Insulin resistance and left ventricular hypertrophy in end-stage renal disease: association between the ENPP1 gene and left ventricular concentric remodelling

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Abstract

Background. Left ventricular hypertrophy (LVH) and insulin resistance (IR) are frequent complications of end-stage renal disease (ESRD). The ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) gene, whose variability has been repeatedly associated with IR, codes for a membrane glycoprotein which inhibits insulin-receptor signalling.

Methods. We investigated the relationship of ENPP1 variability, as indicated by 10 single nucleotide polymorphisms (SNPs) representative of the gene haploblock structure, with left ventricular mass and geometry (by echocardiography) in an ethnically homogeneous series of 238 Caucasian ESRD patients.

Results. ENPP1 rs1974201 and rs9402349 polymorphisms were coherently associated (P ranging from 0.04 to 0.005) with indicators of left ventricular (LV) myocardial hypertrophy (mean wall thickness) and concentric remodelling (relative wall thickness and LV mass-to-volume ratio) but unrelated with the cavitary component of the LV (left ventricular end-diastolic volume). As compared to individuals carrying the alternative genotypes, the risk of LV concentric remodelling was approximately doubled in major allele homozygous for rs1974201 [odds ratio (OR) of GG versus GC + CC: 2.31, 95% confidence interval (CI): 1.30–4.12, P = 0.004] and rs9402349 (OR of AA versus AC + CC: 1.91, 95% CI: 1.02–3.56, P = 0.04) polymorphisms.

Conclusions. Coherent associations exist between echocardiographic parameters of LV myocardial hypertrophy and concentric remodelling and ENPP1 variability in ESRD patients. These data support the hypothesis that IR is a relevant factor in the pathogenesis of myocardiopathy in this population.

Keywords: ENPP1; ESRD; gene polymorphism; insulin resistance; left ventricular hypertrophy

Introduction

Left ventricular hypertrophy (LVH) is an almost universal complication of end-stage renal disease (ESRD) and the strongest risk factor for death and adverse cardiovascular outcomes in these patients [1]. Even though a fairly large set of risk factors have been implicated in this disorder [2], collectively these factors explain <50% in the variability in left ventricular mass (LVM) index in ESRD.

Insulin resistance (IR) is a common disorder in patients with advanced renal insufficiency [3] and a marker of high risk for cardiovascular complications in ESRD [4]. This alteration starts early in the course of chronic kidney disease [5] to gradually affect the majority of uraemic patients [6]. Like LVH, also IR is multifactorial in nature and acidosis, malnutrition and inflammation and accumulation of uraemic toxin all concur to engender acquired defects in the insulin-receptor pathway in this population [7].

The myocardium is an important insulin target tissue. Post-glucose load insulin level explains as much as 40% of the variability of LVM in essential hypertensives [8]. Furthermore, in these same patients, insulin sensitivity as measured by a state-of-the-art glucose clamp technique justifies the 19% in the variability of LVM patterns [9]. In a previous study, we reported that in the setting of mild to moderate CKD, renal function loss and IR interact in determining the severity of LVH [10].

Studies looking at the association between insulin sensitivity and clinical end points in ESRD pose various methodological problems. Firstly, malnutrition and inflammation, two major players in the reverse epidemiology of this population [11], heavily impinge also upon insulin sensitivity [7], which may be a relevant source of confounding. Secondly, the fact that insulin is substantially retained in ESRD may alter achieved insulin levels during glucose clamping, a critical factor in data interpretation.
Thirdly, the euglycaemic hyperinsulinaemic clamp is a very labourious technique that can hardly be applied on a wide scale in epidemiologic studies while surrogates of this technique like the Homeostasis Model Assessment index have not been specifically validated in ESRD patients. These methodological and technical problems can be in part overcome by epidemiologic studies applying genetic markers of insulin sensitivity. Since transmission of genes at mating is a random phenomenon (Mendelian randomization) [12], exploiting genetic variability in insulin sensitivity represents an unbiased approach to the problem because it eliminates confounding by environmental factors in this population. Mendelian randomization is a useful approach for testing causality in epidemiologic studies in ESRD [13]. Variants in the ENPP1 (ectonucleotide pyrophosphatase/phosphodiesterase 1) gene, a gene expressed also in the heart and coding for a plasma membrane enzyme which inhibits insulin sensitivity [14], have now emerged as genetic markers of IR in several large studies [15–17].

