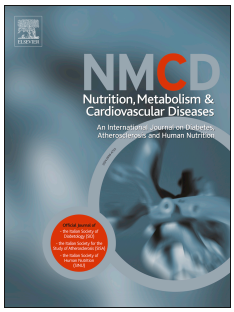
Flinders UNIVERSITY

Accepted Manuscript



Cholesterol lowering treatment restores blood global DNA methylation in chronic kidney disease (CKD) patients

Angelo Zinellu, Salvatore Sotgia, Elisabetta Sotgiu, Stefano Assaretti, Angela Baralla, Arduino A. Mangoni, Andrea E. Satta, Ciriaco Carru

PII: S0939-4753(17)30132-1

DOI: 10.1016/j.numecd.2017.06.011

Reference: NUMECD 1742

To appear in: *Nutrition, Metabolism and Cardiovascular Diseases*

Received Date: 3 March 2017

Revised Date: 19 June 2017

Accepted Date: 19 June 2017

Please cite this article as: Zinellu A, Sotgia S, Sotgiu E, Assaretti S, Baralla A, Mangoni AA, Satta AE, Carru C, Cholesterol lowering treatment restores blood global DNA methylation in chronic kidney disease (CKD) patients, *Nutrition, Metabolism and Cardiovascular Diseases* (2017), doi: 10.1016/j.numecd.2017.06.011.

Cholesterol lowering treatment restores blood global DNA methylation in chronic kidney disease (CKD) patients

Angelo Zinellu[1]*, Salvatore Sotgia[1], Elisabetta Sotgiu[1], Stefano Assaretti[1], Angela Baralla[1], Arduino A. Mangoni[2], Andrea E. Satta[3], Ciriaco Carru[1,4]

[1]Department of Biomedical Sciences - University of Sassari, Sassari, Italy;

[2]Department of Clinical Pharmacology, School of Medicine, Flinders University, Adelaide, Australia;

[3]Department of Surgical, Microsurgical and Medical Sciences - University of Sassari, Sassari, Italy;

[4] Quality Control Unit, University Hospital Sassari (AOU) Sassari, Italy

*Correspondence:

Prof. Angelo Zinellu, e-mail: azinellu@uniss.it

Dept. Biomedical Sciences - University of Sassari,

Viale San Pietro 43/B – 07100 SASSARI (Italy) - fax: +39-079228275

Abbreviations used: All: allantoine; CKD, chronic kidney disease; EZE, ezetimibe; MDA, malondialdehyde; mCyt: methylcytosine; OS: oxidative stress; UA: uric acid.

Keywords: Chronic kidney disease; DNA methylation; ezetimibe; methylcytosine; oxidative stress; simvastatin

Running title: Cholesterol lowering therapy and DNA methylation in CKD

ABSTRACT

Background and Aims: Chronic kidney disease (CKD) is characterized by increased oxidative stress (OS). In consideration of the well-known link between OS and DNA methylation we assessed DNA methylcytosine (mCyt) concentrations in CKD patients at baseline and during cholesterol lowering treatment.

Methods and Results: DNA methylation and OS indices (malonyldialdehyde, MDA; allantoine/uric acid ratio, All/UA) were measured in 30 CKD patients randomized to three cholesterol lowering regimens for 12 months (simvastatin 40 mg/day, ezetimibe/simvastatin 10/20 mg/day, or ezetimibe/simvastatin 10/40 mg/day) and 30 age- and sex-matched healthy controls. DNA methylation was significantly lower in CKD patients vs. controls (4.06±0.20 % vs. 4.27±0.17 % mCyt, p=0.0001). Treatment significantly increased mCyt DNA concentrations in all patients (4.06±0.04 % at baseline; 4.12±0.03% at 4 months; 4.17± 0.03% at 8 months; and 4.20± 0.02% at 12 months, p=0.0001 for trend). A trend for a greater effect on DNA methylation was observed with combined treatment ezetimibe/simvastatin 10/40 mg/day (+5.2% after one year treatment). The treatment-associated mCyt increase was significantly correlated with the concomitant reduction in MDA concentrations and All/AU ratios.

Conclusion: Our results demonstrate that CKD patients have a lower degree of DNA methylation and that cholesterol lowering treatment restores mCyt DNA concentrations to levels similar to healthy controls. The treatment-associated increase in DNA methylation is correlated with a concomitant reduction in OS markers.

The study was registered at clinicaltrials.gov (NCT00861731).

1.INTRODUCTION

There is unequivocal evidence that patients with chronic kidney disease (CKD) develop accelerated atherosclerosis, with a consequent increase in cardiovascular morbidity and mortality [1–3]. The main mechanisms underlying the increased cardiovascular disease (CVD) risk in this population are related to the relatively high prevalence of hypertension, diabetes and obesity [4-5]. Moreover, patients with CKD often have alterations in lipoprotein metabolism, which might result in severe dyslipidemia [6]. Therefore, in view of the cardiovascular risk reduction reached in hypercholesterolaemic patients, the pharmacological management of hypercholesterolaemia represents a promising target to reduce CVD risk in CKD patients [7]. Several clinical studies show that statins, aside from decreasing plasma cholesterol concentrations, may have specific renoprotective properties and, when combined with renin-angiotensin system (RAS) inhibitors, may have additional antiproteinuric effects [8]. The combination of statins with ezetimibe (EZE), a cholesterol absorption inhibitor, exerts additional lipid lowering effects *vs* statins alone [9]. We have recently reported that combined simvastatin/ezetimibe therapy reduces inflammatory status and decreases plasma markers of endothelial dysfunction through a reduction in oxidative stress (OS) in stage III-IV CKD patients [10-11]. OS occurs when reactive oxygen species (ROS) production in the body overcomes the intrinsic antioxidant capacity, leading to oxidative attack of cellular structures such as proteins, lipids and DNA [12]. Furthermore, OS may be associated with aberrant DNA methylation in some diseases such as cancer and cardiovascular disease [13-14]. OS is associated with DNA hypomethylation through several mechanism: i) generation of DNA base adducts, such as 8-hydroxyl-2′-deoxyguanosine (8-OH-dG) and O6-methylguanine, strongly inhibit methylation of adjacent cytosine residues yielding global DNA hypomethylation [15]; ii) redox regulation of S-adenosylmethionine (SAM)-dependent methyltransferases, that have been reported as potentially redox-sensitive enzymes [16]; iii) downregulation of methionine adenosyltransferase, which catalyzes the enzymatic addition of methionine to adenosine for the synthesis of SAM, in an oxidized environment [17]; iv) glutathione (GSH) depletion during chronic oxidative stress, leading

to decreased global DNA methylation through the depletion of SAM in the folate/homocysteine pathway [18]. Under oxidizing conditions, cystathionine-β-synthase principally direct homocysteine (Hcy) metabolism through the transsulfuration pathway for the generation of GSH. This, in turn, reduces the amount of Hcy directed toward the regeneration of methionine, which may result in decreased SAM concentrations [19].

Despite to the well-known presence of OS in CKD and its metabolic link with DNA methylation, little information is currently available on mCyt concentrations in DNA extracted from stage III-IV CKD patients [20-21]. Previous studies have either assessed global DNA methylation patterns in end-stage renal disease (ESRD) [22-23] or focused on site specific DNA methylation [24-25].

