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Elements of Mediterranean diet improve oxidative status in blood of kidney graft recipients

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Patients were fully informed as to the study objectives and benefits, and provided written consent prior to enrolment. The study protocol was approved by the Committee on Human Research at the Pomeranian Medical University, Szczecin, Poland. An intensification of free-radical reactions may contribute to accelerated atherosclerosis in kidney graft recipients. We examined the effect of a Mediterranean-type diet (MD) on the oxidative status of the plasma and erythrocytes of kidney graft recipients. Two patient groups were formed: a study group consuming the MD diet and a control group with a low-fat diet. C-reactive protein levels in plasma, oleic acid C18 : 1*n*-9 and linoleic acid C18 : 2*n*-6 concentrations in triacyloglycerols were determined. To determine the oxidative status, we measured the concentrations of α-tocopherol in plasma, the content of thiobarbituric acid-reactive species (TBARS) in plasma and erythrocytes, and the activities of superoxide dismutase, catalase and glutathione peroxidase in erythrocytes. In the MD group, the activities of erythrocyte enzymes changed significantly: those of superoxide dismutase increased ($P<0{\cdot}001$ after 6 months), catalase decreased ($P<0{\cdot}001$ after 6 months) and glutathione peroxidase decreased ($P<0{\cdot}05$ after 2 months). The oleic acid content of triacylglycerols was increased ($P<0{\cdot}006$) whereas that of linoleic acid was decreased ($P<0{\cdot}00005$), α-tocopherol levels remaining unchanged. TBARS in plasma were decreased after 6 months of MD ($P<0{\cdot}05$). No significant correlations were observed between TBARS, oleic acid, linoleic acid and α-tocopherol levels in plasma. MD appears to protect the erythrocytes against the action of free radicals, as reflected in the modified activities of some enzymes regulating the oxidative status of these blood cells.

Mediterranean diet: Kidney: Transplantation: Superoxide dismutase: Catalase: Glutathione peroxidase: Oleic acid: Linoleic acid: C-reactive protein: α-Tocopherol

Accelerated atherosclerosis caused by lipid disorders associated with immunosuppressive therapy remains a major problem compromising survival in transplant recipients (Berman *et al.* 2003). Hyperlipaemia enhances lipid peroxidation and activates free-radical reactions (Lindholm *et al.* 1995; Berman *et al.* 2003). Protection against the action of free radicals in plasma and erythrocytes is provided by antioxidants of the non-enzymatic system (e.g. vitamin E, GSH) and 'antioxidant' enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) (Debry, 1980).

Vitamin E is one of the most important and efficient antioxidants in plasma. Thanks to its reductive properties, it inhibits the oxidation of polyunsaturated fatty acids protecting cells (e.g. erythrocytes) against free-radical lesions (Debry, 1980). GSH is the main hydrophilic antioxidant of erythrocytes, overwhelmingly present in its reduced form with just 0·2 % GSSG. GSSG is reduced by NADPH to GSH in a reaction catalysed by glutathione reductase (Hussain *et al.* 1995).

SOD contains Cu and Zn as cofactors and frees the cell from superoxide anions (Fang *et al.* 2002). H_2O_2 generated by SOD is neutralised at high concentrations by catalase (Mates *et al.* 1999) and at low concentrations by GPx (Mezzetti *et al.* 1990).

A functioning renal graft contributes to the normalisation of peroxidation reactions in plasma (Reimold, 1980). This beneficial outcome can be strengthened through dietary interventions aimed at delivering antioxidants to the patient (Assmann *et al.* 1997). The Mediterranean diet (MD) has long been recommended for its antiatherosclerotic properties (Expert Panel, 1993). Among effects of the MD is a suppression of lipoprotein peroxidation (Parthasarathy *et al.* 1990) and a normalisation of endothelial (Perez-Jimenez *et al.* 1999; Carluccio *et al.* 2003) and platelet (Larsen *et al.* 1999; Williams, 2001) function. Kidney graft recipients consuming the MD have decreased plasma concentrations of triacylglycerols (TG), total cholesterol and LDL (Barbagallo *et al.* 1999).