With this background in mind, we have therefore investigated whether variants in the ENPP1 gene are independently related with left ventricular (LV) myocardial hypertrophy in the Cardiovascular Risk Extended Evaluation in Dialysis (CREED) study cohort, a carefully characterized ESRD cohort including detailed echocardiographic studies.

**Methods**

The study protocol was in conformity with the ethical guidelines of our institution and informed consent was obtained by each participant.

**Patients**

The original study population was composed by an incident-prevalent cohort of 283 ESRD patients [231 on haemodialysis and 52 on chronic ambulatory peritoneal dialysis (CAPD)] who had been on regular dialysis treatment for at least 6 months, with LV ejection fraction >35% and without cardiac circulatory congestion, major infections (fever, infected vascular access or peritonitis or exit site infection) or intercurrent illnesses requiring hospitalization. These patients represented ~70% of the whole dialysis population treated (between January 1997 and February 1998) in two urban areas (Reggio Calabria and Catania) in southern Italy. The remaining 30% of patients were excluded because of the presence of circulatory congestion or major infections or because they were hospitalized for intercurrent illnesses (20%) or for logistic reasons (10%) to participate in the study (10%). Forty-five out of the 283 patients were excluded from the present analysis because of unavailability of echocardiographic measurements and/or genetic studies. Then, 238 (136 males and 102 females) dialysis patients, all Caucasian (188 on haemodialysis and 50 on CAPD), were recruited into the study.

Haemodialysis patients were being treated thrice weekly with standard bicarbonate dialysis (Na 138 mmol/L, HCO3 5 mmol/L, K 1.5 mmol/L, Ca 1.25 mmol/L; Mg 0.75 mmol/L) either with Cuprophan or semi-synthetic membranes. The average urea Kt/V in these patients was 1.21 ± 0.26. The remaining 50 patients were on CAPD (weekly Kt/ V 1.67 ± 0.32). Thirty-six patients were diabetics and 99 were habitual smokers (22 ± 17 cigarettes/day). One hundred and four patients were treated with various anti-hypertensive drugs (74 on monotherapy with angiotensin-converting enzyme inhibitors, AT-1 antagonists, calcium channel blockers, \( \alpha \)- and \( \beta \)-blockers and the remaining 30 on double or triple therapy with various combinations of these drugs). One hundred and twenty-six patients were on treatment with erythropoietin.

The main clinical and biochemical characteristics of the study population are detailed in Table 1.

**Table 1. Main demographic, somatometric, clinical and biochemical data of patients divided according to a recessive model of ENPP1 rs1974201 polymorphism**

<table>
<thead>
<tr>
<th>ENPP1 rs1974201 polymorphism</th>
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<tbody>
<tr>
<td><strong>GG patients</strong> (n = 107)</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Male sex, n (%)</td>
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<tr>
<td><strong>Diabetes</strong> (n)</td>
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<tr>
<td><strong>Smokers</strong>, n (%)</td>
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<td><strong>Diabetics</strong>, n (%)</td>
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<td><strong>Background CV complications</strong>, n (%)</td>
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<td><strong>On anti-hypertensive treatment</strong>, n (%)</td>
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<td><strong>On EPO treatment</strong>, n (%)</td>
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<tr>
<td><strong>Systolic pressure</strong> (mmHg)</td>
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<tr>
<td><strong>Diastolic pressure</strong> (mmHg)</td>
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<td><strong>Pulse pressure</strong> (mmHg)</td>
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<td><strong>Cholesterol</strong> (mg/dL)</td>
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<td><strong>Haemoglobin</strong> (g/dL)</td>
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<td><strong>Albumin</strong> (g/L)</td>
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<td><strong>Glucose</strong> (mg/dL)</td>
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<td><strong>Phosphate</strong> (mmol/L)</td>
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<td><strong>CRP</strong> (mg/L)</td>
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<tr>
<td><strong>Homocysteine</strong> (mmol/L)</td>
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</table>

*Data are expressed as mean ± SD, median and interquartile range or as percent frequency as appropriate.

