The association of a single-nucleotide polymorphism in CUBN and the risk of albuminuria and cardiovascular disease

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ABSTRACT

Background. Albuminuria is an important risk factor for cardiovascular disease (CVD). We have previously identified a missense single-nucleotide polymorphism (rs1801239) in the CUBN gene that is associated with albuminuria. Whether albuminuria is associated with CVD in the presence of the CUBN mutation is unknown.

Methods. We analyzed participants from the Framingham Heart Study (n = 6399, mean age 47 years, 53.4% women) who underwent genotyping of rs1801239. Cox proportional hazards models were used to test the association between microalbuminuria [UACR ≥17 mg/g (men) and ≥25 mg/g (women)] and incident CVD stratified by the presence or absence of the CUBN risk allele. We tested whether the association between microalbuminuria and CVD was altered by the presence of the risk allele with interaction testing.

Results. Overall, 21.1% of participants carried the risk allele. As expected, carriers of the risk (C) allele had a higher prevalence of microalbuminuria (10.7 versus 8.9%, P = 0.04). During a mean follow-up of 10.4 years, 5.6% (n = 346) of participants experienced a CVD event. Microalbuminuria was associated with an increased risk of CVD [hazards ratio (HR) 1.46, 95% confidence interval (CI) 1.14–1.88]. When stratified by risk allele carrier status, the HR for CVD was 1.95 (95% CI 1.15–3.29) among those with compared to 1.33 (95% CI 1.00–1.76) among those without the risk allele. There was no interaction between microalbuminuria and rs1801239 on CVD (Pinteraction = 0.49).

Conclusions. MA is associated with CVD irrespective of the presence of the CUBN risk allele. These results challenge the concept that albuminuria in the setting of this mutation is benign.

Keywords: epidemiology

INTRODUCTION

Albuminuria is an important manifestation of chronic kidney disease (CKD) that is present in up to 8% of adults in the USA [1]. Albuminuria confers an increased risk of progression to end-stage renal disease [2] and is an independent risk factor for cardiovascular disease (CVD) and all-cause mortality above and beyond diabetes and hypertension [3–6]. Approximately 16% of albuminuria in the general population is heritable, and research has focused on identifying genetic variants that account for this increased risk [7].

Recently, a genome-wide association analysis identified an association between a missense T → C substitution (rs1801239, 12984V, Ile → Val) in the CUBN gene and albuminuria [8]. CUBN encodes cubilin, a proximal tubular epithelial cell (PTEC) protein which, along with megalin, is involved in the retrieval of filtered albumin from the urine. Mutations in CUBN have been associated with tubular albuminuria in animal models [9] and in human disease [10, 11]. Thus, the excess albuminuria observed in participants with the CUBN risk allele is most likely tubular in nature, due to reduced cubilin function resulting in incomplete tubular reabsorption of albumin.

The renal toxicity of albuminuria has been demonstrated in animal models [12, 13]. This nephrotoxicity is present whether the albuminuria is of glomerular or tubular origin, although models of tubular albuminuria remain imperfect and accurately distinguishing between the two is difficult [14, 15]. Nevertheless, it remains uncertain whether tubular albuminuria is associated with deleterious systemic manifestations.
beyond the renal parenchyma. For example, it has been suggested that albuminuria in the setting of the CUBN risk allele might not increase CVD risk in the absence of other systemic or vascular risk factors [16]. However, this is not supported by experimental evidence, which demonstrates increased production of pro-inflammatory and pro-fibrotic cytokines along with increased expression of angiotensin II in the proximal tubule in response to albuminuria [12, 13, 17]. This provides a potential mechanism for the wider systemic toxicity of albuminuria.

Thus, the purpose of the present study was to test the hypothesis that albuminuria in the presence of the CUBN risk allele is associated with CVD similarly to albuminuria in the absence of the risk allele.

MATERIALS AND METHODS

Study sample

Participants were drawn from the Framingham Offspring Study cohort [18] and the Third Generation cohort [19]. The Offspring cohort comprises the children and their spouses of the original Framingham cohort, and the Third Generation cohort are the grandchildren of the original cohort. To be included in the present study, participants were required to have attended either the Offspring sixth examination cycle (1995–98) or the Third Generation first examination cycle (2002–05) and to have provided a urine specimen to measure the albumin/creatinine ratio (UACR). In total, genotype data were available for 6907 participants. A UACR was unavailable for 475 participants and these were therefore excluded. Covariate data were missing in a further 33 participants who were also excluded, leaving 6399 in the final analysis. The number of families in this cohort was 1130 with a mean family size of 5.7 ± 13.7 (range 1–299 individuals).

