ORIGINAL ARTICLE

A high burden of cytomegalovirus marks poor vascular health in transplant recipients more clearly than in the general population

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Abstract

Objectives. Meta-analyses have now confirmed that persistent infections with cytomegalovirus (CMV) can accelerate the onset of diseases of ageing, notably cardiovascular pathologies. We address the circumstances in which the association may be strong enough to warrant intervention to reduce the viral burden. **Results.** We compare markers of the burden of CMV with established indices of vascular pathology in healthy adults ($n = 82$) and in renal transplant recipients (RTR; $n = 81$). Levels of all inflammatory and vascular biomarkers and CMV antibodies were higher in RTR, and flow-mediated dilation (FMD) values were lower indicating inferior endothelial function. In multivariable regression models without adjustment for estimated glomerular filtration rate (eGFR), CMV antibody levels, age and gender were independently associated with FMD in RTR, whilst only CRP associated with FMD in healthy adults. After adjustment for eGFR, associations between CMV antibody and FMD in RTR were reduced. **Methods.** Carotid intima-media thickness, FMD, eGFR and plasma levels of CMV antibodies (reactive with a lysate, CMV IE-1 or CMV gB), ICAM-1, VCAM-1, P-selectin, sIFNαR2, sTNFR1, sCD14 and CRP were determined. **Conclusion.** Levels of CMV antibody predict declining endothelial health in RTR and not in healthy adults, presumably by reflecting a high burden of CMV. The levels of CMV antibodies were a poor reflection of plasma biomarkers thought to reflect 'inflammaging' or vascular damage.

Keywords: cytomegalovirus, inflammatory biomarkers, transplantation, vascular pathology

INTRODUCTION

Cytomegalovirus (CMV) is a β-herpesvirus and replicates in endothelial cells, fibroblasts and monocytes.[1] It is a common virus, where 45–90% of any population are seropositive, depending on age, sex, geographic location and socio-economic factors.[2] CMV has the capacity to become latent and be retained lifelong, with reactivations triggered by inflammation and the viral burden enhanced by immunodeficiency.[3] Acute infections in immunocompetent hosts are usually asymptomatic, but CMV can cause severe disease in transplant recipients, newborns and HIV-infected patients.[4] Moreover, active CMV infections and CMV seropositivity are associated with reduced responses to vaccination[5] and higher incidences of age-related diseases such as vascular dementia[6] and cardiovascular disease.[7,8] Evidence linking CMV with cardiovascular diseases includes the presence of CMV antigen and DNA in smooth muscle cells from patients with aortic aneurysms[9] and in atherosclerotic arteries.[10] These associations have been confirmed in meta-analyses (e.g. Ref. 11) and may reflect chronic immune activation. For example, plasma C-reactive protein (CRP) levels were increased by CMV seropositivity in individuals being evaluated for coronary artery disease.[12] CMV replication in endothelial and smooth muscle cells was associated with atherosclerosis[10] and may upregulate leucocyte adhesion molecules (e.g. VCAM-1 and ICAM-1)[13,14] and pro-inflammatory cytokines.

CMV is a major infectious complication following renal transplantation. Renal transplant recipients (RTR) receive antiviral prophylaxis for several months after transplantation to avoid CMV-related morbidity, including graft rejection.[15] The incidence of cardiovascular events is higher in CMV-seropositive than in seronegative RTR, with the highest risk in RTR who have evidence of CMV replication.[16] Plasma levels of VCAM-1 and ICAM-1 were higher in RTR than in healthy controls and rose during an active CMV infection.[17] We have described an association between CMV seropositivity and flow-mediated dilatation (FMD) of the brachial artery in RTR[18] and use the same cohort to address whether vascular pathology is more effectively predicted by markers of the burden of CMV than by plasma markers of systemic and vascular inflammation. These included soluble (s) IFNαR2, sTNFR1, sCD14, CRP, VCAM-1, ICAM-1 and P-selectin. Large vessel health was assessed by carotid intima-media thickness (cIMT) and FMD. cIMT is a measurement of the thickness of tunica intima and tunica media, the innermost layers of the arterial wall, and is an independent predictor of myocardial infarction and stroke in older adults.[19] Brachial arterial FMD is assessed by high-resolution ultrasound after blood flow is occluded, and reflects endothelium-dependent vasodilator function. It has been validated as a surrogate of endothelial function of the coronary circulation and associates with prevalent and incident cardiovascular disease.[20]

RESULTS

RTR had a higher burden of CMV, elevated levels of inflammatory and vascular biomarkers and reduced FMD values

Demographic, clinical and laboratory assessments of 81 healthy adults and 82 RTR (Table 1) revealed similar ratios of males and females, but RTR were marginally older ($P = 0.07$). CMV antibody levels and the detection of CMV DNA support a higher burden of CMV in RTR. CMV antibody levels remained higher when analyses were restricted to seropositive individuals, and age and transplantation optimally predicted CMV antibody levels in a linear regression.[18]

RTR had higher levels of inflammatory markers (sIFNαR2, sTNFR1, sCD14 and CRP) and vascular markers (ICAM-1, VCAM-1 and P-selectin). The levels of ICAM-1 increased with time since transplant ($r = 0.25$, $P = 0.02$), with a marginal rise in sCD14 ($r = 0.20$, $P = 0.07$). FMD values were lower in RTR than in healthy adults ($P < 0.0001$), but cIMT readings and blood pressure (BP) were similar. Plaques were detected more often in RTR.

