

PWV is a measure of arterial stiffness and has been reported to be associated with an increased mortality risk in RTRs.[17] Some studies have demonstrated a preventive effect of marine n-3 PUFA supplementation on progression of arterial stiffness.[45] In the present study, we found no association between marine n-3 PUFA levels and PWV. This could possibly be due to a threshold effect, since low-dose marine n-3 PUFA supplementation has not shown any effect on PWV.[46] Limited data on use of anti-hypertensive drugs might have influenced the association between marine n-3 PUFA levels and PWV.

### n-6 to n-3 polyunsaturated fatty acid ratio

Levels of n-6 PUFAs were inversely associated with marine n-3 PUFA levels, negatively associated with recipient age positively associated with fPG and rHR. Associations between n-6 to n-3 PUFA ratio and CV risk markers were a little weaker than associations with absolute plasma marine n-3 PUFA levels (Supplemental Table S1).

### The impact of cardiovascular risk markers on the associations between marine n-3 polyunsaturated fatty acid levels and cardiovascular mortality

We have previously reported a negative association between marine n-3 PUFA levels and CV mortality risk in this cohort,[16] which could at least partially be explained by the findings in the present study. Associations between marine n-3 PUFA levels and CV mortality risk were less pronounced after adjustment for either rHR or plasma triglyceride levels, suggesting that marine n-3 PUFAs might offer some cardio-protection after renal transplantation through effects on autonomic nervous function and very low lipoprotein synthesis and / or triglyceride clearance.[4] It should, however, be noted that the lower CV mortality risk with higher marine n-3 PUFA levels remains mostly unexplained and that neither triglycerides nor rHR were significantly associated with CV mortality risk after adjustment for age and gender.

Marine n-3 PUFAs might also lower CV mortality risk in RTRs trough other cardio-protective mechanisms, including effects on inflammation or fibroblast growth factor 23 levels.[8,47,48] Unfortunately, we have no data to substantiate these assumptions.

### Strengths and limitations

The insights provided by the present study are limited by the cross-sectional study design. We assumed that the plasma fatty acid composition determined at a single time-point in patients who participated in the study were representative for their average fatty acid profile over time. Recent reports from Scandinavian populations have shown a strong coherence in marine n-3

PUFA levels over time and a good correlation with fish consumption.[49,50] Unfortunately, we have no information on dietary habits and lifestyle factors like physical activity, educational level and socioeconomic status, which might be associated with marine n-3 PUFA intake. The lower prevalence of diabetes mellitus and smoking in patients with high marine n-3 PUFA levels suggest that higher marine n-3 PUFA levels could be a marker of a healthy life-style. Although diabetes mellitus and smoking were included as covariates in the multivariable linear regression analysis, the impact of these variables on associations between marine n-3 PUFA levels and CV risk markers might not have been fully adjusted. Any residual confounding from a healthier life-style associated with fish consumption cannot be ruled out. At our center, all RTRs receive regular dietary advice from a nutritionist after transplantation, including advice to eat fish at least twice a week. Possibly, high marine n-3 PUFA levels reflect good adherence to dietary advice from health personnel. Patients who were adherent to dietary advice might also be more likely to have a good drug adherence, which clearly would influence the results.

During the ten weeks between transplantation and measurements performed in this study, the patients had gone from a uremic phase, via a post-operative phase to a new stable post-transplantation phase. Their dietary habits might have been influenced by post-operative complications as well as intended dietary changes motivated and made possible by the renal transplantation. Slowly progressive CV markers, e.g. PWV, are not likely to respond to dietary changes on the short run. Dietary changes after the time of transplantation might therefore underestimate associations between marine n-3 PUFA levels and CV risk markers in the present study.

We have data on the number, but not the type or dose of anti-hypertensive drugs, and lack data on the use of glucose-lowering and anti-thrombotic drugs. The majority of patients in this Norwegian study were Caucasian, hence results may not be applicable to other ethnic groups and the results may not apply to regions with a lower intake of marine n-3 PUFAs.

This study also have several strengths, including a well-defined and large study population, a high inclusion rate from a single center representing an entire country and several CV risk factors included as candidate variables in the multivariable linear regression models.

