Increased calcium intake reduces plasma cholesterol and improves vasorelaxation in experimental renal failure


Increased calcium intake reduces plasma cholesterol and improves vasorelaxation in experimental renal failure. Am J Physiol Heart Circ Physiol 285: H1882–H1889, 2003; 10.1152/ajpheart.01148.2002.—Chronic renal failure (CRF) is associated with abnormal lipid metabolism and high prevalence of vascular complications. Calcium salts are commonly used in CRF as phosphate binders. Increased calcium intake may also lower plasma cholesterol and beneficially influence vascular tone. Therefore, we investigated the influence of increasing dietary calcium from 0.3% to 3.0% for 8 wk after 5/6 nephrectomy (NTX) on plasma cholesterol and mesenteric resistance vessel tone in male Sprague-Dawley rats. The groups were Sham, Sham-Calcium, NTX, and NTX-Calcium (n = 10–11). Blood pressure was modestly elevated after NTX, whereas the plasma creatinine, urea nitrogen, phosphate, and parathyroid hormone levels were clearly increased. The high-calcium diet suppressed plasma phosphate and parathyroid hormone but was without effect on blood pressure. The NTX resulted in 1.6-fold elevation in plasma total cholesterol and 40% reduction in high-density-to-low density lipoprotein ratio (HDL/LDL). However, the lipid profile in NTX rats on the high-calcium diet did not differ from sham-operated controls. The endothelium-mediated relaxations induced by acetylcholine were impaired in NTX rats, whereas the response was normalized by a high-calcium diet. No differences in vasorelaxations by the endothelium-independent vasodilator nitroprusside were detected. In conclusion, improved vasorelaxation after a high-calcium diet could be due to reduced plasma total cholesterol and ameliorated HDL/LDL ratio, although decreased plasma phosphate and parathyroid hormone may also play a significant role in the vascular effects of increased calcium intake.
CRF, and whether these changes are reflected as alterations in the control of resistance artery tone.

**METHODS**

*Animals and experimental design.* Male Sprague-Dawley rats (*n* = 42; *n* = 18 in additional extended follow up) were housed two to a cage with access to water and food (AnalyCen; Lindköping, Sweden). Systolic blood pressures (rat age 8 wk) were measured at +28°C with a tail-cuff blood pressure meter (model 129; IITC; Woodland Hills, CA). Surgical procedures were performed under ketamine/diazepam anesthesia (75 and 2.5 mg/kg ip, respectively). In the 5/6 nephrectomized (NTX) groups, the upper and lower poles of the left kidney were cut off, and the right kidney was removed (38). In the sham groups, both kidneys were decapsulated. Antibiotics (60 mg/kg metronidazole and 225 mg/kg cefuroxim) were given postoperatively, and pain was relieved with buprenorphine (0.2 mg/kg subcutaneously) three times a day during the first 3 days. Four weeks after NTX (rat age 12 wk), the rats were divided into four groups (Sham, Sham-NTX, 5/6-NTX, and NTX-Calcium; *n* = 10–11) of equal mean systolic blood pressures and body weights. The Sham and NTX rats continued on chow containing 0.3% calcium, whereas the Sham-Calcium and NTX-Calcium rats were on 3% calcium. Extra calcium was supplied as the carbonate salt, and otherwise the chows were identical.

High calcium intake continued for 8 wk, and 24-h fluid consumption and urine output were measured at *study week 20*. The rats were weighed and anesthetized (1.3 g/kg urethane), and blood samples from cannulated carotid artery for plasma electrolyte, creatinine, urea nitrogen, phosphorus, PTH, 1,25-dihydroxyvitamin D [1,25(OH)2D3], cholesterol, and hemoglobin measurements were drawn into chilled CRF, and whether these changes are reflected as alterations in the control of resistance artery tone.

**Methods**

*Calcium, NTX, and NTX-Calcium;* 12 wk), the rats were divided into four groups (Sham, Sham-NTX, 5/6-NTX, and NTX-Calcium; *n* = 10–11) of equal mean systolic blood pressures and body weights. The Sham and NTX rats continued on chow containing 0.3% calcium, whereas the Sham-Calcium and NTX-Calcium rats were on 3% calcium. Extra calcium was supplied as the carbonate salt, and otherwise the chows were identical.