Markers of the burden of CMV correlate weakly with inflammatory and vascular markers

As most parameters were affected by RTR status, correlations were examined separately in healthy adults and RTR (Table 2). The levels of CMV lysate antibody increased with time since transplant. This did not simply reflect increasing age, as antibody levels correlated with age in healthy adults, but not in RTR.

We noted weak positive correlations between CMV antibody levels and sIFNαR2, ICAM-1 and VCAM-1 in RTR. CMV antibody levels also correlated with VCAM-1 in healthy adults. These

Table 1. Demographic, clinical and laboratory assessments of healthy adults and renal transplant recipients (RTR)

	Healthy adults (n = 81)	RTR (n = 82)	P-value[a]
Age (years)	54 (21–86)[b]	57 (31–76)	0.07
Gender (Male/Female)	38/43	46/36	0.27
Time since transplant (years)	–	7 (2–37)	
Creatinine (μmol L^{-1})	69 (46–109)	108 (51–439)	< 0.0001
eGFR (mL min^{-1})	97 (58–130)	60 (11–111)	< 0.0001
CMV antibody and DNA in plasma			
CMV lysate (AU mL^{-1})	56 (0.0–1496)	606 (0.0–7611)	< 0.0001
CMV gB (AU mL^{-1})	5.6 (0.0–40.1)	24.6 (0.0–293)	< 0.0001
CMV IE-1 (AU mL^{-1})	6.3 (0.9–156)	9.6 (0.5–477)	< 0.0001
CMV DNA			
Undetectable	10	61	0.20
< 20 copies per mL	–	9	
> 20 copies per mL	–	7	
Not done	71	5	
Inflammatory markers in plasma			
sIFNαR2 (ng mL^{-1})	4.3 (2.5–7.9)	8.7 (4.3–21)	< 0.0001
sTNFR1 (ng mL^{-1})	0.6 (0.4–1.2)	1.4 (0.5–3.8)	< 0.0001
sCD14 (μg mL^{-1})	1.7 (1.0–2.7)	2.0 (1.4–3.6)	< 0.0001
CRP (μg mL^{-1})	0.8 (0.1–7.1)	1.5 (0.1–20)	0.0003
Vascular markers in plasma			
ICAM-1 (ng mL^{-1})	125 (76–214)	142 (86–330)	0.03
VCAM-1 (ng mL^{-1})	308 (184–552)	447 (249–1143)	< 0.0001
P-selectin (ng mL^{-1})	44 (17–117)	50 (27–107)	0.03
Clinical assessments			
BMI	26.2 (18.2–39.8)	27.0 (17.0–58.0)	0.08
FMD	7.9 (1.4–18.0)	3.9 (0.0–15.8)	< 0.0001
Left cIMT (mm)	0.64 (0.44–0.93)	0.66 (0.44–1.30)	0.17
Right cIMT (mm)	0.64 (0.42–1.19)	0.65 (0.43–1.33)	0.22
Average cIMT (mm)	0.64 (0.43–0.93)	0.67 (0.44–1.31)	0.15
Systolic BP (mmHg)	121 (90–178)	124 (97–189)	0.18
Diastolic BP (mmHg)	71 (52–98)	72 (58–96)	0.30
Plaques (Yes/No)	1/80	10/63	0.003

BMI, body mass index; BP, blood pressure; cIMT, carotid intima-media thickness; CMV, cytomegalovirus; eGFR, estimated glomerular filtration rate; FMD, flow-mediated dilation; RTR, renal transplant recipient.
[a]Mann–Whitney rank sum test for continuous data, Fisher's exact test for categorical data.
[b]Median (range).

correlations remained evident when CMV antibodies were assessed using CMV gB (r = 0.19, P = 0.09) or CMV IE-1 (r = 0.24, P = 0.04) antigens.

Plasma CMV DNA was detectable in 16 CMV-seropositive RTR. Amongst CMV-seropositive RTR, those with CMV DNA had more recent transplants (P = 0.01), higher levels of VCAM-1 (P = 0.04) and lower levels of P-selectin (P = 0.01) (Supplementary table 1). However, the presence of CMV DNA did not affect vascular pathology.