Therefore, the aim of our study was to assess a) the concentrations of mCyt in DNA of CKD patients, and b) whether OS improvement during cholesterol-lowering treatment is associated with a modification of DNA methylation pattern. Moreover, since hyperhomocysteinaemia, a raised concentration of the sulphur aminoacid homocysteine in plasma that often occurs in CKD, has been found to be associated with DNA hypomethylation in ESRD [23], quantification of Hcy was also performed.

2.METHODS

2.1. Study Population

Patients recruitment was conducted as previously described [10-11]. In brief, 30 CKD patients (age 60.2±10.5 years, 19 males) were identified at the Istituto di Patologia Medica - Azienda Ospedaliero Universitaria, University of Sassari, with the following inclusion criteria: age >18 years, plasma LDL-cholesterol concentrations > 100 mg/dL (without concomitant hypolipidemic drugs), presence of proteinuric CKD defined as creatinine clearance >20 ml/min/1.73 m^2 combined with urinary protein excretion rate > 0.3 g/24h, without evidence of urinary tract infection or overt heart failure (New York Heart Association class III-IV). Patients

were stage III-IV CKD not receiving dialysis. Exclusion criteria were: previous or concomitant treatment with steroids, anti-inflammatory and/or immunosuppressive agents, vitamin B6, B12, folate or statins; evidence or clinical suspicion of obstructive uropathy, type 1 diabetes, vasculitis and renovascular disease. The latter was ruled out by renal artery echo-Doppler or by following the American College of Cardiology/American Heart Association guidelines on Peripheral Artery Disease, that propose that diagnostic testing for renal artery stenosis should be performed in the presence of one of the following: onset of severe hypertension (blood pressure ≥180 mmHg systolic and/or 120 mmHg diastolic) after the age of 55 years; unexplained deterioration of kidney function during antihypertensive therapy, especially an acute and sustained elevation (>50%) in serum creatinine concentrations within one week of treatment with an angiotensin-converting enzyme (ACE) inhibitor or angiotensin II receptor blocker (ARB); severe hypertension in patients with diffuse atherosclerosis, particularly those aged >50 years; severe hypertension in a patient with an unexplained atrophic kidney or asymmetry in renal sizes of >1.5 cm; a unilateral small kidney (≤9 cm); severe hypertension in patients with recurrent episodes of acute pulmonary edema or refractory heart failure with impaired renal function; a systolic-diastolic abdominal bruit that lateralizes to one side [26].

All patients were on stable treatment with RAS inhibitor therapy (ACE inhibition by benazepril plus angiotensin II antagonism by valsartan) for at least six months. Enrolled patients were randomized to a 12-month treatment with either 40 mg/day simvastatin (group 1, n=10), ezetimibe/simvastatin 10/20 mg/day (group 2, n=10) or ezetimibe/simvastatin 10/40 mg/day (group 3, n=10). Patients were evaluated at baseline, 4, 8 and 12 months.

A control group including 30 age- and sex-matched subjects (age 59 ±10 years, 19 males) was also recruited. Exclusion criteria for control subjects were a history of diabetes, hypertension, cardiovascular or cerebrovascular disease, renal failure, blood dyscrasias, cancer, retinal vascular disorders, age <18 years, and current medication with vitamin B6, B12, or folic acid. Serum

creatinine concentrations in controls were also measured at baseline, 4, 8 and 12 months, to rule out renal impairment [27].

Informed consent was obtained from each subject. The study was approved by the Ethics Committee of our Institution. The study complied with the principles of the Helsinki Declaration and was registered at clinicaltrials.gov (NCT00861731).

2.2.Biochemical analysis

Whole blood DNA methylation was determined as follows. Genomic DNA extraction was performed by using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the instructions supplied by the manufacturer. After extraction DNA was hydrolyzed by 90% formic acid. Hydrolyzed samples were evaporated and the dry residue containing free bases was dissolved in ultrapure water and immediately analyzed by capillary electrophoresis as previously described [28]. The percentage of methylated to total cytosine (mCyt/tCyt) was calculated using the formula: {[mC] / [mC] + [C]}*100. The inter-assay CV for mCyt/tCyt measurements was 3.3%.

OS indices allantoine/uric acid (All/UA) ratio and malondialdehyde (MDA) were assessed by capillary electrophoresis UV detection as previously described [29-30]. Plasma Hcy was measured by laser induced fluorescence (LIF) capillary electrophoresis [31].

Total plasma cholesterol, LDL, HDL and trygliceride concentrations were assayed by enzymatic methods using commercial kits (Boehringer-Mannheim, Mannheim, Germany).

eGFR was calculated by using the CKD-EPI creatinine equation.

2.3.Statistical analysis

All results are expressed as mean values (mean ± SD) or median values (median and interquartile range). The variables distribution was assessed by the Kolmogorov-Smirnov test. Differences between groups after randomization were tested by one-way ANOVA or Kruskal-Wallis test as appropriate. Correlation analysis between variables was performed by Pearson's correlation or Spearman’s correlation. Multiple linear regression analysis was used to assess the

contribution of different variables to DNA methylation at baseline. The effect of drug treatment was evaluated by one-way repeated measures ANOVA or Friedman test as appropriate.

We evaluated that a sample size of 37 patients and 37 controls is needed to ensure a power of 0.8 to detect a meaningful difference ($p<0.05$) in DNA methylation levels.

Statistical analyses were performed using MedCalc for Windows, version 12.5 64 bit (MedCalc Software, Ostend, Belgium) and SPSS for Windows, version 14.0 32 bit (IBM Corporation; Armonk, NY, USA).

3.RESULTS

Baseline clinical characteristics of controls and CKD patients are described in Table 1. As previously reported [10-11], CKD patients showed higher concentrations of plasma triglycerides, total cholesterol and LDL cholesterol vs. controls. CKD patients also exhibited higher concentrations of MDA, All/UA ratio and homocysteine. As reported in figure 1, CKD patients had lower mCyt concentrations in DNA extracted from blood when compared with healthy controls (4.06±0.20% vs. 4.27±0.17%, $p=0.0001$). At baseline, no significant correlations were observed between mCyt and OS markers, Hcy or presence of diabetes. Moreover, when serum lipid (LDL, HDL and triglycerides) concentrations were categorized into tertiles (with tertile I as the lowest and tertile III as the highest tertile) DNA methylation showed a trend towards a reduction only with increasing serum LDL concentrations (Figure 2). This was also confirmed by multiple linear regression analysis that revealed that baseline DNA methylation was negatively correlated only with LDL cholesterol ($\beta=-0.41$, $p<0.05$) after correction for age, gender, MDA, Hcy and GFR.

After randomization, no significant differences were found among the three treatment groups. As previously described [10-11], a significant improvement in lipid profile was observed in all groups after 4 months of therapy: a mean decrease of 38% in total cholesterol (median 40%, IQR: 32-44%), 54% in LDL cholesterol (median 56%, IQR: 36-59%), and 15% in triglyceride concentration (median 20%, IQR: 0.8-36%), and an increase of 2% in HDL concentration

(median 2%, IQR:-13-12%). A relatively greater improvement in lipid profile was observed in group 3: a decrease of 44% in total cholesterol (median 43%, IQR: 40-46%), 65% in LDL cholesterol (median 64%, IQR: 56-71%), and 21% in triglyceride concentration (median 19%, IQR:3-47%), and an increase of 6% in HDL concentration (median 8%, IQR:-3-19%).

Moreover, while a significant decrease in OS parameters (MDA and All/UA ratio) was observed, indicating a reduction of oxidative stress during drug treatment, Hcy plasma concentrations remained virtually unchanged [10-11].