In summary, plasma marine n-3 PUFA levels were associated with lower resting heart rate, lower levels of fasting plasma glucose, lower plasma triglyceride levels and higher plasma HDL cholesterol levels. Since both plasma triglyceride levels and resting heart rate influenced the association between plasma marine n-3 PUFA levels and cardiovascular mortality risk, we speculate that triglyceride-lowering and anti-arrhythmic effects of marine n-3 PUFAs might prevent deaths due to cardiovascular disease after renal transplantation. Associations with plasma HDL cholesterol levels differed between plasma levels of the marine n-3 fatty acids EPA and DHA. Further clinical investigations are needed to clarify whether dietary supplementation of marine n-3 PUFAs might have clinical beneficial effects in renal transplant recipients, and whether EPA and DHA exert different effects on cardiovascular risk factors in these patients.

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# Plasma levels of marine n-3 fatty acids and cardiovascular risk markers in renal transplant recipients. Supplementary appendix.

## Statistical approach to analyze the impact of cardiovascular risk markers on the association between plasma marine n-3 polyunsaturated fatty acid levels and cardiovascular mortality

We used Cox proportional hazard regression to 1) Estimate age- and gender adjusted CV mortality hazard ratios for CV risk markers included in this study and 2) Evaluate the impact of CV risk markers on associations between marine n-3 PUFA levels and CV mortality, by including these CV risk markers as covariates in a multivariable Cox regression model that also included the following variables: Recipient age, gender, donor age, diabetes mellitus, smoking status, use of tacrolimus, use of cyclosporine A, n-6 PUFA levels and eGFR. These variables were selected based on univariate associations with marine n-3 PUFA levels (Table 1). For fPG only non-diabetic patients were included in the analysis, and for rHR, SBP, DBP and PWV only patients transplanted after the 1st of January 2007 were included. The observational time started at the time of transplantation and surviving patients were censored at the 1st of January 2015. We have previously described the CV mortality definition used in this cohort.[1] A formal hypothesis test (Schoenfeld residuals) was used to check model assumptions. Potential competing risk and choice of a Cox regression model over a subdistribution hazard model have previously been discussed.[1,2] We were particularly interested in CV risk markers associated with marine n-3 PUFA levels; plasma triglycerides, HDL cholesterol, fPG and rHR, and results are presented in the main article. Plasma LDL cholesterol, SBP, DBP and PWV were not associated with marine n-3 PUFA levels and adjustment for these factors had no impact on the association between marine n-3 PUFA levels and CV mortality.

## Associations with cardiovascular risk markers for n-6 polyunsaturated fatty acid levels and n-6 to n-3 polyunsaturated fatty acid ratio

We evaluated associations between n-6 PUFA levels (the sum of arachidonic, linoleic, gammalinoleic, eicosadienoic, dihomogammalinolenic and adrenic acid levels) and the ratio of n-6 PUFA to n-3 PUFA levels (the sum of marine n-3 PUFAs and alpha linolenic acid) and CV risk markers in multivariable linear regression analysis. We also performed similar analyses including only arachidonic and linoleic acids as n-6 PUFAs and excluded the n-3 PUFA alpha linolenic acid derived from plants. This approach produced nearly identical results (data not shown).

There was an inverse association between n-6 PUFAs and marine n-3 PUFAs, indicating different dietary profiles. As opposed to marine n-3 PUFA levels, n-6 PUFA levels were negatively associated with recipient age. In multivariable linear regression, n-6 PUFA levels were positively associated with fPG and rHR (Supplemental Table S1). Associations with

plasma triglyceride levels, rHR and fPG were a little weaker for n-6 to n-3 PUFA ratio compared with plasma marine n-3 PUFA levels, and n-6 to n-3 PUFA ratio was not associated with plasma HDL cholesterol levels. We therefore concluded with no additional value of using the n-6 to n-3 PUFA ratio, consistent with previous reports,[3] suggesting that direct effects of marine n-3 PUFAs might be of greater importance for CV risk markers than the balance between n-6 PUFAs and n-3 PUFAs. We have previously presented associations with patient survival for both marine n-3 PUFA levels and n-6 to n-3 PUFA ratio in this cohort, where we also failed to demonstrate any additional value of using the n-6 PUFA to n-3 PUFA ratio instead of or in addition to plasma marine n-3 PUFA levels.[1]