The Institutional Review Board at Boston University approved this study and all patients provided written informed consent.

Genotyping

Direct genotyping of the single-nucleotide polymorphism (SNP) (rs1801239) was performed at KBiosciences (Herts, UK) using a TaqMan real-time polymerase chain reaction assay (Applied Biosciences, Carlsbad, CA). The call-rate for the SNP was 98.6%.

Albuminuria measurement

UACR was measured on spot morning urine samples. Urinary albumin concentration was measured using immunoturbidimetry (Tina Quant albumin assay, Roche diagnostics). The intra-assay coefficient of variation of this test was 1.7–3.8%. Urinary creatinine levels were measured using the modified Jaffe method. Microalbuminuria was defined using sex-specific cutoffs as a UACR ≥ 17 mg/g in men and UACR ≥ 25 mg/g in women.

Incident CVD

Incident CVD was defined as cardiovascular death, myocardial infarction, coronary insufficiency, angina pectoris, stroke, transient ischemic attack, intermittent claudication or congestive heart failure during the period of follow-up through 31 December 2011 (median follow-up 9.0 years, IQR 8.0–14.1). All events were adjudicated by a panel of three reviewers using predefined criteria.

Covariates

Covariates obtained at the baseline examination were used in the analysis. Total cholesterol, high-density lipoprotein (HDLc) and glucose were measured in blood samples obtained following an overnight fast. Diabetes mellitus was defined as a fasting plasma glucose of at least 126 mg/dL (5.9 mmol/L) or treatment with insulin or a hypoglycemic agent. Systolic and diastolic blood pressure was recorded as the average of two physician-performed measurements. Hypertension was defined as a systolic blood pressure of ≥ 140 mmHg, a diastolic blood pressure of ≥ 90 mmHg or the use of antihypertensive medications. Participants were considered current smokers if they smoked ≥ 1 cigarette daily in the previous year. Serum creatinine levels were measured using the modified Jaffe method. CKD was defined as an estimated glomerular filtration rate (eGFR) ≤ 60 mL/min/1.73 m² using the CKD-Epi equation [20].

Biomarkers of tubular function

Urinary biomarkers [α1-microglobulin, β2-microglobulin, kidney injury molecule-1 (KIM1) and neutrophil gelatinase-associated lipocalin (NGAL)] were measured using an immunoassay (Rules Based Medicine, Austin, TX) and indexed to urinary creatinine to account for urine concentration. Intra-assay coefficients of variation ranged from 7.4 to 12.5%.

Statistical analysis

The demographic characteristics and cardiovascular risk factors of the participants were grouped according to the presence or absence of carriers of at least one copy of the CUBN risk allele, a missense (T → C) substitution. Dichotomous variables were compared using logistic regression, while continuous variables were compared using linear regression adjusting for age and sex. Logistic regression models, adjusted for familial relatedness, were constructed to test the association between the risk allele and microalbuminuria. For the purpose of creating a continuous variable for the model, the UACR was log-transformed prior to analysis. The multivariable adjusted model included age, sex, systolic blood pressure, hypertension treatment, smoking status, diabetes and HDLc. These covariates were previously shown to independently predict microalbuminuria in the Framingham Study [21].

Next, a Cox proportional hazards model that incorporated a generalized estimating equation to account for familial correlation was used to estimate the association between microalbuminuria and CVD. Participants with CVD at baseline were excluded from the longitudinal analysis. In this model, participants were censored at death or incident CVD. Two model adjustments were used: age and sex (model 1) and a multivariable
model that included age, sex, systolic blood pressure, hypertension treatment, lipid treatment, smoking status, diabetes and the ratio of total cholesterol to HDL cholesterol (total/HDL) (model 2). We next evaluated whether the presence of the CUBN risk allele was associated with incident CVD using a Cox proportional hazard model censored for death or incident CVD. Then, we evaluated the association between microalbuminuria and CVD when participants were stratified according to the presence or absence of the risk allele in order to test whether the association between microalbuminuria and CVD was modified by the presence of the risk allele. Finally, we tested whether there was an association between CVD risk and the multiplicative interaction between the presence of microalbuminuria and carriage of the minor C risk allele of rs1801239 by adding this factor into the multivariable Cox proportional hazard model. This was done to test whether the association between MA and incident CVD was modified by the presence of the CUBN risk allele.