As reported in Figure 3 drug treatment significantly increased mCyt content of DNA in all patients (4.06±0.04% at baseline; 4.12± 0.03% at 4 months; 4.17± 0.03% at 8 months; and 4.20± 0.02% at 12 months) as well as in individual treatment groups: group 1 (4.08±0.07% at baseline; 4.15±0.07% at 4 months; 4.20±0.04% at 8 months; and 4.23±0.03% at 12 months); group 2 (4.07±0.04% at baseline; 4.11±0.04% at 4 months; 4.13±0.05% at 8 months; and 4.16±0.05% at 12 months); group 3 (4.02±0.08% at baseline; 4.10±0.07% at 4 months; 4.16±0.06% at 8 months; and 4.22±0.03% at 12 months). After 12-month treatment, the DNA methylation values in CKD patients were similar to those of healthy subjects (4.20±0.02% vs 4.27±0.17%, $p>0.05$).

Figure 4 describes the ratio between the DNA methylation mean values of CKD and control subjects after one year of therapy in the three treatment groups. The greatest effect on DNA methylation was observed in group 3 (+5.2% after one year treatment), even if the differences with group 2 and group 1 (+2.3 and +3.6%, respectively) were not statistically significant.

As shown in figure 5, the increase in mCyt during therapy was significantly correlated to the reduction in MDA concentrations ($r=-0.987$, $p=0.013$) and All/AU ratios ($r=-0.983$, $p=0.017$). By contrast, no associations were observed between the increase in DNA methylation and changes in either LDL cholesterol (or other lipid parameters) or plasma Hcy.

4.DISCUSSION

Previous findings suggest a role for DNA methylation in some diseases such as cancer and cardiovascular disease [13-14]. Moreover, some clinical studies reported altered mCyt concentrations in DNA of ESRD patients [22-23]. By contrast, relatively little information is available on DNA methylation patterns in stages III and IV CKD patients [20-21]. In contrast with previous observations, reporting no differences in DNA methylation between stage III-IV CKD patients and healthy subjects [20-21], we observed significantly lower concentrations of mCyt in DNA of CKD patients vs. healthy controls. Discrepancies in the observed findings may be due to different procedures used to detect DNA methylation. Notably, in our study DNA methylation values showed a relatively narrow distribution with a particularly low biological variation (inter-individual CV: 4.0% in controls and 4.9% in CKD). It has been previously reported that the statistical power decreases significantly with increasing measurement imprecision, and this effect is already apparent with a CV of 3% [32]. This implies that high measurement precision is key to detect differences between groups. For these reasons, we used a method that ensured an inter-assay CV of 3.3%, near to values suggested for analytes with a narrow distribution [32]. Previous studies have reported lower DNA methylation in ESRD patients vs. controls [20, 23]. The similar trend in patients with mild/moderate CKD mandates the use of high-precision assays to maximise the chance of detecting statistically significant between-group differences.

Multiple correlation analysis at baseline showed the presence of inverse associations between DNA methylation and serum LDL cholesterol concentrations. The latter is in agreement with previous reports of lower DNA methylation levels in Long Interspersed Nuclear Elements (LINE-1), a surrogate marker of global methylation analysis, in Samoan Islanders with higher LDL cholesterol concentrations [33]. Conversely, there was no significant association between DNA methylation degree and total cholesterol, HDL cholesterol or triglycerides concentrations in CKD patients. The lower DNA methylation in CKD patients, characterized by a cardiovascular risk that is threefold higher than that in the general population, is consistent with recent observations that

healthy men with lower levels of global DNA methylation are more likely to develop cardiovascular disease [34]. This suggests that DNA methylation might represent a novel marker of cardiovascular risk, both in CKD and in other patient groups, that is independent of traditional risk factors, particularly dyslipidaemia. However, it remains to be established whether altered DNA methylation is a cause or a consequence of cardiovascular disease, and whether treatment-induced changes in methylation impact on hard end-points such as cardiovascular morbidity and mortality.

Also in consideration of the relatively high CVD risk, pharmacological interventions to manage dyslipidaemia in CKD patients have focused on lowering LDL, with statins showing some beneficial effects in patients with mild-to-moderate CKD (SHARP study) [35]. However, the recent evidence that CKD patients with low plasma HDL concentrations have a particularly poor prognosis, is likely to be addressed in future, HDL-targeted, trials in these patients [36].

Since statins are effective antioxidant agents, we hypothesised that statin therapy could also induce an increase in DNA methylation levels in CKD patients through improvement in OS markers. As previously reported [10-11], a significant amelioration in lipid profile was observed in all groups already after 4 months of therapy, with a relatively greater improvement in group 3. This was associated with a concomitant reduction in both MDA and All/UA ratios, particularly in group 3 patients. In this study we observed that cholesterol lowering treatment increased DNA mCyt concentrations in the whole study group (+3.72%), with a relatively greater effect in group 3 (+5.2%). As a result, the level of DNA methylation after 12 months of treatment was similar to that of healthy people. The increase in DNA methylation levels was associated with a concomitant reduction in OS markers (MDA and All/AU). This suggests a close relationship between DNA methylation and OS pathways. A previous study failed to show a significant effect of statin treatment on DNA mCyt concentrations in CKD [21]. However, the treatment with statin (6 months) was shorter than that in our study (12 months). This might have led to a less prominent effect on OS when compared with our study. In addition, the statin employed in this study, pravastatin, has been recently reported to have modest effects on OS indexes [37]. It is therefore

possible that the effect magnitude of pravastatin on OS was not sufficient to improve DNA methylation pattern.

Ingrosso et al. [23] reported that folate treatment in ESRD reduces Hcy plasma concentrations. This would force the homocysteine-methionine cycle through the remethylation pathway, increasing the intracellular pool of the methyl donor S-adenosylmethionine and, as a consequence, DNA methylation. However, in our study homocysteine concentrations did not change during statin therapy. Furthermore, no associations were observed between the increase in mCyt DNA levels and homocysteine concentrations.

Our findings of a global DNA methylation alteration in CKD patients vs. controls at baseline confirm previous observations about site-specific methylation modifications of important target genes in CKD [25, 38-39]. It has been hypothesized that modifications of DNA methylation pattern may play an important regulatory role in loss of kidney function through expression or suppression of key pathway genes [39]. However, the observation of a restoration of the methylation degree after lipid lowering treatment in our study might pave the way to the identification of novel treatment strategies and therapeutic targets to prevent the progression of CKD.

Some limitations of this study deserve mention. As the study was under-powered, our results require confirmation in larger cohorts. All patients were on stable treatment with RAS inhibitor therapy (for at least six months) before starting cholesterol lowering therapy. It is currently unknown whether valsartan and/or benazepril treatment might affect DNA methylation. The lack of assessment of the methyl donor SAM does not allow evaluating its impact on baseline mCyt levels, and their treatment-induced changes. Furthermore, the lack of data about proteinuria in controls, limits the assessment of the impact of renal function on the degree of DNA methylation in this group.

In conclusion, our study provides experimental evidence that CKD patients in stage III and IV have lower DNA mCyt levels than healthy subjects. A 12-month cholesterol lowering treatment restored the methylation pattern to levels similar to those observed in healthy controls. The

improvement in DNA methylation was associated with a concomitant reduction in OS indexes. The potential synergistic effect of the simvastatin/ezetimibe combination *vs* statin monotherapy warrants further investigations in larger study cohorts.

Acknowledgements

Arduino A. Mangoni has participated to this work during a Visiting Professorship at the University of Sassari.

Disclosures

The authors declare that there is no conflict of interest regarding the publication of this paper.