Elevated plasma levels of C-reactive protein (CRP), a recognised marker of inflammation, correlate with accelerated atherosclerosis and increased risk of death due to stroke (Illingworth, 1999; Ford & Giles, 2002).

The aim of this work was to determine the effect of simple MD principles on the rate of inflammatory reactions (as measured by CRP levels in plasma) and oxidative status in the plasma and erythrocytes of kidney graft recipients.

Abbreviations: CRP, C-reactive protein; GPx, glutathione peroxidase; MD, Mediterranean diet; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive species; TG, triacylglycerols.

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Material and methods

Patient data

A low-fat diet is standard therapy for graft recipients. Our own patients started a low-fat diet at the time of grafting or before transplantation (dialysed patients). Kidney graft recipients with stable graft function, non-smokers, were randomly assigned to two groups: the study and control groups. Patients in the study group had been on a low-fat diet for 29·1 (0–32) months before the study, whereas patients from the control group had been on a low-fat diet for 29·5 (1–38) months before the study. In this study, the diet was changed only for the study (MD) group, the control group patients continuing the low-fat diet.

A qualified dietician instructed patients on either the MD or the low-fat diet. The study group consisted of 21 patients whose diet complied with MD principles in the form of 4-week, all-day menus. The control group included sixteen patients on a low-fat diet, isocaloric with the study diet (isocaloric intake being determined by estimation of kcal/kg and then verified by dietary record and computer software). Patients were asked to take home and complete a 24 h diet diary. The diet diary booklet contained menus, pages to record foods, and photographs of food that depicted portion choices for a common food item. The dietician indicated that the patient should record the food brand and portion size. The amounts consumed were recorded in household units, by volume or by measuring with a ruler. Each subject was interviewed about their dietary pattern in the previous month. Nutrient analyses were carried out using the corresponding Polish food table (Kuhanowicz *et al.* 2001) and the nutrient database developed in Poland (Dietetyk, Jumar, Poland). In addition, dietary compliance was assessed with the chromatographic measurement of oleic C18 : 1*n*-9 and linoleic acid C18 : 2*n*-6 content in plasma TG.

Patients continued their immunosuppressive (cyclosporin, prednisone, azathioprine) and hypotensive (diuretics, calcium channel blockers, selective inhibitors of angiotensin I convertase) regimens. No antilipaemics were administered, either prior to or during the study. Measurements were made before and after 1, 2 and 6 months of the experiment. Details of the patients are presented in Table 1.

Main assumptions concerning diet

Daily energy intakes were 10 460 kJ (2500 kcal) for men and 8368 kJ (2000 kcal) for women in the study and control group. In the study group, daily energy intake was attributed as follows: 47 % carbohydrates, 38 % fatty acids (including 10 % saturated, 22 % monounsaturated and 6 % polyunsaturated species) and 15 % protein (Table 2). Cholesterol and fibre supply was 165 (SD 17) mg/d and 47 (SD 9)g/d respectively. The dominating fatty acid was oleic acid from olive oil and erucic acid-poor rapeseed oil. Patients consumed 30 ml cold-pressed olive oil per day (fresh salads) and prepared their cooked meals exclusively with rapeseed oil. Patients consumed approximately 30 g daily of products rich in α-tocopherol and α-linolenic acid C18 : 3*n*-3 (grains, flax-seed, nuts). The carbohydrate component contained less glucose (low glycaemic index). Allowable products included cereals, pulses, wholemeal bread, vegetables (fresh and cooked), oat flakes (cooked) and spaghetti. The patients were advised to consume fresh vegetables with every meal. The daily animal protein consumption was 25–50 g for men and 23–46 g for women, representing one-third of the total protein. No additional vitamin supplementation was offered.

In the control group, daily energy intake was attributed as follows: 57 % carbohydrates, 26 % fatty acids and 17 % protein (Table 2). Cholesterol and fibre supply was 257 (SD 15) mg/d and 24 (SD 13) g/d respectively. The carbohydrate component was poor in cellulose and rich in starch (white bread, potatoes, rice). The fat content was lower than in the study group, with polyunsaturated (mainly C18 : 2*n*-6) fats dominating. Questionnaires revealed that butter and sunflower oil were the main source of fat in this group. Daily animal protein consumption was higher than in the study group (approximately 70 g for men and 50 g for women), whereas the consumption of fruit and fresh vegetables was lower. The diet was not supplemented with vitamins.