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Haplotype structure and SNP selection

A genomic region ranging from ~600 bp up-stream to ~4 kb downstream of ENPP1 gene was targeted to define the haploblock structure of the gene for the Central European population using Haploview (http://www.broadinstitute.org/haploview/haploview) (version 2.0 release n ¼ 24, accessed June 2009; Whitehead Institute for Biomedical Research, USA). Defining a solid spine of linkage disequilibrium (LD) as D’ > 0.90, seven haploblocks in the ENPP1 locus were identified. Using a minor allele frequency >5%, a pairwise approach and setting an r² > 0.80, nine SNPs (rs6569759; rs1409181; rs2021966; rs858339; rs7775376; rs858342; rs9402349; rs1974201; rs975909), which were not in LD between them, were sufficient to tag the seven haploblocks considered capturing almost the whole common variation in the region. In addition, we determined the ENPP1 non-synonymous K121Q SNP (rs1044498), which was in LD with the rs9402349. This polymorphism has been repeatedly associated with IR and cardiovascular disease in previous large studies [14, 16, 17] and determines a gain of function with the Q121 variant resulting in a stronger inhibitor of insulin-receptor signalling [14].

Genotyping of the selected SNPs

Allelic discrimination was performed by TaqMan SNP Genotyping Assays provided by Applied Biosystems. Genomic DNA was extracted from peripheral blood leukocytes by salting-out technique [18]. The reaction system contained 20 ng of genomic DNA, 12.5 µL of 2X TaqMan Universal PCR Master Mix No AmpErase UNG, 1.25 µL of 40× Assay Mix [including unlabelled polymerase chain reaction (PCR) primers, FAM and VIC dye-labelled TaqMan MGB probes] and H₂O for a total volume of 25 µL. The genotyping was performed on an Applied Biosystems 7900HT Fast Real-Time PCR System and a random 10% of samples were independently repeated to confirm genotyping results. The genotype results for these samples were completely consistent.

**Laboratory measurements**

For haemodialysis patients, fasting blood sampling was performed during the midweek non-dialysis day and for CAPD patients on an empty
stomach. Blood was drawn and put into tubes containing EDTA, and plasma supernatants were stored at ~80°C until batch analyses. All analyses were done blinded to clinical information. Serum cholesterol, albumin, calcium and phosphate and haemoglobin measurements were made using standard methods in the routine clinical laboratory. High-sensitivity C-reactive protein (CRP) was measured by a commercially available kit [intra-assay coefficient of variation (CV): 3.5%; inter-assay CV: 3.4% (Dade Behring, Marburg, Germany)]. Plasma total homocysteine was measured as previously reported [19].

**Blood pressure measurement**

In haemodialysis patients, predialysis and postdialysis blood pressures (BPs) were calculated as the average value of all recordings [12 measurements (i.e. three/week)] taken during the month preceding the study. Because predialysis BPs approaches that of 24 h ambulatory BP monitoring better than that of predialysis and postdialysis BPs [20], we considered this average value for global statistical assessment. In CAPD patients, BP values were obtained by averaging home BP measurements (10–20 measurements/month).

**Echocardiography**

These studies were performed on a non-dialysis day for haemodialysis patients or on an empty stomach for those on CAPD within 2 h before sampling. All echocardiographic measurements were carried out according to the recommendations of the American Society of Echocardiography by an observer unaware of biochemical results. LVM was calculated according to the Devereux formula and indexed to height$^2.7$ (LVMI), as detailed in a previous study [21]. LVH was defined by an LVMI of >47 g/m$^2$ in women or >50 g/m$^2$ in men. LV end-diastolic volume (LVEDV) was calculated by the standard formula [(1.047 + LVEDD) / body surface area]. The relative wall thickness (RWT: 2 * posterior wall thickness/LV end-diastolic diameter (LVEDD)] and the LV mass-to-volume ratio, a ratio specifically applied in ESRD patients [22], were calculated as indexes of LV concentric geometry. Values indicative of concentric LV geometry were established on the basis of age-specific reference standards according to RWT [23]. Mean wall thickness (MWT) was calculated by the cavitary component of the LVM and the muscular component of the LVM (MWT, RWT and LV mass-to-volume ratio) but no association with the cavitary component of the left ventricle (LVEDV) (P > 0.27) thus pointing to a peculiar link between the ENPP1 gene and concentric LV geometry. Associations between the other polymorphisms and these measurements were either much weaker or not significant. We, therefore, used rs1974201 and rs9402349 polymorphisms to deeper investigate the relationship between ENPP1 polymorphisms and cardiac geometry.