High calcium intake continued for 8 wk, and 24-h fluid consumption and urine output were measured at *study week 20*. The rats were weighed and anesthetized (1.3 g/kg urethane), and blood samples from cannulated carotid artery for plasma electrolyte, creatinine, urea nitrogen, phosphorus, PTH, 1,25-dihydroxyvitamin D [1,25(OH)2D3], cholesterol, and hemoglobin measurements were drawn into chilled tubes, and for ionized Ca2+ and hemoglobin measurements were drawn into chilled tubes, and for ionized Ca2+ and hemoglobin measurements.

Eighteen more rats in three groups (Sham, NTX, and NTX-Calcium; *n* = 6 in all) were followed for a further 8 wk, so that the follow up after subtotal nephrectomy or sham operation was altogether 20 wk. Then plasma creatinine and aortic tissue calcification were examined.

The Mulvany multimyograph (model 610A, J.P. Trading; Aarhus, Denmark) was employed for vascular studies. In this isometric system the force transducer is directly linked to a computer with Myodaq software (J.P. Trading). Vessels were mounted as ring preparations (length 1.9 mm) on two 40-gauge stainless steel wires, each of which was attached to a myograph jaw. The physiological salt solution (PSS; pH 7.4) contained (in mM) 119.0 NaCl, 25.0 NaHCO3, 1.11 glucose, 1.6 CaCl2, 4.7 KCl, 1.2 KH2PO4, and 1.2 MgSO4, and aerated with 95% O2-5% CO2. Normalization of the vascular preparations was then performed so that the internal diameter of the vessel was set at 90% of that obtained when exposed to an intraluminal pressure of 100 mmHg in the relaxed state (19).

The experimental design of the study was approved by the Animal Experimentation Committee of the University of Tampere, Finland, and the Provincial Government of Western Finland Department of Social Affairs and Health, Finland. Moreover, the investigation conforms to the American Physiological Society’s “Guiding Principles for Research Involving Animals.”

**Morphological analyses of small arteries and thoracic aorta.** Morphology of small arteries was examined with a pressure myograph (Living Systems Instrumentation; Burlington, VT) as previously reported, and the development of myogenic tone was inhibited by Ca2+-free solution containing 30 mmol/L EDTA (30). Three 5-μm aortic cross sections from the 18 rats that were followed for another 8 wk were stained with von Kossa stain and processed for light microscopic evaluation. An expert who was blinded to the treatment of the rats quantified aortic calcification from three aortic cross sections from each animal. The calcification was expressed as the percentage of the aortic cross section.

**Mesenteric arterial responses in vitro.** Presence of intact endothelium in vascular preparations was confirmed by a clear relaxation to 1 μM ACh in 5 μM norepinephrine (NE)-precontracted rings and the absence of endothelium by the lack of this response. The endothelium was removed by perfusing air through the lumen.

**Arterial contractions to NE and endothelium-mediated relaxations in endothelium-intact small arteries.** Contractions to NE (0.1–10 μM) were first cumulatively determined. Thereafter relaxations to ACh (1 nM-10 μM) were examined in arteries precontracted with 5 μM NE, which resulted in ~80% of the maximal contractile response attained in both groups. The rings were allowed a 30-min equilibration period in PSS between each cumulative response.

**Arterial contractions to KCl and endothelium-independent relaxations to exogenous nitric oxide (endothelium-denuded small arteries).** Contractions to increasing concentrations of KCl were determined, and in solutions containing high concentrations of KCl (20–125 mM) NaCl was replaced with KCl on an equimolar basis. Relaxations to 1 nM-100 μM nitroprusside [nitric oxide (NO) donor] were examined in rings precontracted with 5 μM NE. The preparations were allowed a 30-min equilibration period in PSS between each relaxation.