REFERENCES

1. Gusbeth-Tatomir P, Covic A. Causes and consequences of increased arterial stiffness in chronic kidney disease patients. Kidney Blood Press Res 2007;30:97–107
2. Hamm LL, McCullough PA, Kasiske BL, Kelepouris E, Klag, Parfrey P, Pfeffer M, Raij L, Spinosa DJ, Wilson PW: Kidney disease as a risk factor for development of cardiovascular disease: A statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention. Circulation 2003;108: 2154 –2169
3. Go AS, Chertow GM, Fan D, McCulloch CE, Hsu CY: Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. N Engl J Med 2004;351: 1296 –1305
4. Weiner DE, Tabatabai S, Tighiouart H, Elsayed E, Bansal N, Griffith J, Salem DN, Levey AS, Sarnak MJ. Cardiovascular outcomes and all-cause mortality. Exploring the interaction between CKD and cardiovascular disease. Am J Kidney Dis 2006;48:392–401.
5. Sarnak MJ, Levey AS, Schoolwerth AC, Coresh J, Culleton B, Hamm LL, McCullough PA, Kasiske BL, Kelepouris E, Klag MJ, Parfrey P, Pfeffer M, Raij L, Spinosa DJ, Wilson PW; American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention: Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on kidney in cardiovascular disease, high blood pressure research, clinical cardiology, and epidemiology and prevention. Circulation 2003;108: 2154–2169

6. Vaziri ND. Dyslipidemia of chronic renal failure: the nature, mechanisms, and potential consequences. Am J Physiol Renal Physiol 2006; 290: 262–272
7. Sever PS, Dahlöf B, Poulter NR, Wedel H, Beevers G, Caulfield M, Collins R, Kjeldsen SE, Kristinsson A, McInnes GT, Mehlsen J, Nieminen M, O'Brien E, Ostergren J; ASCOT investigators. Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial-Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial. Lancet 2003; 361: 1149 – 1158
8. Zoja C, Corna D, Gagliardini E, Conti S, Arnaboldi L, Benigni A, Remuzzi G. Adding a statin to a combination of ACE inhibitor and ARB normalizes proteinuria in experimental diabetes, which translates into full renoprotection. Am J Physiol Renal Physiol 2010; 299: 1203-1211
9. Dembowski E, Davidson MH. Statin and ezetimibe combination therapy in cardiovascular disease: Curr Opin Endocrinol Diabetes Obes 2009; 16:183–188
10. Zinellu A, Sotgia S, Loriga G, Deiana L, Satta AE, Carru C. Oxidative stress improvement is associated with increased levels of taurine in CKD patients undergoing lipid-lowering therapy. Amino Acids. 2012;43:1499-1507.
11. Zinellu A, Sotgia S, Mangoni AA, Sotgiu E, Ena S, Satta AE, Carru C. Effect of cholesterol lowering treatment on plasma markers of endothelial dysfunction in chronic kidney disease. J Pharm Biomed Anal. 2016;129:383-388.
12. Young IS, Woodside JV. Antioxidants in health and disease. J Clin Pathol. 2001;54:176–86
13. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet. 2002;3:415–428.
14. Muka T, Koromani F, Portilla E, O'Connor A, Bramer WM, Troup J, Chowdhury R, Dehghan A, Franco OH. The role of epigenetic modifications in cardiovascular disease: A systematic review. Int J Cardiol 2016;212:174-183.

15. Franco R, Schoneveld O, Georgakilas AG, Panayiotidis MI. Oxidative stress, DNA methylation and carcinogenesis. Cancer Lett. 2008;266:6–11
16. Fomenko DE, Xing W, Adair BM, Thomas DJ, Gladyshev VN. High-throughput identification of catalytic redox-active cysteine residues. Science 2007; 315:387-389.
17. Pajares MA, Durán C, Corrales F, Pliego MM, Mato JM. Modulation of rat liver S-adenosylmethionine synthetase activity by glutathione. J Biol Chem 1992;267:17598-17605.
18. Hitchler MJ, Domann FE. An epigenetic perspective on the free radical theory of development. Free Radic Biol Med 2007;43:1023-1036
19. Mosharov E, Cranford MR, Banerjee R. The quantitatively important relationship between homocysteine metabolism and glutathione synthesis by the transsulfuration pathway and its regulation by redox changes. Biochemistry 2000;39:13005-13011
20. Stenvinkel P, Karimi M, Johansson S, Axelsson J, Suliman M, Lindholm B, Heimbürger O, Barany P, Alvestrand A, Nordfors L, Qureshi AR, Ekström TJ, Schalling M. Impact of inflammation on epigenetic DNA methylation - a novel risk factor for cardiovascular disease? J Intern Med. 2007;261:488-499.
21. Nanayakkara PW, Kiefte-de Jong JC, Stehouwer CD, van Ittersum FJ, Olthof MR, Kok RM, Blom HJ, van Guldener C, ter Wee PM, Smulders YM. Association between global leukocyte DNA methylation, renal function, carotid intima-media thickness and plasma homocysteine in patients with stage 2-4 chronic kidney disease. Nephrol Dial Transplant. 2008;23:2586-2592.
22. Pushpakumar S, Kundu S, Narayanan N, Sen U. DNA hypermethylation in hyperhomocysteinemia contributes to abnormal extracellular matrix metabolism in the kidney. FASEB J. 2015;29:4713-4725
23. Ingrosso D, Cimmino A, Perna AF, Masella L, De Santo NG, De Bonis ML, Vacca M, D'Esposito M, D'Urso M, Galletti P, Zappia V. Folate treatment and unbalanced methylation

and changes of allelic expression induced by hyperhomocysteinaemia in patients with uraemia. Lancet 2003;361:1693-1699

24. Yang J, Fang P, Yu D, Zhang L, Zhang D, Jiang X, Yang WY, Bottiglieri T, Kunapuli SP, Yu J, Choi ET, Ji Y, Yang X, Wang H. Chronic Kidney Disease Induces Inflammatory CD40+ Monocyte Differentiation via Homocysteine Elevation and DNA Hypomethylation. Circ Res. 2016;119:1226-1241.
25. Smyth LJ, McKay GJ, Maxwell AP, McKnight AJ. DNA hypermethylation and DNA hypomethylation is present at different loci in chronic kidney disease. Epigenetics 2014;9:366-376
26. Rooke TW, Hirsch AT, Misra S, Sidawy AN, Beckman JA, Findeiss LK, Golzarian J, Gornik HL, Halperin JL, Jaff MR, Moneta GL, Olin JW, Stanley JC, White CJ, White JV, Zierler RE; Society for Cardiovascular Angiography and Interventions; Society of Interventional Radiology; Society for Vascular Medicine; Society for Vascular Surgery. ACCF/AHA Focused Update of the Guideline for the Management of Patients With Peripheral Artery Disease (updating the 2005 guideline): a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol. 2011;58:2020-2045.
27. National Kidney Foundation. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. Am J Kidney Dis. 2002;39:S1–S266.
28. Sotgia S, Carru C, Franconi F, Fiori PB, Manca S, Pettinato S, Magliona S, Ginanneschi R, Deiana L, Zinellu A. Rapid quantification of total genomic DNA methylation degree by short-end injection capillary zone electrophoresis. J Chromatogr A. 2008;1185:145-150.
29. Zinellu A, Sotgia S, Deiana L, Carru C. Field-amplified online sample stacking capillary electrophoresis UV detection for plasma malondialdehyde measurement. Electrophoresis 2011; 32:1893-1897