**Statistical analysis**

Data are summarized as mean ± SD (normally distributed data), median and interquartile range (non-normally distributed data) or as percent frequency and comparisons between to groups were made by t-test, Mann–Whitney U-test or chi-square test, as appropriate.

The independent relationship between ENPP1 polymorphisms (recessive model) and echocardiographic indicators of LVM and geometry (RWT, MWT, LVMI and LV mass-to-volume ratio) was investigated by multiple linear regression analysis. In these models, we included ENPP1 polymorphisms as well as Framingham risk factors (age, gender, smoking, diabetes, cholesterol and systolic pressure), anti-hypertensive treatment, previous CV complications, factors peculiar to ESRD (treatment modality, dialysis vintage, haemoglobin, albumin and phosphate) and emerging risk factors (CRP and homocysteine). By this strategy, we constructed models of adequate statistical power (at least 15 patients for each variable in the model).

The relationship between ENPP1 polymorphisms and concentric LV geometry was also investigated by multiple logistic regression analysis adjusting for the same set of variables listed in Table 2. The effect modification of ENPP1 polymorphisms on the relationship between circulating levels of glucose and insulin with LVMI was investigated by including multiplicative terms in the multiple linear regression model as suggested by Altman [24]. In brief, we included into the model glucose, insulin and ENPP1 polymorphism as well as all combinations of their multiplicative terms and adjusted for a set of potential confounders. Since glucose and insulin had a positively skewed distribution, they were log transformed before multivariate modelling. The estimated LVMI (mean ± SE) in GG and GC + CC genotypes across predefined values of insulin and glucose were derived by the multiple linear regression model in which all terms, but glucose and insulin, were set to the corresponding average values. Data are expressed as standardized regression coefficient ($\beta$), odds ratio (OR) and 95% confidence interval (CI) and P-value. Data analysis was performed by a standard statistical package (SPSS for Windows, Version 9.01, Chicago, IL).

**Results**

Among the 10 SNPs (rs1974201; rs9402349; rs659759; rs1409181; rs2021966; rs858339; rs7775376; rs858342; rs997509, rs1044494) tested in this study and all in Hardy–Weinberg Equilibrium (P ranging from 0.31 to 0.98), the rs1974201 and the rs9402349 showed coherent and strong (P < 0.01) relationships with echocardiographic measurements of the muscular component of the LVM (MWT, RWT and LV mass-to-volume ratio) but no association with the cavitary component of the left ventricle (LVEDV) (P > 0.27) thus pointing to a peculiar link between the ENPP1 gene and concentric LV geometry. Associations between the other polymorphisms and these measurements were either much weaker or not significant. We, therefore, used rs1974201 and rs9402349 polymorphisms to deeper investigate the relationship between ENPP1 variability and myocardial hypertrophy in ESRD in multivariate models.

**Demographic, clinical and biochemical characteristics of ESRD patients as characterized by the ENPP1 rs1974201 and rs9402349 polymorphisms**

Homozygote for the G allele of the rs1974201 polymorphism had higher CRP and pulse pressure than GC and CC patients (Table 1). The two groups did not otherwise differ as for demographic, somatometric and clinical data including a series of pertinent biochemical parameters (Table 1). The same analysis carried out for the rs9402349 polymorphism showed only a difference in serum cholesterol (212 ± 58 versus 196 ± 47 mg/dL) between AA patients and the group combining AC and CC patients (P = 0.03).
ENPP1 rs1974201 and rs9402349 polymorphisms and concentric LV geometry