**Plasma electrolytes, urea nitrogen, phosphorus, PTH, 1,25(OH)2D3, cholesterol, creatinine, protein, pH, and hemoglobin measurements.** Plasma sodium and potassium concentrations were measured by potentiometric direct dry chemistry, urea nitrogen by colorimetric enzymatic dry chemistry, and phosphorus by colorimetric end-point dry chemistry analyzer (Vitros 950, Johnson & Johnson Clinical Diagnostics; Rochester, NY). Plasma PTH was measured by immunometric assay (IRMA, Immutopics; San Clemente, CA). 1,25(OH)2D3 was quantified by radioreceptor assay (24). Total fasting plasma triglycerides, and total and high-density lipoprotein (HDL) cholesterol concentrations were analyzed with the use of an autoanalyzer (Cobas Integra 700, Hoffman-La-Roche, Basel, Switzerland). LDL cholesterol concentration was calculated with the use of Friedewald’s formula. Plasma creatinine was determined by the kinetic colorimetric assay according to Jaffe and plasma protein by colorimetric end-point measurement according to Biuret (Cobas Integra analyzer). Plasma-ionized Ca2+ and pH were measured with the use of an ion-selective electrode (model 634 Ca2+/pH Analyzer, Ciba Corning Diagnostics; Sudbury, UK). Hemoglobin was determined by photometric analysis with the use of a cyanide-free hemoglobin reagent (H2, Technicon Instruments; Tarrytown, NY).

**Data presentation and analysis of results.** The maximal wall tensions to NE and KCl were expressed in milli-Newtons per millimeter. The EC50 for NE and KCl in each ring was calculated as a percentage of maximal response and presented as the negative logarithm (pD2), which was used in the statistical analysis. The relaxations in response to ACh and nitroprusside were presented as a percentage of preexisting contractile force. The EC50 for ACh in each ring was also presented as the negative logarithm (pD2), and scatterplots were generated to emphasize the possible relationships.
between plasma levels of cholesterol, LDL, HDL-to-LDL (HDL/IDL ratio), PTH, phosphate, ionized calcium, and the pD2 values for ACh in subtotally nephrectomized rats. The results from sham-operated animals were excluded from the scatterplots because increased calcium intake had no effect on arterial tone in these animals.

Statistical analysis was carried out by one- and two-way ANOVA supported by the Bonferroni test when carrying out pairwise comparisons between the test groups. ANOVA for repeated measurements was applied for data consisting of repeated observations at successive time points. Results are expressed as means ± SE, and the differences were considered significant when \( P < 0.05 \). Unless otherwise indicated, the \( P \) values in the text refer to ANOVA for repeated measurements. In the subtotally nephrectomized rats, correlation analyses were also performed between plasma levels of creatinine, urea, and the pD2 values for ACh. Spearman’s correlation coefficient \( (r) \); two tailed) was calculated, which was considered significant when \( P < 0.05 \).

**Drugs.** The following drugs were used: ketamine (Parke-Davis Scandinavia; Solna, Sweden), cefturoxime, diazepam (Opec Pharma; Espoo, Finland), metronidazole (Braun; Melsungen, Germany), buprenorphine (Reckitt and Colman; Hull, UK), acetylcysteine, norepinephrine bitartrate (Sigma; St. Louis, MO), and sodium nitroprusside (Fluka Chemie; Buchs, Switzerland). Stock solutions were created by dissolving the compounds in distilled water. All solutions were freshly prepared before use and protected from light.

**RESULTS**

**Blood pressure, body and heart weights, total renal mass, drinking fluid, and urine volumes.** Systolic blood pressures and body weights in individual study groups did not differ during the 12-wk follow up. However, when analyzed by two-way ANOVA, a modest but significant increase in blood pressure was observed in subtotally nephrectomized rats versus sham-operated controls: the mean systolic blood pressure values were 150.3 ± 2.7 mmHg in the two NTX groups, and 140.4 ± 2.9 mmHg in the two Sham groups \( (P = 0.018) \). The outcome of two-way ANOVA was also that increased calcium intake did not significantly influence blood pressure in this study \( (P = 0.12) \). Renal mass was 30% lower in both NTX groups than the weight of the two kidneys in sham-operated rats. At the end of the study, the intake of drinking fluid and the output of urine were higher in the NTX rats when compared with the Sham rats (Table 1).