30. Zinellu A, Sotgia S, Deiana L, Carru C. Field-amplified sample injection combined with pressure-assisted capillary electrophoresis UV detection for the simultaneous analysis of allantoin, uric acid, and malondialdehyde in human plasma. Anal Bioanal Chem 2011;399: 2855-2861
31. Zinellu A, Carru C, Galistu F, Usai MF, Pes GM, Baggio G, Federici G, Deiana L. N-methyl-D-glucamine improves the laser-induced fluorescence capillary electrophoresis performance in the total plasma thiols measurement. Electrophoresis 2003;24:2796-2804
32. Teerlink T. Measurement of asymmetric dimethylarginine in plasma: methodological considerations and clinical relevance. Clin Chem Lab Med. 2005;43:1130-1138.
33. Cash HL, McGarvey ST, Houseman EA, Marsit CJ, Hawley NL, Lambert-Messerlian GM, Viali S, Tuitele J, Kelsey KT. Cardiovascular disease risk factors and DNA methylation at the LINE-1 repeat region in peripheral blood from Samoan Islanders. Epigenetics 2011;6:1257-1264.
34. Baccarelli A, Wright R, Bollati V, Litonjua A, Zanobetti A, Tarantini L, Sparrow D, Vokonas P, Schwartz J. Ischemic heart disease and stroke in relation to blood DNA methylation. Epidemiology 2010;21:819-828.
35. Baigent C, Landry M. Study of Heart and Renal Protection (SHARP). Kidney Int Suppl 2003; 63: S207–10.
36. Baragetti A, Norata GD, Sarcina C, Rastelli F, Grigore L, Garlaschelli K, Uboldi P, Baragetti I, Pozzi C, Catapano AL. High density lipoprotein cholesterol levels are an independent predictor of the progression of chronic kidney disease. J Intern Med. 2013;274:252-262.
37. Adema AY, van Ittersum FJ, Hoenderop JG, de Borst MH, Nanayakkara PW, Ter Wee PM, Heijboer AC, Vervloet MG; NIGRAM consortium.. Reduction of Oxidative Stress in Chronic Kidney Disease Does Not Increase Circulating α-Klotho Concentrations. PLoS One 2016;11:e0144121

38. Wing MR, Devaney JM, Joffe MM, Xie D, Feldman HI, Dominic EA, Guzman NJ, Ramezani A, Susztak K, Herman JG, Cope L, Harmon B, Kwabi-Addo B, Gordish-Dressman H, Go AS, He J, Lash JP, Kusek JW, Raj DS; Chronic Renal Insufficiency Cohort (CRIC) Study. DNA methylation profile associated with rapid decline in kidney function: findings from the CRIC study. Nephrol Dial Transplant. 2014;29:864-872.

39. Young GH, Wu VC. KLOTHO methylation is linked to uremic toxins and chronic kidney disease. Kidney Int 2012;81:611-612.

LEGENDS

Figure 1. % of mCyt in DNA extracted from blood in healthy subjects (n=30) and CKD patients (n=30). The central horizontal line on each box represents the median, the ends of the boxes are the 25 and 75 percentiles, and the error bars indicated the 5% and 95% values. P-values derived from Student-Newman-Keuls test.

Figure 2. DNA methylation levels according to tertiles of lipid parameters. (A) LDL cholesterol: tertile I range 104-143 mg/dL; tertile II range 146-173 mg/dL; tertile III range 175-264 mg/dL. (B) HDL cholesterol: tertile I range 31-40 mg/dL; tertile II range 43-51 mg/dL; tertile III range 56-95 mg/dL. (C) Triglycerides: tertile I range 41-116 mg/dL; tertile II range 118-153 mg/dL; tertile III range 160-296 mg/dL

Figure 3. Effect of drug treatment on DNA methylation in all patients (**A**) and after categorization for therapy type: **B** Group 1 (n = 10), **C** group 2 (n = 10), **D** group 3 (n = 10). p values were evaluated by one-way repeated measures ANOVA with Bonferroni correction.

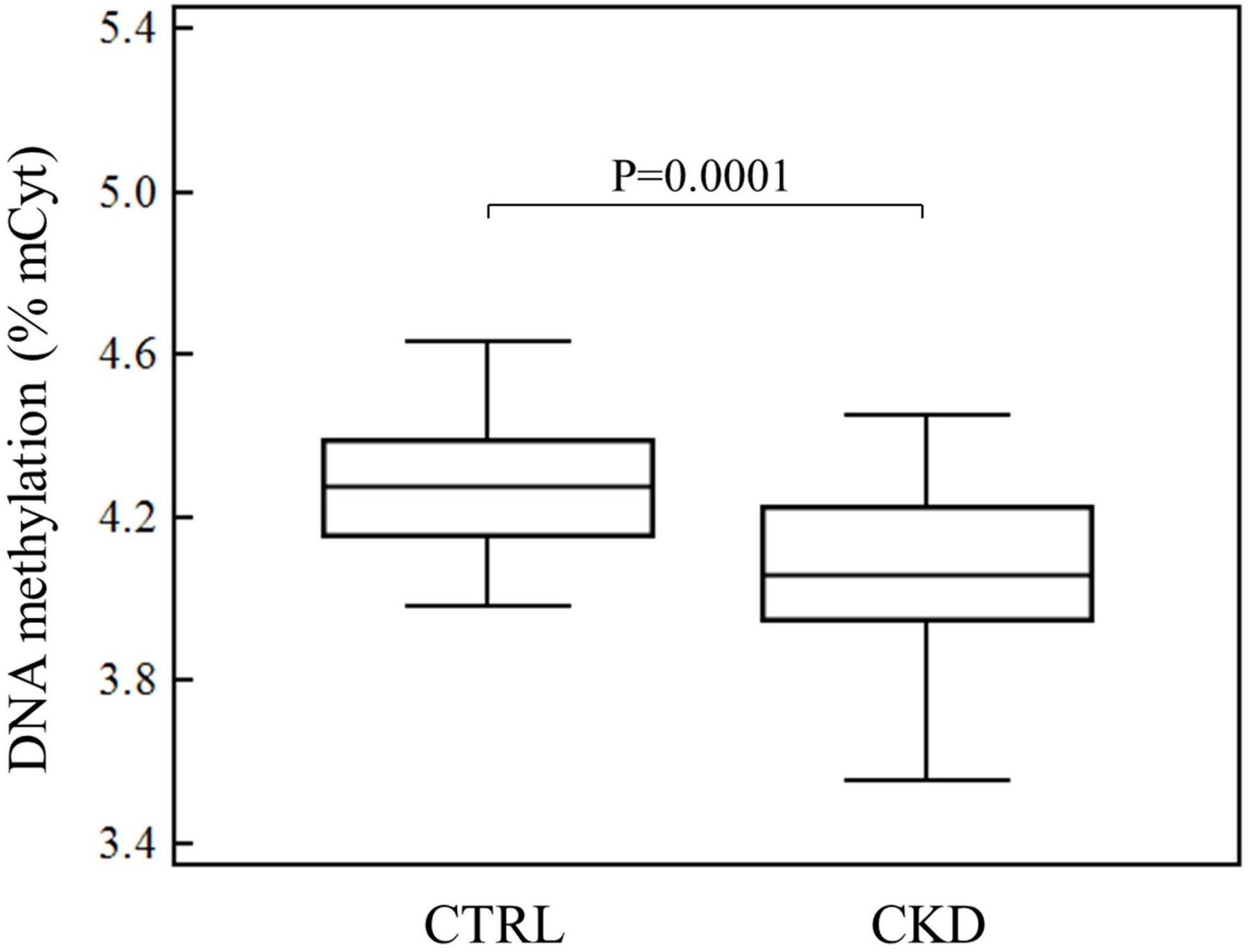
Figure 4. Ratio between DNA methylation mean values in CKD and controls after one year of therapy in the three treatment groups. The central horizontal line on each box represents the median,