Plasma lipids

Total, LDL and HDL cholesterol fractions, and TG, were measured in fasting plasma with commercial test kits (bio-Merieux, Marcy l'Etoile, France).

Fluorometric determination of thiobarbituric acid-reactive substances in plasma and erythrocytes

The method for measuring thiobarbituric acid-reactive substances (TBARS) was based on the reaction between malondialdehyde and thiobarbituric acid (Wasowicz *et al.* 1993). The coloured complex was measured with a spectrofluorometer (LS 50 B; Perkin-Elmer Wellesley, MA, USA).

Table 1. Clinical data of patients at the time of enrolment

	Study Group			Control Group		
	Mean	Range	SD	Mean	Range	SD
Number of patients		21*			16†	
Age (years)	41		12.5	4.6		9.5
Pre-graft dialysis history (months)	2.84	0–32		18.2	1–38	
Time from grafting to enrolment (months)	10.7	2–24		11.3	1–31	
Creatinine in serum (mg/dl)	1.62		0.57	1.73		0.054
Systolic blood pressure (mmHg)	134.7		17.4	139.4		22.6
Diastolic blood pressure (mmHg)	84.2		9.6	8		13.5
BMI (kg/m^2)	25.0		4.1	26.2		4.4
Immunosuppressive and hypotensive treatment		+			+	

* 6 females, 15 males.
† 6 females, 10 males.

Table 2. Basic features of the diet in the study and control groups (Mean values and standard deviations)

	Study Group		Control Group	
	Mean	SD	Mean	SD
Meals per day	5		3	
Dietary protein content (%)	15		17	
Dietary carbohydrate content (%)	47		57	
Dietary fatty acid content (%)	38		26	
Carbohydrate content (g/d)	307	16	369	55
Starch consumption (g/d)	204.3	28	313	18
Fibre consumption (g/d)	47	9	24	13
Cholesterol consumption (mg/d)	165	17	257	15

C-reactive protein test (highly sensitive)

The ELISA automated microparticle enzyme immunoassay test kit (Euroimmun, Lubeck, Germany), providing a quantitative *in vitro* assay for highly sensitive CRP, was used. Measurements were carried out in EDTA-anticoagulated plasma.

Chromatography of triacylglycerols and fatty acids

Plasma (1 ml) was extracted according to the method of Folch *et al.* (1957). Aliquots were separated on TLC plates (Merck, Hauar, Germany), and the TG band was scraped off, saponified with methanolic KOH and methylated in the presence of BF_3 (15 min, 65°C). Fatty acid methyl esters were extracted with hexane, concentrated under N_2 and injected into a gas chromatograph (8500; Perkin-Elmer) equipped with a 30 m capillary column (RTX 5; Restek, Bellefonte PA, USA). Chromatographic conditions were: injector temperature 220°C, detector temperature 260°C, oven temperature 50–245°C, ramp rate was 4°C/min and oven temperature was then held at 245°c for 5 min. Fatty acids were identified by comparing their retention times with those of high-purity standards (>99 %, Sigma-Aldrich USA, Cayman, USA). Peak areas were integrated using the Chromed Pl software (Chromed, Poznan, Poland).

Determination of α-tocopherol in plasma

Concentrations of α-tocopherol were measured using HPLC. Blood was anticoagulated with EDTA and centrifuged (850 ***g***, 10 min) at 4°C. Plasma was stored at − 80°C in the dark in plastic tubes until assayed. Volumes of 250 μl plasma were mixed with 250 μl ethanol containing 0·5 mg/l *trans*-β-apo-8′-carotenal (internal standard) and 100 mg/l di-tet-butryl-4-methylphenol (antioxidant) and vortexed for 30 s. Next, 1 ml hexane was added, and the mixture was vortexed for 2 min (Epler *et al.* 1993). Following centrifugation (1000 ***g***, 0°C, 5 min), 800 μl of the upper layer was transferred to a glass tube and evaporated to dryness under N_2. The sediment was dissolved in 150 μl ethanol with intense vortexing for 30 s. The temperature at all stages was 0°C, and samples were protected from light.