To determine whether the presence of the risk allele was associated with other markers of proximal tubular function, we compared the levels of four urinary biomarkers: α1-microglobulin, β2-microglobulin, KIM1 and NGAL in those who did and did not carry at least one copy of the risk allele after adjustment for age and sex. For the purpose of this analysis, the biomarkers values were log-transformed to ensure normality.

All statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC) and R statistical software (version 2.15) and a two-tailed P-value of <0.05 was considered significant.

**RESULTS**

**Baseline characteristics of the participants**

In total, 6399 participants were included in the study and the baseline characteristics of the participants stratified by the presence of the risk allele are shown in Table 1. The mean age of the participants was 47.2 years and slightly more than half were women. Baseline characteristics were similar in those with and without the risk allele with the exception of albuminuria.

**The SNP rs1801239 in association with albuminuria**

Overall, 1352 (21.1%) of the participants carried at least one copy of the risk allele. The presence of the risk allele was associated with a higher median baseline UACR (4.7 versus 4.5 mg/g, P = 0.04). Similarly, the proportion of participants with microalbuminuria was higher in those carrying the risk allele (10.7 versus 8.9%, P = 0.04). In a multivariable logistic regression analysis, the risk allele was associated with increased MA [odds ratio (OR) 1.27, 95% confidence interval (CI) 1.03–1.56, P = 0.02, Table 2].

**The SNP rs1801239 in association with markers of proximal tubular function**

There was no difference in levels of β2-microglobulin or NGAL in carriers of the minor allele compared with those who did not carry the risk allele. However, higher levels of KIM1, a marker of proximal tubular damage [22], were noted in participants who carried at least one copy of the risk allele (51 versus 58 pg/g, P = 0.03). Similarly, levels of α1-microglobulin, a known cubulin ligand [11], were higher in carriers of the risk allele (0.14 versus 0.11 µg/g, P < 0.0001).

**Microalbuminuria in association with incident CVD**

Among participants free of CVD at baseline (n = 6193), 346 (5.6%) developed CVD during the follow-up period (mean 10.4 ± 3.4 years). As expected, the risk of CVD was higher in participants with microalbuminuria [hazards ratio (HR) 1.47, CI 95% 1.15–1.88, P = 0.002, Table 4]. The presence of the risk allele was not associated with an increased risk of incident CVD (HR per copy of the minor allele 1.06, 95% CI 0.82–1.36, P = 0.67, Table 3). When stratified by the presence of the risk allele, the HR for CVD in participants with microalbuminuria was 1.98 (95% CI 1.10–3.54, P = 0.02) in those carrying at least one copy of the risk allele, compared with 1.33 (95% CI 1.00–1.76, P = 0.04) in participants carrying no copies of the risk allele (Table 4). Finally, the interaction term between microalbuminuria and the risk allele on CVD was not significant (P-value for the interaction 0.49), suggesting that the association between microalbuminuria and CVD was not altered by the presence of the CUBN risk allele. However, the power of this study to identify a positive multiplicative interaction was limited (16.8%).

**DISCUSSION**

The findings in this study are four-fold. First, the presence of the CUBN risk allele rs1801239 was associated with an increased risk of microalbuminuria. Secondly, levels of the
cubulin ligand, α1-microglobulin, were elevated in carriers of the minor allele. Thirdly, microalbuminuria was associated with an increased risk of incident CVD whether or not the CUBN risk allele was present. Finally, the presence of the risk allele did not alter the association between microalbuminuria and CVD. Taken together, these findings suggest that the association between microalbuminuria and incident CVD is similar irrespective of the presence of the CUBN microalbuminuria risk allele.

In the context of the current literature

In the normal kidney, the glomerulus acts as a barrier to the filtration of albumin. Despite this, ~3 g of albumin is filtered daily. The majority is reabsorbed in the proximal tubule and a small proportion is excreted unchanged in the urine [23]. The process of albumin reabsorption can be overloaded by excessive filtration even in non-disease states [24]. This suggests that the removal of albumin from the urine is rate-limited and explains how albuminuria can develop in the setting of both glomerular and tubular disorders.