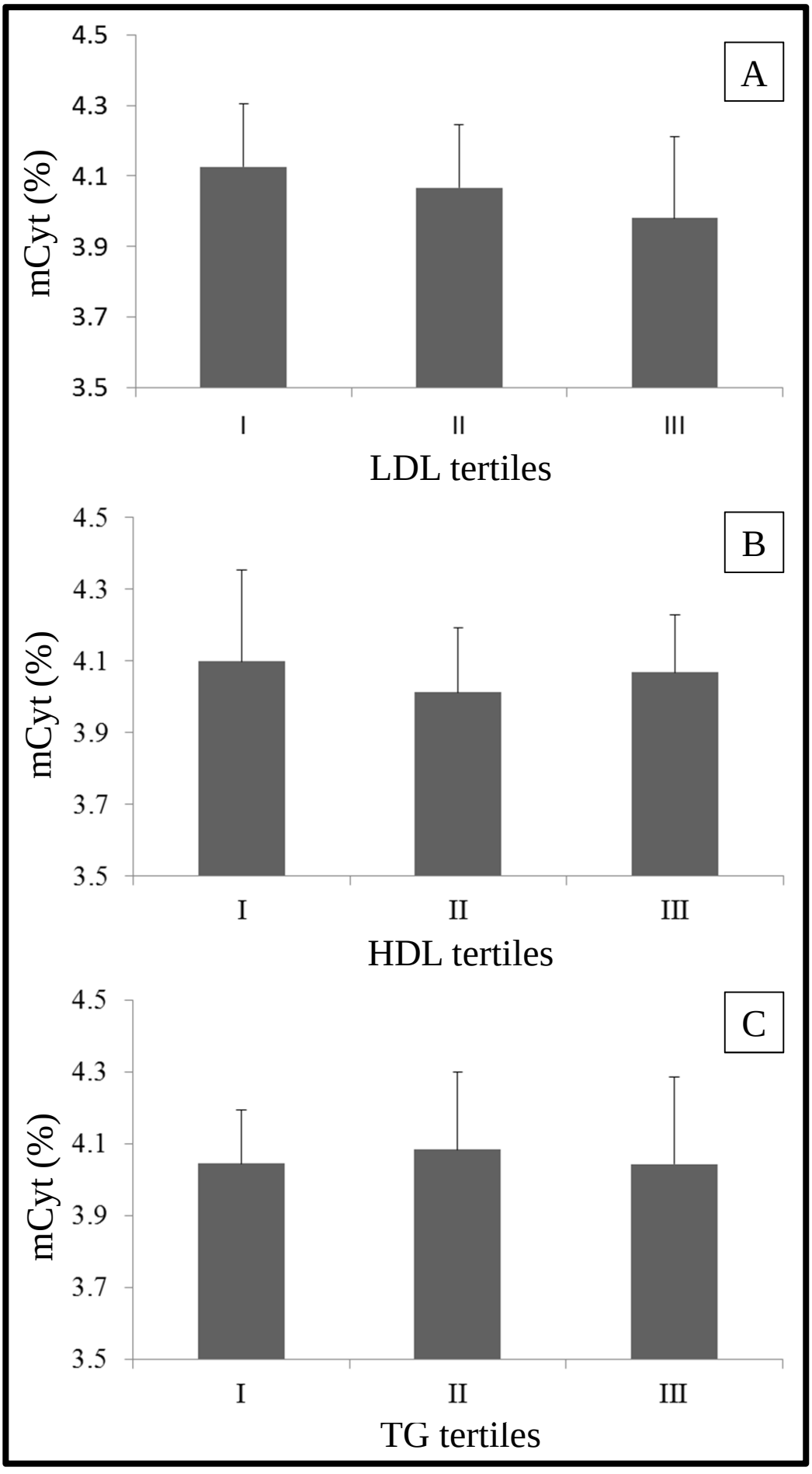
the ends of the boxes are the 25 and 75 percentiles, and the error bars indicated the 5% and 95% values.

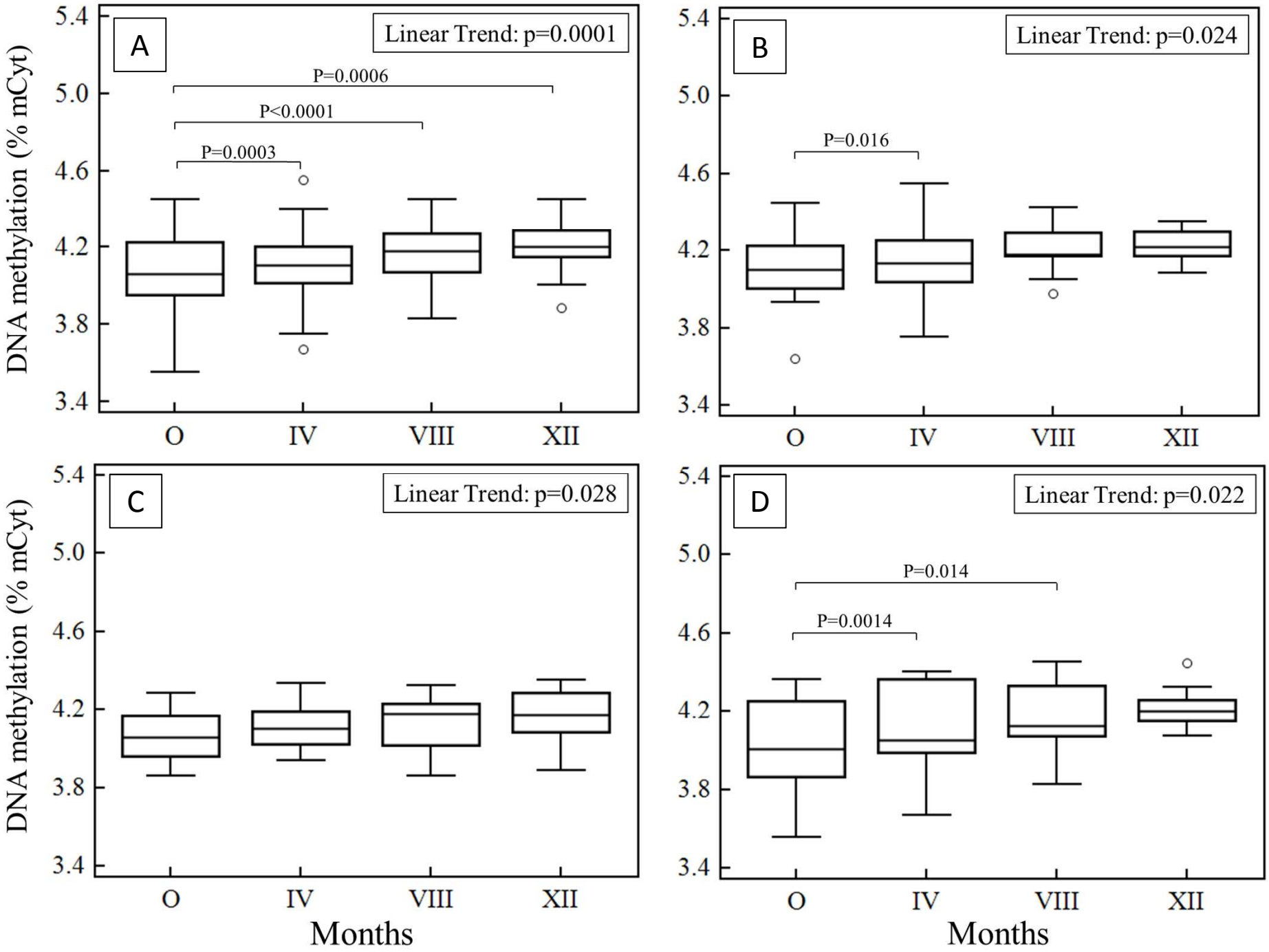
Figure 5. Trends of DNA methylation and plasma concentrations of oxidative stress indices, in all patients, during cholesterol lowering treatment. MDA plasma levels *vs* DNA methylation (A) and All/UA ratio *vs* DNA methylation (B).