Reversed phase HPLC was carried out with an HP1050 chromatograph (Hewlett-Packard; now Agilent PaloAlto, CA, USA) equipped with a UV/VIS variable wavelength detector and thermostatted column compartment. A 201TP54 (250 × 4·6 mm) column and a 201GK54 precolumn (Vydac, Warsaw, Poland, USA) were mounted. Tocopherols were separated isocratically at 21°C. The mobile phase was methanol–tetrahydrofurane–acetonitrile (88·5:7·5:5·0 v/v) flowing at 1·5 ml/min. All reagents were of HPLC-grade purity (LiChrosolv; Merck). The detection of α-tocopherol was achieved at 295 nm, whereas the internal standard was detected at 450 nm. The sample volume was 20 μl and the time between runs 10 min. Calibration was performed with an ethanolic solution of α-tocopherol (Fluka; Sigma-Aldrich, Poznan, Poland) added instead of ethanol.

Determination of activities of superoxide dismutase E.C. 1.11.1.1, *catalase* E.C. 1.11.1.6 *and glutathione peroxidase* E.C. 1.11.1.9

Heparinised blood (5 ml) samples were collected from each patient. Blood was centrifuged at 804 g for 10 min, the supernatant was discarded, and the erythrocyte pellet was rinsed three times in PBS pH 7·4. Erythrocytes were suspended in deionised water (200 μl erythrocytes + 100 μl water) and rapidly frozen to − 80°C. Haemolysates from thawed erythrocytes were adjusted to 5 g/dl Hb and used for enzyme determinations. All reagents for spectrophotometric measurements were from Sigma-Aldrich.

SOD activity was determined with a spectrophotometric method (Mirsa & Fridovich, 1972). The enzyme was extracted from the haemolysate with chloroform–ethanol (3:5 v/v) and intense shaking for 5 min. The extract was centrifuged (3200 **g**, 10 min, 4°C). Supernatant (50 μl) was added to 2·85 ml bicarbonate buffer (0·05 M, pH 10·2) containing 100 μl adrenaline (16·5 mg in 0·1 M HCl). The absorbance change during 10 min was measured with a UV/VIS Lambda 40P (Perkin-Elmer) spectrophotometer at 320 nm and 30°C against a blank containing the buffer and SOD extract only.

Catalase activity was determined with the method of Aebi (1984). Immediately prior to measurement, the haemolsyate was diluted 1:500 with phosphate buffer (50 mM, pH 7·0). The removal of H_2O_2 by catalase from samples containing 2 ml diluted haemolysate and 1 ml H_2O_2 (30 mM) was assessed, against a blank containing haemolysate and 1 ml buffer, by measuring the change in absorbance over 30 s. Measurements were made at 240 nm and 20°C. Catalase activity was expressed as k/gHb, where k is first-order reaction rate constant.

GPx activity was determined according to Wendel (1981). Haemolyates (3 mg Hb/ml) were supplemented with the transforming reagent (4·5 mM KCN and 0·45 nM $K_3[Fe(CN)_6]$). Following an incubation of 5 min at room temperature, 0·5 ml haemolysate was added to the reaction solution containing phosphate buffer (50 mM, pH 7·0), glutathione reductase (0,30/ml), GSH (10 mM) and NADPH (2·5 mM) and incubated for 10 min at 37°C. The reaction was started with tert-buthyl hyperoxide (t-BOOH) (12 mM), and the linear decrease in NADPH concentration over 4 min was followed by measuring the change in absorbance against a blank free of NADPH. Measurements were carried out at 340 nm and 37°C.