Cubulin is a 460 kDa proximal tubular membrane protein that binds and internalizes filtered proteins including vitamin B12 and albumin [25]. Albumin reabsorption in the proximal tubule is dependent on normal cubilin function. Cubilin function is in turn dependent on its interaction with megalin, a 600 kDa transmembrane protein that is part of the low-density lipoprotein receptor family. Cubilin forms a complex with megalin on the surface of epithelial cells, thus allowing ligand internalization to occur [25]. Disruption of cubilin function in utero results in embryonic death [26] but milder, functional CUBN variants have been associated with familial albuminuria [10] while up to 2 g proteinuria has been reported in two siblings with a frame-shift mutation in CUBN [27]. Cubilin has also recently been shown to be expressed on podocytes, although the relative contribution of podocyte-cubilin to ligand reabsorption remains to be determined [28]. A genome-wide association study of albuminuria identified a missense variation in CUBN which was associated with albuminuria [8]. The allele rs1801239 encodes a variant in domain 22 of CUBN which is known to be involved in the binding of cubilin to megalin [29]. Thus, the presence of this risk allele may interfere with the formation of the cubilin–megalin complex. Functionally, this could reduce the capacity for the reabsorption of filtered albumin in the proximal tubule, thereby leading to tubular albuminuria. It is notable that in this study, levels of urinary α1-microglobulin were increased in participants who carried at least one copy of the risk allele. Alpha-1-microglobulin is a ligand of cubilin that is filtered at the glomerulus and almost completely reabsorbed in the proximal tubule [11]. The presence of increased α1-microglobulin levels suggests that this variant may be functional. Albuminuria is an independent risk factor for CVD [3–5]. However, because glomerular albuminuria is generally associated with systemic disease while tubular albuminuria often results from local pathology, there is a perception that tubular albuminuria may not increase CVD risk to the same extent [16, 30]. It is difficult clinically to differentiate glomerular from tubular albuminuria, and previous studies have limited and explains how albuminuria can develop in the setting of both glomerular and tubular disorders.

Table 2. Odds of prevalent albuminuria in participants carrying at least one copy of the CUBN risk (C) allele

<table>
<thead>
<tr>
<th>Model</th>
<th>OR</th>
<th>CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>The risk of albuminuria in participants carrying at least one copy of the risk allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1a</td>
<td>1.24</td>
<td>0.99–1.56</td>
<td>0.06</td>
</tr>
<tr>
<td>Model 2b</td>
<td>1.27</td>
<td>1.03–1.56</td>
<td>0.02</td>
</tr>
</tbody>
</table>

aAdjusted for age and sex.
bAdjusted for age, sex, systolic blood pressure, hypertension treatment, lipid treatment, smoking status, diabetes and total/HDL cholesterol.

Table 3. Risk of incident CVD in participants carrying at least one copy of the CUBN risk (C) allele

<table>
<thead>
<tr>
<th>Model</th>
<th>HR</th>
<th>CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1a</td>
<td>1.07</td>
<td>0.83–1.38</td>
<td>0.61</td>
</tr>
<tr>
<td>Model 2b</td>
<td>1.06</td>
<td>0.82–1.36</td>
<td>0.67</td>
</tr>
</tbody>
</table>

aAdjusted for age and sex.
bAdjusted for age, sex, systolic blood pressure, hypertension treatment, smoking status, diabetes and total/HDL cholesterol.

Table 4. Risk of incident CVD in participants with albuminuria stratified by the presence or absence of the CUBN risk (C) allele

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>MA (%)</th>
<th>CVD events (%)</th>
<th>Age- and sex-adjusted model</th>
<th>Multivariable-adjusted modela</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HR</td>
<td>CI</td>
</tr>
<tr>
<td>MA overallb</td>
<td>6193</td>
<td>539 (8.7)</td>
<td>346 (5.6)</td>
<td>1.74</td>
<td>1.37–2.22</td>
</tr>
<tr>
<td>MA in the presence of the risk allelec</td>
<td>1310</td>
<td>135 (10.3)</td>
<td>77 (5.9)</td>
<td>2.32</td>
<td>1.35–3.97</td>
</tr>
<tr>
<td>MA in the absence of the risk alleled</td>
<td>4883</td>
<td>404 (8.3)</td>
<td>269 (5.5)</td>
<td>1.59</td>
<td>1.21–2.10</td>
</tr>
<tr>
<td>Interaction (risk allele×MA)e</td>
<td></td>
<td></td>
<td></td>
<td>0.36</td>
<td></td>
</tr>
</tbody>
</table>

aAdjusted for age, sex and systolic blood pressure, hypertension treatment, lipid treatment, smoking status, diabetes and total/HDL cholesterol.
bExcluding participants with CVD at baseline.
cThe association of microalbuminuria with CVD risk in participants carrying at least one copy of the risk allele.
dThe association of microalbuminuria with CVD risk in participants carrying no copies of the risk allele.