**Supplemental Table S1.**

Associations between n-6 polyunsaturated fatty acid levels of and n-6 to n-3 polyunsaturated fatty acid ratio and cardiovascular risk markers in renal transplant recipients

| **n-6 polyunsaturated fatty acids** | | | | | |
|---|---|---|---|---|---|
| **Cardiovascular risk markers** | **Unstd. β-coeff. (95% CI)** | | **Std. β-coeff.** | **p** | **adj. $R^2$** |
| Triglycerides, *mg/dL* [a] | 0.981 | (-0.534, 2.496) | 0.030 | 0.20 | 0.06 |
| HDL cholesterol, *mg/dL* [b] | -0.012 | (-0.263, 0.239) | -0.002 | 0.92 | 0.13 |
| LDL cholesterol, *mg/dL* [c] | 0.423 | (-0.532, 1.377) | 0.021 | 0.39 | 0.06 |
| Fasting plasma glucose, *mg/dL* [d] | 0.699 | (0.137, 1.260) | 0.063 | 0.02 | 0.04 |
| Resting heart rate, *bpm* [e] | 0.372 | (0.101, 0.642) | 0.099 | 0.01 | 0.08 |
| Systolic blood pressure, *mmHg* [f] | 0.144 | (-0.233, 0.521) | 0.026 | 0.45 | 0.21 |
| Diastolic blood pressure, *mmHg* [g] | -0.011 | (-0.263, 0.241) | -0.003 | 0.93 | 0.11 |
| Pulse wave velocity, *m/sec* [h] | 0.035 | (-0.025, 0.096) | 0.035 | 0.26 | 0.40 |
| **n-6 to n-3 polyunsaturated fatty acid ratio** | | | | | |
| **Cardiovascular risk markers** | **Unstd. β-coeff. (95% CI)** | | **Std. β-coeff.** | **p** | **adj. $R^2$** |
| Triglycerides, *mg/dL* [i] | 5.304 | (2.874, 7.735) | 0.101 | <0.001 | 0.07 |
| HDL cholesterol, *mg/dL* [b] | -0.161 | (-0.561, 0.240) | -0.018 | 0.43 | 0.13 |
| LDL cholesterol, *mg/dL* [c] | 0.167 | (-1.365, 1.700) | 0.005 | 0.83 | 0.06 |
| Fasting plasma glucose, *mg/dL* [j] | 1.279 | (0.354, 2.203) | 0.071 | 0.01 | 0.04 |
| Resting heart rate, *bpm* [k] | 0.609 | (0.204, 1.014) | 0.108 | 0.003 | 0.08 |
| Systolic blood pressure, *mmHg* [f] | 0.389 | (-0.191, 0.968) | 0.047 | 0.19 | 0.21 |
| Diastolic blood pressure, *mmHg* [g] | 0.053 | (-0. 336, 0.442) | 0.010 | 0.79 | 0.11 |
| Pulse wave velocity, *m/sec* [h] | 0.031 | (-0.062, 0.124) | 0.020 | 0.51 | 0.40 |

Associations between plasma phospholipid levels of n-6 polyunsaturated fatty acids and the ratio of n-6 polyunsaturated fatty acid levels to n-3 polyunsaturated fatty acid levels and cardiovascular risk markers were evaluated by multivariable linear regression analysis. In addition to adjusted explained variance ($R^2$) for the final model, unstandardized regression coefficients (*Unstd. β-coeff.*), 95% confidence intervals (*CI*), standardized regression coefficients (*Std. β-coeff.*) and p-values are shown. In addition to n-6 polyunsaturated fatty acid levels and the n-6 to n-3 polyunsaturated fatty acid ratio the following variables were included in the fully adjusted final models (p<0.10 for inclusion): Recipient age (a-k), gender (a,b,e,h,i,k), donor age (d,g,h,j), atherosclerotic disease (a,b,d,f,h,i,j), diabetes mellitus (c,f,g,h), smoking status (f), body mass index (a,b,c,d,e,g,h,i,j,k), number of anti-hypertensive drugs (a,b,c,e,f,g,i,k), first renal transplant (a,g,h,i), time in dialysis therapy (a,b,g,h,i), living donor (b,e,g,k), number of human leukocyte antigen DR mismatches (e,k), use of tacrolimus (a,b,c,d,h,i,j),

use of cyclosporine A (e,g,h,k), albumin (b,c,d,e,f,j,k) and estimated glomerular filtration rate (a,c,i). *HDL*: High-density lipoprotein. *LDL*: Low-density lipoprotein.

## References


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