**Laboratory findings.** Plasma total cholesterol and HDL and LDL cholesterol levels were elevated in the NTX group but normalized by calcium supplementation (Fig. 1). Plasma concentrations of creatinine, urea nitrogen, PTH, and phosphate values were increased, whereas hemoglobin, ionized Ca\(^{2+}\), and protein concentrations were decreased in the NTX group when compared with the Sham group (Table 1 and Fig. 2). A high-calcium diet elevated plasma ionized Ca\(^{2+}\) and suppressed plasma phosphate and PTH in both NTX and Sham rats (Fig. 2). No changes were detected in plasma potassium or sodium concentrations (Table 1). Although plasma levels of active vitamin D in individual study groups did not significantly differ, analyses by two-way ANOVA showed that plasma 1,25(OH)\(_2\)D\(_3\) was slightly but significantly lower in the NTX groups than the Sham groups \( (34.8 ± 1.8 \text{ vs. } 40.4 ± 2.0 \text{ pg/ml}, \text{respectively, } P = 0.049) \).

**Morphology of small arteries and thoracic aorta.** The resistance arteries showed no significant differences in lumen diameter or wall thickness between the study groups (Table 2). The cross sections of thoracic aortas from rats that were followed for another 8 wk showed increased calcification in the NTX group, whereas aortic calcification in the NTX-Calcium group did not differ from that in the Sham group (Table 2). The plasma levels of creatinine continued to rise in the NTX groups during the extended follow up (Table 2).

**Responses of small mesenteric arterial preparations.** The relaxations induced by ACh in endothelium-intact NE \((5 \mu M)\)-precontracted preparations were impaired in the NTX group, whereas the NTX-Calcium and Sham-Calcium groups did not differ from the Sham group (Fig. 3A). The relaxations induced by nitroprussi-
side, a vasodilator acting via formation of exogenous NO, were similar in all study groups (Fig. 3B). The vascular rings of the study groups exhibited similar contractile sensitivity (i.e., pD2 values) and maximal wall tensions to NE and KCl (Table 2). Therefore, no changes in vasoconstrictor responses were observed in the NTX-Calcium rats that could explain the enhanced vasorelaxation in the arterial rings of these animals.
Table 2. Parameters of contractile responses and lumen diameters of isolated mesenteric arterial rings in experimental groups, and data from extended followup in 12 NTX and 6 sham rats that were followed for further 8 wk (altogether for 20 wk)

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham-Calcium</th>
<th>NTX</th>
<th>NTX-Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrine (+E)</td>
<td>pD2, −log M</td>
<td>5.61 ± 0.07</td>
<td>5.71 ± 0.05</td>
<td>5.68 ± 0.02</td>
</tr>
<tr>
<td>Maximal wall tension, mN/mm</td>
<td>4.70 ± 0.30</td>
<td>4.58 ± 0.21</td>
<td>4.71 ± 0.23</td>
<td>4.82 ± 0.55</td>
</tr>
<tr>
<td>Potassium chloride (−E)</td>
<td>pD2, −log M</td>
<td>1.72 ± 0.02</td>
<td>1.70 ± 0.03</td>
<td>1.73 ± 0.01</td>
</tr>
<tr>
<td>Maximal wall tension, mN/mm</td>
<td>4.92 ± 0.67</td>
<td>5.06 ± 0.61</td>
<td>5.42 ± 0.87</td>
<td>5.29 ± 0.61</td>
</tr>
<tr>
<td>Morphology at 90 mmHg</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Wall thickness, μm</td>
<td>22.9 ± 1.5</td>
<td>23.5 ± 1.4</td>
<td>25.8 ± 1.8</td>
<td>25.0 ± 1.8</td>
</tr>
<tr>
<td>Lumen diameter, μm</td>
<td>277 ± 7</td>
<td>271 ± 3</td>
<td>281 ± 8</td>
<td>275 ± 5</td>
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<tr>
<td>Data from extended 20-wk followup</td>
<td></td>
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<tr>
<td>Plasma creatinine, μmol/l</td>
<td>69.0 ± 2.7</td>
<td>Not studied</td>
<td>142.0 ± 14.5*</td>
<td>137.0 ± 6.7*</td>
</tr>
<tr>
<td>Aortic calcification, % of the area of aortic cross-section</td>
<td>0.0 ± 0.0</td>
<td>Not studied</td>
<td>7.2 ± 2.4*</td>
<td>0.0 ± 0.0†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10–11 for other groups, but n = 6 in the extended 20-wk followup. +E and −E, endothelium intact and endothelium-denuded arterial rings, respectively. pD2 is the negative logarithm of the concentration of agonist producing 50% of maximal contractile force. *P < 0.05 compared with the sham group; †P < 0.05 compared with the NTX group.