ORIGINAL ARTICLE

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employed indirect measures. These include the use of dextran clearance as a surrogate for glomerular permeability [31], the simultaneous measurement of the excretion of low-molecular weight proteins that are normally reabsorbed in the proximal tubule [32], or the inhibition of proximal tubular albumin reabsorption with L-arginine [33]. However, these methods are not suitable for population-based studies. The presence of specific cubilin ligands in the urine in the absence of other markers of glomerular damage is suggestive of tubular rather than glomerular pathology. However, given that these ligands are generally filtered at the glomerulus, this is not entirely specific and could represent over-filtration or overproduction. Thus, the relative contribution of tubular and glomerular albuminuria to CVD risk remains to be well-characterized.

**Potential mechanisms**

How tubular albuminuria could increase CVD risk remains unclear. Recent studies have found experimental evidence for the local and systemic toxicity of increased urinary albumin excretion. Albuminuria induces apoptosis in cells of the proximal and distal nephron [14] and also induces the production of pro-inflammatory cytokines in PTECs as well as pro-fibrotic transforming growth factor-β (TGF-β) [12, 13]. The secretion of TGF-β is not dependent on albumin endocytosis, suggesting that albumin acts as a ligand for a receptor-mediated process [15]. TGF-β, in turn, inhibits albumin reabsorption through its effects on the cubilin/megalin complex [34]. It is thought that albumin may signal its effects via the epidermal growth factor receptor which in turn plays a role in mediating intracellular signaling induced by angiotensin II/angiotensin receptor interactions and renal fibrosis [17]. Increased urinary catecholamines have been noted in patients with albuminuria independent of blood pressure and particularly in those with normal renal function [35]. Thus, there is significant interplay between albumin reabsorption in PTECs, TGF-β expression and angiotensin effects, potentially leading to wider systemic effects. However, these models, while useful, are imperfect and may not adequately distinguish tubular versus glomerular albuminuria.

It is uncertain what role other cubilin ligands may play in this process. In Dent’s disease, caused by a defect in proximal tubular endocytosis, it is believed that signaling by parathyroid hormone in the distal part of the proximal tubule may be responsible for some of the systemic effects noted in patients with this disorder [36]. Similarly, there may be other, unidentified mediators of renal toxicity apart from albumin in participants carrying the CUBN risk allele.

**Implications**

The major implication of this study is that albuminuria is associated with increased CVD risk irrespective of the presence of the cubilin risk allele. This suggests several potential avenues for future research. Current techniques for distinguishing glomerular and tubular albuminuria are not yet ready to be applied to population-based studies [31, 32]. The accurate differentiation of albuminuria could allow us to study tubular albuminuria as an entity and provide insights into the relationship with CVD risk as well as identifying causal pathways. There is growing recognition of the importance of tubular albuminuria in a variety of renal disorders, particularly early diabetic nephropathy [37, 38]. Ultimately, a better understanding of the relationship between albuminuria and systemic disease may lead to the development of targeted therapies in the future. The CUBN risk allele rs1801239 explains a relatively small proportion of the variance in albuminuria seen in the population. It may be that there are other, rare variants which remain to be identified that contribute more to albuminuria.

**Strengths and limitations**

Strengths of this study include the well-characterized Framingham Heart Study and well-defined cardiovascular risk factors. However, there are some limitations that should be mentioned. The participants are predominantly of European ancestry, which may limit the generalizability of the results. However, the association between the CUBN risk allele and albuminuria was shown to be robust across ethnicities [8]. UACR was measured on a single sample; there is significant intra-individual variability in UACR measurements and this could lead to misclassification in some cases. The power of the study to detect a positive interaction between the risk allele and albuminuria was low and this should be considered when interpreting the negative result. The relative contribution of tubular versus glomerular albuminuria to the increased CVD risk noted in this study remains uncertain due to the lack of a marker that categorically distinguishes between them. For this reason, we cannot be certain that albuminuria in the presence of the CUBN risk allele was solely due to a cubilin-related etiology. The clinical correlates of tubular albuminuria remain to be determined, and may include risk factors traditionally associated with glomerular albuminuria.

**CONCLUSION**

Microalbuminuria is associated with CVD irrespective of the presence of the CUBN risk allele. These results challenge the concept that albuminuria in the setting of the CUBN mutation is benign.

**ACKNOWLEDGEMENTS**

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**CONFLICT OF INTEREST STATEMENT**

None declared.
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