One hundred and eighty-five patients of 238 (78%) displayed LVH that was eccentric in 92 cases and concentric in the remaining 93 cases. Seventeen patients had concentric LV remodelling. As shown in Figure 1, in a recessive model analysis, GG patients for the ENPP1 rs1974201 polymorphism had higher MWT, RWT, LV mass-to-volume ratio but similar LVEDV as compared to GC + CC patients. LVMI tended to be higher in GG (67 ± 19 g/m²) than in GC + CC patients (62 ± 20 g/m²), but the difference was not significant (P = 0.07). In multiple linear regression analyses adjusting for the full series of potential confounders, the ENPP1 rs1974201 polymorphism was independently related to MWT, RWT, LV mass-to-volume ratio (Table 2). Forcing into multivariate models KT/V, it did not modify the strength of the relationship between ENPP1 polymorphism and echocardiographic indicators of LV remodelling (data not shown). Similarly, coherent associations with parameters of concentric LV geometry were found for the ENPP1 rs9402349 polymorphism. Indeed, homozygous AA patients for this polymorphism had higher MWT, RWT and LV mass-to-volume ratio but similar LVEDV in comparison to the other genotypes (Figure 2). These associations except that with LVMI were again confirmed by multiple linear regression analyses (β adjusted for the set of covariates given in Table 2: MWT (β = 0.15, P = 0.009), RWT (β = 0.13, P = 0.037) and LV mass-to-volume ratio (β = 0.16, P = 0.01)).

Fig. 1. MWT, RWT, LV mass-to-volume ratio and LVEDV in GG patients for the ENPP1 rs1974201 polymorphism as compared to GC + CC patients. All echocardiographic measurements are expressed as mean and SD.

ENPP1 rs1974201 and ENPP1 rs9402349 polymorphisms and the risk for LV concentric remodelling

GG patients (ENPP1 rs1974201 polymorphism) had higher prevalence (P = 0.006) of concentric LV remodelling (56%) than GC and CC patients (38%) and this was also true for the ENPP1 rs9402349 polymorphism (AA: 51% versus AC + CC: 35%, P = 0.03). In multiple logistic regression analyses adjusting for the same set of variables listed in Table 2, the odds of LV concentric geometry was two times higher (OR: 2.31, 95% CI: 1.30–4.12, P = 0.004) in GG than in GC and CC patients of the ENPP1 rs1974201 polymorphism. Similarly, AA patients (ENPP1 rs9402349 polymorphism) had higher odds ratio of concentric LV geometry when compared to AC + CC patients (OR: 1.91, 95% CI: 1.02–3.56, P = 0.04).

Effect modification of ENPP1 polymorphisms on the relationship between glucose and insulin with LVMI

The effect modification of ENPP1 rs1974201 polymorphism on the relationship between circulating levels of glucose and insulin with LVMI was investigated in a multiple linear regression model adjusting for the same set of variables listed in Table 2. As shown in Figure 3, for relatively higher levels of glucose (>120 mg/day) and insulin (>30 UI/L), the relationship between these metabolic risk factors with LVMI was closely dependent on the ENPP1 rs1974201 genotype. Indeed, the relationship was significantly steeper (P = 0.038) in GG than in GC + CC patients.

Fig. 2. MWT, RWT, LV mass-to-volume ratio and LVEDV in AA patients for the ENPP1 rs9402349 polymorphism as compared to AC + CC patients. All echocardiographic measurements are expressed as mean and SD.
patients. No such an effect modification was found for the ENPP1 rs9402349 polymorphism.

Discussion

This study shows coherent associations between echocardiographic parameters of LV myocardial hypertrophy and concentric remodelling and two polymorphisms in the gene coding for the synthesis of ENPP1, a modulator of insulin sensitivity in health and disease states. Moreover, the ENPP1 rs1974201 polymorphism significantly modifies the relationship between glucose and insulin with LVH in ESRD patients. Overall, these data support the hypothesis that IR in ESRD is a relevant factor in the pathogenesis of myocardial hypertrophy and LV concentric remodelling in ESRD patients.

LVH is a strong integrator of cardiovascular risk in patients with cardiovascular diseases and community-based studies have solidly established the prognostic value of this alteration at population level [25]. LVH is pervasive in ESRD where it represents a powerful predictor of adverse clinical outcomes [21] concentric remodelling being the riskiest geometric pattern in ESRD patients with LVH [22]. IR commonly occurs in ESRD [6, 7] and, independently of other risk factors, it portends a high risk for cardiovascular sequelae in these patients [4]. Since IR is also a solid correlate of LV myocardial hypertrophy [8–10], the coexistence of these two highly prevalent disorders in ESRD generates the hypothesis that disturbed insulin signalling is a factor contributing to LVH in this population.