Measurement of glutathione concentration in erythrocyte suspension

The concentration of GSH was measured spectrophotometrically (Beutler *et al.* 1963). A volume of 100 μl washed frozen erythrocytes in suspension were added to 900 μl distilled water. The resulting haemolyate was precipitated by vortexing with a solution containing metaphosphoric acid, EDTA and NaCl. Centrifugation (3214***g***, 4°C, 10 min) followed, and phosphate buffer (0·3 M, pH 7·5) containing dithio-*bis*-2-nitrobenzene was added to the supernatant. Extinction of the product formed by aithio-

Table 3. Patient data (Mean values and standard deviations) including total cholesterol, HDL cholesterol, LDL cholesterol and triacylglycerol concentrations in plasma

	Study group				Control group			
	At the begining		After 6 months		At the beginning		After 6 months	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Total cholesterol (mg/dl)	230	58	210	53*	265	37	259	51
HDL cholesterol (mg/dl)	51	15	52	16	67	22	64	20
LDL cholesterol (mg/dl)	123	38	112	33	143	25	135	34
Triacylglycerols (mg/dl)	194	76	152	63†	201	67	207	81

* Significant difference in relation to starting value (Wilcoxon's test) $P<0{\cdot}05$.
† Significant difference in relation to starting value (Wilcoxon's test) $P<0{\cdot}0007$.

bis-2-nitrobenzene and GSH proportionally to GSH concentration was measured at 412 nm and 25°C.

Statistics

As the distribution in most cases deviated from normal (Shapiro–Wilk test), non-parametric tests were used. Significance was first checked with Friedmann's ANOVA, and significant results were next subjected to the Wilcoxon matched-pairs test. Groups were compared with the Mann–Whitney U test. Correlations were checked with Spearman's rank correlation coefficient.

Results

Plasma concentrations of lipids (total cholesterol, LDL cholesterol, HDL cholesterol and TG) in the study and control groups are shown in Table 3.

Total cholesterol levels in the study group decreased from 230 mg/dl to 210 mg/dl during the study ($P<0{\cdot}05$), whereas in the control group cholesterol levels remained unchanged. No significant differences in LDL and HDL cholesterol levels were noted over time or between groups. A decline in the TG level was observed in the study group (from 194 mg/dl to 152 mg/dl; $P<0{\cdot}0007$). In the control group, TG levels did not change.

Concentrations of CRP in graft recipients are presented in Table 4. No statistically significant changes induced by the diet or differences between groups of patients were revealed.

The content of oleic and linoleic acid in plasma triacylglycerols of both groups is also presented in Table 4. The concentration of oleic acid increased in the study group during the experiment ($P<0{\cdot}006$ after 1, 2 and 6 months; Table 4), whereas no statistically significant changes were noted in the control group. Significant differences between both groups in the concentration of oleic acid appeared after 1 month of the diet and persisted thereafter ($P<0{\cdot}00005$ after 6 months; Table 4). The linoleic acid content in the triacylglycerols of the study group fell during the experiment. In contrast, a significant increase was noted in the controls (Table 4). The MD produced a statistically significant difference in the content of linoleic acid between the study and control groups ($P<0{\cdot}00\,005$ after 6 months).

The plasma concentration of TBARS prior to the experiment was similar in both groups. In the study group, concentrations after 6 months of the diet approached the values seen in healthy individuals (0·78 μmol/l; E. Stachowska *et al.* unpublished results). In the control group, TBARS increased, and the group ended the experiment with significantly higher concentrations than the study group ($P<0{\cdot}05$; Table 4).

Rising concentrations of oleic acid and decreasing concentrations of linoleic acid in TG in the study group did not correlate with TBARS levels in the plasma at any point during the experiment. No correlation was ascertained in the control group either.

No statistically significant changes or differences between groups in α-tocopherol plasma concentrations and concentration normalised to blood lipids (Table 5) were observed. Levels of α-tocopherol did not correlate with TBARS in either group of patients.

Significant changes in the activities of antioxidant enzymes in the erythrocytes were observed (Table 6). SOD activity increased in both groups. In the study group, the change was statistically significant after 2 ($P<0{\cdot}05$; n 18) and 6 ($P<0{\cdot}001$; n 19) months of the diet. In the control group, the change was significant after 6 months ($P<0{\cdot}01$; n 11). Values were higher in the study (S) than the control (C) groups after 1 month ($P<0{\cdot}05$; nS 20, nC 15; Mann–Whitney U-test), and this discrepancy remained noticeable until the end of the experiment ($P<0{\cdot}01$; nS 19, nC 11). Catalase activity changed only in the study group, revealing lower values after 6 months of the diet ($P<0{\cdot}001$; n17). GPx activity was decreased in the study group after 1 ($P<0{\cdot}05$; n 17) and 2 ($P<0{\cdot}05$; n 17) months of the diet. No statistically significant differences were noted after 6 months (n 16). In the control group, no significant changes in GPx activity were seen throughout the experiment (Table 6). No statistically significant difference in the GSH content of erythrocytes was noted between both groups (Table 6). We believe that this finding can be attributed to the use of sedimented frozen erythrocytes (differences could be revealed by studying fresh erythrocytes). However, this erythrocyte material also served to determine activities of GPx and the remaining enzymes.