The present results showed that increased calcium intake, which reduced plasma phosphate and PTH and elevated plasma ionized calcium in NTX rats as expected, was also associated with favorable changes in the lipid profile and endothelium-dependent relaxation of resistance arteries. The scatterplots depicting the variables of plasma lipid profile and calcium-phosphorus metabolism in relation to the sensitivity of resistance arteries to endothelium-dependent vasorelaxation are illustrated in Fig. 4. These scatterplots showed clear clustering of data points in separate subgroups in the NTX and NTX-Calcium groups. Renal function was not different in the two NTX groups, and plasma creatinine and urea showed no correlation to the sensitivity to ACh in the NTX and NTX + Calcium groups (r = −0.255 and −0.186; P = 0.307 and P = 0.445, respectively).

DISCUSSION

In the present study, renal failure was induced in rats by 5/6 nephrectomy, and the subsequent 1.6- and 1.7-fold elevations in plasma creatinine and urea nitrogen, respectively, were comparable to those observed previously (13). The intake of drinking fluid and the output of urine were increased in the renal failure rats, suggesting a deteriorated urine concentration capacity in the remnant kidney.

Plasma total cholesterol and LDL levels were significantly increased in the NTX group, whereas a high-calcium diet normalized the total and LDL cholesterol levels. The HDL/LDL ratio was also reduced in experimental renal failure but was normalized by high-calcium intake. Plasma triglyceride levels were comparable between the groups. These results correspond to previous findings concerning calcium intake and plasma cholesterol levels in both humans and experimental animals (14, 34, 37). The lipid-lowering effect of dietary calcium probably results from the ability of Ca²⁺ to bind bile acids and saturated fatty acids in the intestine and form inabsorbable chelates thereby increasing the fecal excretion of these compounds (7, 34).
The mechanisms of the detrimental effect of CRF on plasma cholesterol have also been elucidated previously. It is known that in the rat CRF is associated with down-regulation of hepatic lecithin:cholesterol acyltransferase (LCAT) gene expression and reduction of plasma LCAT activity, as well as upregulation of hepatic acyl-CoA:cholesterol acyltransferase-2 (ACAT-2) expression and total ACAT activity, both of which may contribute to the observed lipid disorders (15, 35).

Secondary hyperparathyroidism is a common complication of CRF. In this study, subtotal nephrectomy markedly raised plasma PTH and phosphate concentrations in the NTX group, whereas high-calcium intake effectively lowered plasma PTH and phosphate levels, as expected. Plasma levels of 1,25(OH)2D3 were slightly but significantly reduced by NTX but were not affected by increased calcium intake. The main regulators of 1,25(OH)2D3 synthesis by 1-a-hydroxylase in the kidney are phosphate, PTH, calcium, and 1,25(OH)2D3 itself (5). Modulation of phosphate concentration within the normal range can alter serum 1,25(OH)2D3: high level of phosphate inhibits and low level stimulates the synthesis. In addition, high PTH concentration increases, whereas hypercalcemia decreases active vitamin D production. 1,25(OH)2D3 also regulates its own synthesis by a negative feedback mechanism (5, 28). In this study, the long-term changes in plasma phosphate, PTH, and calcium provide an explanation why active vitamin D levels were not affected by increased calcium intake in NTX or Sham rats (5, 28).

In previous studies, a high level of LDL cholesterol has been shown to inhibit endothelium-dependent vasodilatation in both humans (25) and rats (18). LDL can impair the vascular production of NO (25) or increase the generation of superoxide anion, which reacts with NO to form peroxynitrate, which in turn may play a significant role in oxidative tissue damage (20, 36). To examine endothelial function, we compared the ACh-induced relaxations between the study groups. The relaxations to ACh in the resistance arteries were clearly impaired in the NTX rats but were normalized by concomitant high-calcium diet. In subsequent analyses, we observed scattering of data points in separate subgroups when the plasma levels of total cholesterol, LDL-cholesterol, HDL/LDL cholesterol ratio, ionized calcium, phosphate, and PTH were depicted in relation to the sensitivity of resistance arteries to endothelium-mediated relaxation in the nephrectomized rats. Importantly, the variables reflecting renal function did not differ in the NTX and NTX-Calcium groups.