Chronic insulin infusion increases LVM and triggers concentric remodelling in the rat, an effect largely mediated by angiotensin II receptors [26]. In accordance with these experimental findings, insulin sensitivity as measured by whole body glucose disposal explained as much as 25% of the variability in the muscular component of the LV but was unrelated with the cavitary component of the LV (end-diastolic diameter) in essential hypertensives [9]. Furthermore, the relevance of IR in LVH is also highlighted by the fact that regression of myocardial hypertrophy goes strictly along with improvement in IR in these patients [27].

Insulin sensitivity and LVH show a parallel partial regression in ESRD patients after renal transplantation [28] suggesting a causal implication of IR in LVH in ESRD. However, the relationship between insulin sensitivity and LVM has never been investigated in this population. As mentioned, large-scale insulin sensitivity studies in ESRD pose various methodological problems, which may help to explain why the issue has not received sufficient attention in this population so far. In this respect, genetic variants involved in the regulation of insulin sensitivity represent unconfounded estimators of this physiologic function since they are inherited and are not modified by environmental factors [13].

The ENPP1 gene, a regulator of insulin sensitivity whose variability has been repeatedly associated [14, 16, 17] with IR and adverse cardiovascular outcomes in several large studies, is expressed in various tissues including the myocardium. Therefore, ENPP1 genetic variability may well be exploited as an instrumental variable to test the relevance of IR in myocardial hypertrophy/remodelling in ESRD. By targeting 10 genetic polymorphisms capturing almost the whole common variation in the ENPP1 gene, we could identify two polymorphisms which are consistently associated with the main parameters defining the muscular component of the LV in ESRD patients, namely rs1974201 and rs9402349. Associations between these polymorphisms and myocardial hypertrophy and concentric remodelling were apparent both at crude and adjusted analyses. Thus, IR emerges as a relevant player in myocardial hypertrophy in ESRD patients.

The relatively small sample size is an objective limitation of our study. In general terms, Mendelian randomization studies require very large sample sizes [12]. Yet, this applies to studies with no or weak biological ‘a priori’. Our study was based on an ethnically homogeneous cohort and had a strong a priori. Furthermore, the association between concentric LV remodelling and ENPP1 gene variants was quite specific and conformed to experimental findings indicating that chronic insulin infusion induces concentric LV remodelling in the rat [26], i.e. a geometric pattern identical to that which we associated with the ENPP1 gene variants in ESRD patients. Confirmatory observations in other ESRD cohorts and mechanistic studies specific to gene variants (rs1974201 and rs9402349) identified in the present study will help to clarify the biology underlying observed associations. Furthermore, the association between an SNP [the rs1044498 (K121Q)] which was previously associated with diabetes, cardiovascular disease [14] and LVH [29] in other populations but that was unrelated to LV myocardial hypertrophy in the present study needs to be investigated in larger ESRD populations. Another novel observation in our study is that ENPP1 rs1974201 polymorphism modifies the relationship between circulating levels of glucose and insulin with LVM. This finding provides further support to the hypothesis that this polymorphism is involved in the pathogenetic pathway leading to alterations in LV geometry in ESRD patients.

In conclusion, in a sizable series of ESRD patients, we show that LV concentric myocardial hypertrophy is consistently associated with genetic variability of ENPP1, a modulator of insulin sensitivity. Overall, these data support the hypothesis that IR in ESRD is a relevant player in the
pathogenesis of myocardial hypertrophy and LV concentric remodelling in ESRD patients.

Acknowledgements. Funding. This study was supported by grants of the CNR and Regione Calabria and is part of the Syskid project which is supported through European Union’s FP7, Grant agreement number HEALTH-F2-2009-241544

Conflict of interest statement. The author C.Z., who was serving as Editor-in-Chief, was blinded to the review of his manuscript and had no part in the review or decision process. All other authors: none declared.

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Received for publication: 18.1.11; Accepted in revised form: 22.4.11