The TBARS content of the erythrocytes did not exhibit any significant fluctuations in either group.

No significant correlations between α-tocopherol concentration in plasma and SOD or catalase activities in the erythrocytes of either group were seen. Nevertheless, a positive correlation of α-tocopherol with GPx activity was noted when the groups were pooled (Table 7). An even stronger correlation was revealed between GPx activity and the ratio of α-tocopherol to TG (in both groups), reflecting the content of α-tocopherol in the TG fraction (Table 7). The same correlation was observed in the pooled groups after 2 months of the experiment. No such correlation could be seen in the case of SOD and catalase.

Table 4. Changes in the concentrations of C-reactive protein, oleic acid, linoleic acid and thiobarbituric acid-reactive species (TBARS) in the plasma of kidney graft recipients

(Mean values and standard deviations)

	Study group								Control group							
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Time (months)	0		1		2		6		0		1		2		6	
C-reactive protein (mg/l)	1·05	0·9	1·31	0·1	1·76	2·0	2·07	2·2	1·10	0·9	3·47	4·0	1·49	1·3	2·84	4·8
Oleic acid in triacylglycerols (μg/ml serum)	0·020	0·001	0·028	0·007‡**	0·030	0·005‡¶	0·029	0·005‡**	0·019	0·0014	0·018	0·004	0·019	0·004	0·019	0·005
Linoleic acid in triacylglycerols (μg/ml serum)	0·015	0·005	0·014	0·004‡**	0·012	0·002‡**	0·011	0·003‡**	0·015	0·006	0·020	0·005*	0·020	0·005‡	0·020	0·005*
TBARS (μmol/l)	0·83	0·1	0·77	0·1	0·75	0·1*	0·78	0·1‖	0·78	0·08	0·88	0·2	0·91	0·3	1·01	0·2

Statistically significant differences (Wilcoxon's test) within groups in comparison to starting values: *$P<0·05$; ‡$P<0·01$; §$P<0·001$.
Statistically significant differences (Mann–Whitney *U*-test) between groups at given time points: ‖$P<0·05$; ¶$P<0·01$; **$P<0·001$.

Table 5. α-Tocopherol concentration in plasma normalised to blood lipids concentration

(Arithmetic means ± S.D.)

	Study group								Control group							
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Time (months)	0		1		2		6		0		1		2		6	
α-Tocopherol concentration (mg/l)	14·6	3·9	18·4	0·6	16·5	1·7	14·9	2·2	14·6	5·5	13·9	1·7	14·7	4·4	17·6	4·2
α-Tocopherol/: TG (mg/mg)	0·0067	0·0017	0·0065	0·0019	0·0075	0·0007	0·0092	0·0014	0·0055	0·0003	0·0052	0·0019	0·0048	0·0023	0·0073	0·0016
α-Tocopherol/: (TCH+TG) (mg/mg)	0·0028	0·00001	0·0031	0·0002	0·0030	0·001	0·0030	0·00008	0·0028	0·0001	0·0028	0·0008	0·0026	0·0011	0·0034	0·00006
α-Tocopherol/: LDL CH (mg/mg)	0·0091	0·0027	0·1003	0·0024	0·0085	0·0017	0·0092	0·0034	0·0097	0·0007	0·0116	0·0015	0·0105	0·0042	0·0123	0·0026

TG, triacylglycerol; TCH, total cholesterol; LDL CH, LDL cholesterol.
No statistically significant differences were noted between groups during the study.

Table 6. Changes in the activities of antioxidative enzymes, GSH and thiobarbituric acid-reactive species (TBARS) in the erythrocytes of kidney graft recipients (Arithmetic means± S.D.)