The detrimental effect of high plasma cholesterol (29), and also the beneficial effect of elevated HDL, on endothelium-dependent vasodilatation are well known.
from previous studies (20). In addition, HDL is an important antioxidant, which may improve the bioavailability of NO by decreasing the formation of superoxide anion and thus oppose the negative effect of LDL in the vasculature (20, 25). Moreover, oxidative stress is a known feature of CRF, which has been evidenced by the elevation of lipid peroxidation products, depression of antioxidant capacity and impairment of antioxidant enzyme activity in both human and experimental forms of CRF (36). In the present study, the plasma level of HDL cholesterol was increased in the NTX group but so were the levels of total and LDL cholesterol. In addition, a significant reduction in HDL/LDL ratio was observed, whereby this model of renal failure was associated with clear unfavorable changes in plasma lipid profile. All of these changes were reversed by increased calcium intake, which also induced a slight elevation in the HDL/total cholesterol ratio when compared with the Sham group. Therefore, improved plasma lipid profile may play an important role in the modulation of vascular function after increased calcium intake in CRF.

Because the high-calcium diet suppressed plasma phosphate and PTH in the NTX rats, these changes may also explain the observed improvement of endothelium-mediated vasodilator properties in the resistance artery. Previous studies (11, 26, 28) suggest that secondary hyperparathyroidism and hyperphosphatemia both contribute to vascular pathology in CRF. PTH excess increases cytoplasmic Ca2+ and alters the production of endothelium-derived vasoactive substances (26, 27). Elevated phosphate may directly influence the metabolism of vascular smooth muscle and induce phenotypic changes in smooth muscle cells that predispose the vessel wall to calcification (12). We found that increased calcium intake actually reduced signs of calcification in aortic cross sections of NTX rats. This underlying mechanism of the reduction of vascular calcification by increased calcium intake is a subject for future studies, but a very probable explanation is the effective suppression of plasma phosphate in the NTX rats by the high-calcium diet. Altogether, the present results suggest that alterations in the calcium-phosphate balance may significantly contribute to both morphological vascular changes and impairment of vasodilatation during reduced kidney function.

Earlier studies (22, 31) have demonstrated the detrimental effect of hypertension on the endothelium-dependent and -independent relaxations of the resistance arteries. The previous findings concerning the development of hypertension in rats with renal failure are inconsistent and depend on the rat strain and the renal ablation procedure (3, 8, 13, 32). Recently, reduction of functioning renal tissue by infarction, i.e., ligation of renal arteries, was shown to increase blood pressure (measured by a telemetric method) in rats, whereas surgical excision of an equal amount of renal parenchyma did not result in the development of hypertension during the 6-wk follow up (9). In the present investigation, renal failure was associated with a small (10 mmHg) increase in blood pressure during the 12-wk follow up. This modest elevation of blood pressure in the NTX rats suggests that the observed functional alterations of the resistance arteries potentially resulted from the metabolic alterations induced by CRF and not exclusively from increased blood pressure. Nevertheless, the beneficial effects of increased calcium intake on arterial tone could not be explained by reduction of blood pressure. It is of course possible that the tail plethysmography method employed in the present study failed to detect slight changes in blood pressure.

In conclusion, CRF in rats was associated with deteriorated plasma lipid profile, secondary hyperparathyroidism, and impaired endothelium-dependent vasorelaxation of the resistance arteries. Increased calcium intake improved the lipid profile, lowered plasma PTH and phosphate, and normalized the deficient resistance artery relaxation in experimental renal failure. The improved vasorelaxation after a high-calcium diet could thus be due to reduced plasma total cholesterol and ameliorated HDL/LDL ratio, but the decreased plasma phosphate and PTH levels may also play a significant role in the vascular effects of increased calcium intake.

DISCLOSURES

This study was supported by the Finnish Kidney Foundation, the Medical Research Fund of Tampere University Hospital, the Paavo Nurmi Foundation, the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, the Satakunta Regional Fund of the Finnish Cultural Foundation, the Finnish Medical Foundation, the Einar and Karin Stroom Foundation, the Aarne and Aili Turunen Foundation, the Sigrid Juselius Foundation, the Maud Kuistila Memorial Foundation, and the University of Tampere.

REFERENCES