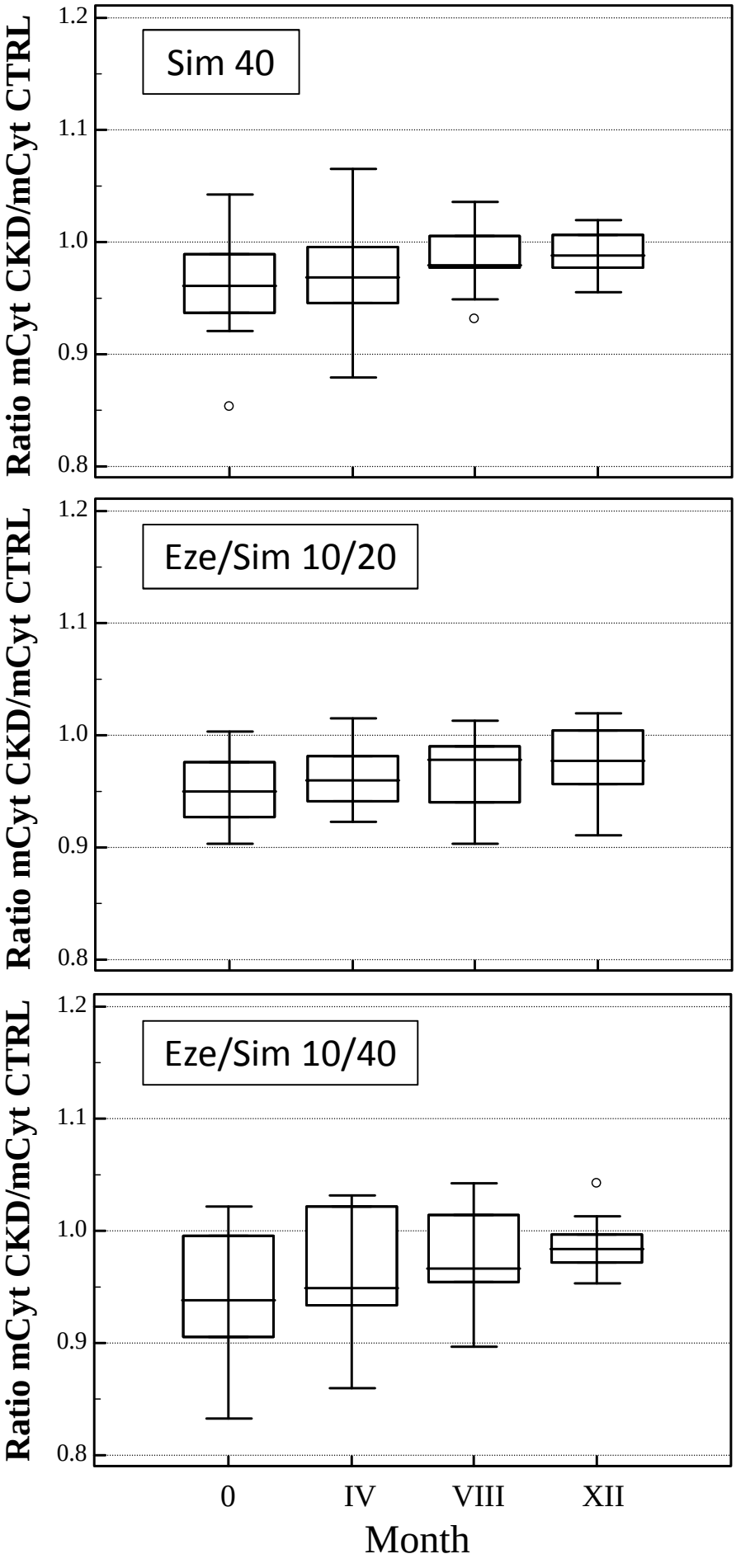
Table 1. Demographic and clinical characteristics of patients and controls

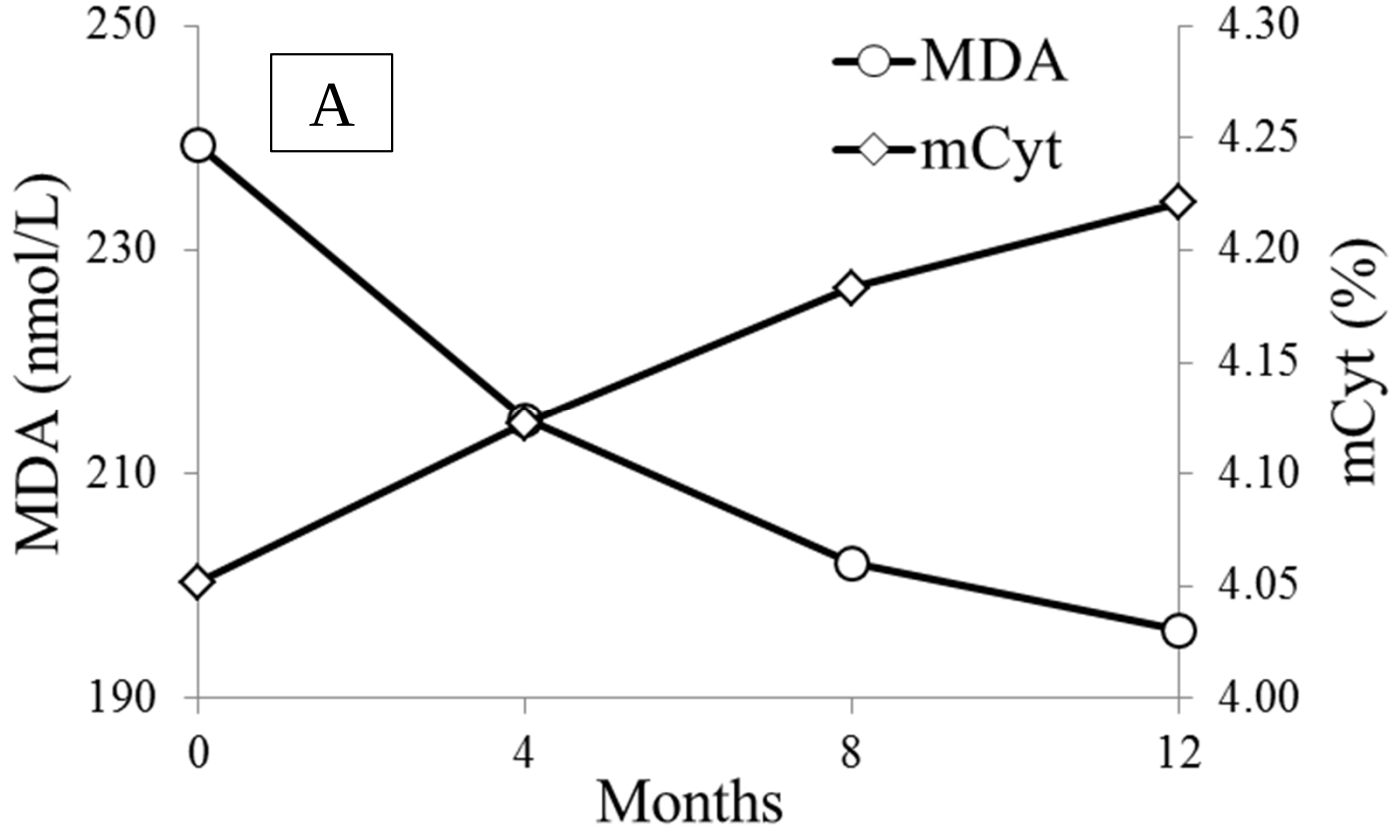
	Controls Mean ± SD or Median (range)	**CKD** Mean ± SD or Median (range)	**p-value**
Number	30	30	
Gender, F	11	11	ns
Age, Years	59±10	60±11	ns
BMI	25.2±4.3	27.9±4.4	P<0.05
Diabetes	3/30	7/30	ns
Systolic BP, mmHg	124±8	130 ± 9	ns
Diastolic BP, mmHg	80 (70-90)	80 (60-95)	ns
Creatinine, mg/dL	0.85±0.22	1.75 ± 0.77	P<0.001
eGFR, ml/min per 1.73 m^2	89±17	48 ± 25	P<0.001
Proteinuria, g/24h	--	0.99 ± 1.27	
Total cholesterol, mg/dL	207±42	239 ± 43	P<0.01
LDL-C, mg/dL	130±39	160 ± 37	P<0.01
HDL-C, mg/dL	55±19	49 ±15	ns
Triglycerides, mg/dL	106 ± 55	143 ± 69	P<0.05