	Study group						Control group					
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Time (months)	0		1		2		0		1		6	
TBARS/ER (nmol/g Hb)	31·6	7·9	20	11·2	27	16·5	43	18	31	14	35	22
SOD (*U*/g Hb)	1550	414	1676	361‖	1882	271*‖	1417	447	1419	459	1918	177‡
CAT (k/g Hb)	411	64	414	51	328	48	371	73	391	73	305	69
GPx (*U*/g Hb)	12·6	3·3	9·17	2·6*	10·9	2·6*	11·6	2·3	11·3	2·8	10·3	3
GSH (μmol/gHb)	0·97	0·5	1·45	0·5	1·47	0·4	1·28	0·3	1·04	0·4	1·12	0·2
					1·45	0·2	1·28§ ¶	0·3	1·19	0·3		

SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.
Statistically significant differences (Wilcoxon's test) within groups in comparison to starting values: *$P<0.05$; ‡$P<0.01$; §$P<0.001$.
Statistically significant differences (Mann–Whitney *U*-test) between groups at given time points: ‖$P<0.05$; ¶$P<0.01$; **$P<0.001$.

Table 7. Spearman rank correlation coefficients (Rs) for α-tocopherol content in plasma and triacylglycerols and glutathione peroxidase activity at the beginning and after 2 months of the study

Correlated parameters	*n*	Rs	Significance
α-Tocopherol concentration in plasma at the beginning of the study with Glutathione peroxidase activity at the beginning of the study	28	0·42	$P<0.027$
α-Tocopherol concentration in triacylglycerols at the beginning of the study with Glutathione peroxidase activity at the beginning of the study	27	0·64	$P<0.0002$
α-Tocopherol concentration in triacylglycerols after 2 months with glutathione peroxidase activity after 2 months	27	0·45	$P<0.035$

Discussion

Accelerated atherosclerosis caused by dyslipaemia and hypercholesterolaemia following organ transplantation is one of the factors adversely affecting graft survival (Barbagallo *et al.* 1999; Salen *et al.* 1999). The MD, rich in olive oil, natural cereals, vegetables and fruits (Visoli *et al.* 1998), has emerged as an interesting possibility for reducing atherosclerotic risk. Olive oil as the chief source of fat protects the vasculature against atherogenesis, exerts an antioxidant action and suppresses the release of arachidonic acid from the lipid constituents of cell membranes (Expert Panel, 1993). In countries of the Mediterranean basin notable for their consumption of olive oil, the rate of atherosclerosis is the lowest in Europe (Expert Panel, 1993).

CRP is a recognised marker of inflammation. Levels of CRP are elevated in patients with atherosclerosis (Ford & Giles, 2002). Several large-scale prospective studies have demonstrated that high-sensitivity CRP is a strong independent predictor of future myocardial infarction and stroke among healthy subjects (Ridker, 2001). Elevated CRP levels in plasma correlate with an increased risk of death due to stroke. With levels of CRP above 5·5 mg/l, the risk is 1·67 higher than at levels below 2·1 mg/l; moreover, this is independent of other risk factors (Ridker, 2001; Ford & Giles, 2002). In our study, however, CRP levels in plasma were not affected by the diet (see Table 4).

It is known from the literature that plasma lipoproteins are protected against oxidative stress. Protection is afforded by three components. Polyphenols from the diet (e.g. from cold-pressed olive oil) and monounsaturated fatty acids (including oleic acid) incorporated into the lipoprotein structure effectively suppress oxidative modifications (Caruso *et al.* 1999; Ford & Giles, 2002). Rising levels of oleic acid in the study group (see Table 4) were accompanied by lower concentrations of TBARS, reflecting suppressed peroxidation. In the control group oleic acid levels did not increase and, consequently, significantly higher TBARS levels were noted in this group at the end of the experiment. No significant correlations could be revealed between TBARS, oleic acid, linoleic acid and α-tocopherol levels when Spearman's rank coefficients were applied (data not shown). This finding suggests that the key role in protecting lipoproteins against peroxidation is played by some factors present in the MD other than oleic acid or α-tocopherol. Such a role has been ascribed by several authors to polyphenols con-

tained, for example in cold-pressed olive oil (Caruso *et al.* 1999; Galli & Visioli, 1999).