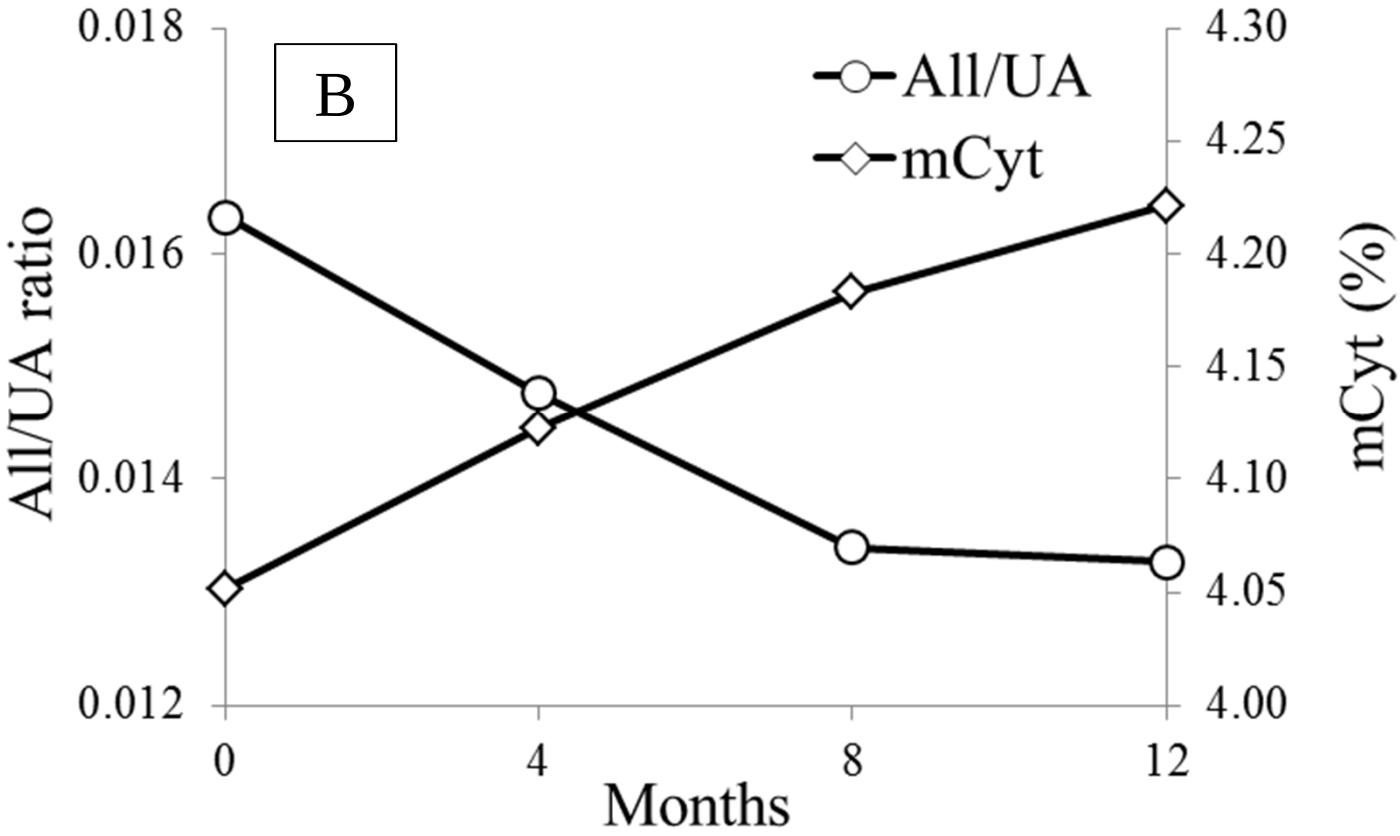



A
4.5
4.3
4.1
3.9
3.7
3.5
mCyt (%)
I
II
III
LDL tertiles
B
4.5
4.3
4.1
3.9
3.7
3.5
mCyt (%)
I
II
III
HDL tertiles
C
4.5
4.3
4.1
3.9
3.7
3.5
mCyt (%)
I
II
III
TG tertiles


A
Linear Trend: p=0.0001
P=0.0006
P<0.0001
P=0.0003
B
Linear Trend: p=0.024
P=0.016
C
Linear Trend: p=0.028
D
Linear Trend: p=0.022
P=0.014
P=0.0014
DNA methylation (% mCyt)
Months
O
IV
VIII
XII
5.4
5.0
4.6
4.2
3.8
3.4


Sim 40
Eze/Sim 10/20
Eze/Sim 10/40
Ratio mCyt CKD/mCyt CTRL
1.2
1.1
1.0
0.9
0.8
0
IV
VIII
XII
Month


A
MDA
mCyt
MDA (nmol/L)
mCyt (%)
250
230
210
190
4.30
4.25
4.20
4.15
4.10
4.05
4.00
0
4
8
12
Months


B
All/UA
mCyt
All/UA ratio
mCyt (%)
0.018
0.016
0.014
0.012
4.30
4.25
4.20
4.15
4.10
4.05
4.00
0
4
8
12
Months

Highlights

Chronic kidney disease is characterized by increased oxidative stress (OS).

DNA methylation was significantly lower in CKD patients vs. controls.

Treatment significantly increased mCyt DNA concentrations in CKD patients.

Methylcytosine rise was significantly correlated with the reduction of OS indices.