The cardioprotective properties of the diet largely depend on the right profile (qualitative and quantitative) of fatty acids (Lichtenstein, 1997; Kris-Etherton, 1999; Kris-Etherton *et al.* 2001), which in turn determines which fatty acids will be incorporated into plasma lipoproteins. The findings of de Lorgeril *et al.* (1994, 1999) led to the hypothesis that the content of oleic (C18 : 1*n*-9), linoleic (C18 : 2*n*-6) and α-linolenic (C18 : 3*n*-3) acid in cholesterol esters is important for reducing mortality. Higher concentrations of oleic acids, much lower concentrations of linoleic acid and unexpectedly high concentrations of α-linolenic acid have been disclosed in inhabitants of Crete (which has the lowest mortality from coronary heart disease in Europe) compared with other European cohorts (Sandker *et al.* 1993).

We focused attention on the content of oleic and linoleic acids in plasma. The content of linoleic acid in TG demonstrated a downward course in the study group, unlike the control group, in which a slight increase was seen. Although there was no correlation between the reduced content of linoleic acid and the inhibition of plasma peroxidation processes (as reflected by TBARS levels), decreased plasma levels of linoleic acid may act as a cardioprotective factor due to the reduced availability of this acid for the synthesis of *n*-6 eicosanoids (Ulbricht & Southagate, 1991).

We observed that patients on the MD demonstrated increased activities of SOD, a key antioxidant enzyme in the erythrocyte. This blood cell is equipped with a robust antioxidant system consisting of antioxidant enzymes like SOD, catalase and GPx with relatively high activities, as well as non-enzymic components (GSH, α-tocopherol, carotenoids; Smith & Berkseth, 1990; Siems *et al.* 2000). GSH concentrations are known to rise sharply after kidney grafting compared with controls and preoperative values (Hussain *et al.* 1995).

We used TBARS in the plasma and erythrocytes as a marker of oxidative stress. Although TBARS in erythrocytes did not change significantly in either group throughout the study (see Table 6), TBARS in plasma was significantly reduced in the MD group after 6 months of the diet (see Table 4).

However, we failed to show a reduction in free-radical reactions in the erythrocytes, although we have no doubt that this reduction took place in the plasma of MD patients. This finding allowed us to conclude that the MD may protect against the generation of free radicals in kidney graft recipients.

The activities of SOD, catalase and GPx changed during the experiment (see Table 6). The rising activity of superoxide dismutase may reflect a lower rate of free-radical reactions in the erythrocyte. It is believed that elevated SOD activity reflects a sparing effect on that enzyme when free radical reactions have been quenched (Vanella *et al.* 1983; Paul *et al.* 1993; Chen *et al.* 1997). SOD catalyses the dismutation of O_2^- to H_2O_2 and O_2. A decreased activity of SOD following haemodialysis is due to consumption of the enzyme involved in the breakdown of O_2^- and in other reactions with reactive oxygen species such as $OH^\bullet$ and $^1O_2$ generated during interactions between O_2^- and H_2O_2 (D'Haese & DeBroe, 1996).

The other two enzymes, catalase and GPx, catalyse the breakdown of H_2O_2 produced by SOD. Unlike the situation in the control group, we observed significantly decreased activities of catalase (after 6 months of the diet) and GPx in the study group. Presumably, the MD contributed to a reduction in the production of H_2O_2 and in this way suppressed both enzymes for which H_2O_2 serves as a substrate.

Changes in SOD activities observed in both groups may be attributed to environmental factors. Our study was carried out during summer and early autumn when the availability of fruits and vegetables may favour an improvement in the oxidative status of the plasma in study and control patients alike. It is worth mentioning that it was only the activity of SOD that changed in control patients, with no affect on the other two antioxidative enzymes: catalase and GPx.

It is worth noting the positive correlation between the activity of GPx and the plasma concentration of α-tocopherol. According to the literature (Sneddon *et al.* 2003), α-tocopherol protects the cellular membrane of the erythrocyte and prevents haemolysis. This correlation revealed by us irrespective of the diet may reflect a protective function of α-tocopherol as a factor that stabilises the environment of GPx (Table 7).

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