Vascular health correlates with age, CMV antibodies, plasma biomarkers and eGFR in controls, and with age and VCAM-1 in RTR

FMD and cIMT deteriorated with age in RTR and healthy adults (Table 3). Amongst healthy adults,

FMD correlated with plasma levels of sTNFR1, whilst cIMT correlated with sIFNαR2, sTNFR1, ICAM-1, VCAM-1 and CMV lysate antibody. These associations were not seen in RTR. VCAM-1 correlated inversely with FMD (i.e. with impaired endothelial function) in RTR but this was not found with other markers and so should be interpreted with caution.

Also amongst healthy adults, eGFR correlated directly with FMD and inversely with cIMT. As the eGFR algorithm incorporates age, it is notable that creatinine levels also suggest a link between renal function and vascular pathology in healthy adults, but not RTR. Accordingly, eGFR correlated inversely with levels of sIFNαR2, sTNFR1, ICAM-1 and VCAM-1 (r = −0.55 to −0.22, P < 0.0001 to 0.055) in healthy adults and with sIFNαR2, sTNFR1,

Table 2. Levels of CMV lysate antibody correlate with age, eGFR, VCAM-1 and cIMT in healthy adults, and with selected inflammatory markers in RTR

	Healthy adults (*n* = 81)		RTR (*n* = 82)	
	r[a]	*P*-value	*r*	*P*-value
Demographic and renal factors				
Age	0.23	0.04	0.04	0.72
Time since transplant	–	–	0.28	0.01
eGFR	−0.24	0.03	−0.20	0.06
Other measures of CMV antibody				
CMV gB	0.82	< 0.001	0.66	< 0.001
CMV IE-1	0.42	< 0.001	0.70	< 0.001
Vascular and inflammatory markers				
sIFNαR2	0.06	0.59	0.24	0.03
sTNFR1	0.07	0.56	0.14	0.21
sCD14	0.01	0.95	0.13	0.24
CRP	−0.23	0.04	0.00	1.00
ICAM-1	0.16	0.16	0.21	0.06
VCAM-1	0.30	0.007	0.21	0.06
P-selectin	−0.16	0.16	0.03	0.80

cIMT, carotid intima-media thickness; CMV, cytomegalovirus; eGFR, estimated glomerular filtration rate; RTR, renal transplant recipient.
[a]Spearman correlation coefficients.

sCD14, ICAM-1 and VCAM-1 ($r = −0.83$ to $−0.21$, $P < 0.0001$ to 0.057) in RTR.

No plasma biomarkers were associated with the detection of atherosclerotic plaque in CMV-seropositive RTR, but the levels of sCD14 were marginally higher ($P = 0.06$; data not shown). Only one healthy adult had detectable plaque. BP readings showed few associations (data not shown).

Multivariable analyses link CMV lysate antibody with FMD values in RTR

Multivariable regression models were used to assess the independent effects of CMV antibody levels, plasma biomarkers, cIMT or BP on FMD with adjustments for age, gender and body mass index (BMI). Analyses were performed with and without adjustment for covariate eGFR as these data correlate with several other metrics and the calculation of eGFR incorporates age (Table 4).

All participants

In optimal models without eGFR, CMV lysate antibody levels ($P = 0.022$), age ($P = 0.024$), gender ($P < 0.001$) and transplant status ($P < 0.001$) were independently associated with FMD (Table 4). This

Table 3. cIMT and FMD correlate with a range of biomarkers in healthy adults, but not in RTR

	Healthy adults (*n* = 81)		RTR (*n* = 82)	
	r[a]	*P*-value	*r*	*P*-value
FMD versus...				
Age	−0.22	0.05	−0.29	0.01
Time since transplant	–	–	0.12	0.32
Creatinine	−0.33	0.003	−0.17	0.16
eGFR	0.23	0.04	0.17	0.15
CMV lysate	−0.19	0.09	−0.09	0.44
sIFNαR2	−0.12	0.30	−0.16	0.19
sTNFR1	−0.25	0.02	−0.10	0.39
sCD14	0.11	0.34	−0.04	0.75
CRP	0.06	0.58	−0.19	0.12
ICAM-1	−0.18	0.12	−0.06	0.63
VCAM-1	−0.14	0.23	−0.24	0.04
P-selectin	-0.19	0.10	0.17	0.15
cIMT versus...				
Age	0.72	< 0.001	0.46	< 0.001
Time since transplant	–	–	−0.02	0.86
Creatinine	0.26	0.017	−0.18	0.14
eGFR	−0.64	< 0.001	0.09	0.45
CMV lysate	0.28	0.01	−0.11	0.36
sIFNαR2	0.22	0.05	−0.16	0.17
sTNFR1	0.28	0.01	−0.11	0.37
sCD14	0.12	0.29	−0.06	0.62
CRP	0.08	0.48	−0.08	0.49
ICAM-1	0.23	0.04	0.06	0.62
VCAM-1	0.28	0.01	−0.05	0.65
P-selectin	−0.02	0.84	−0.14	0.25

cIMT, carotid intima-media thickness; CMV, cytomegalovirus; eGFR, eGFR,estimated glomerular filtration rate; FMD, FMD, flow-mediated dilation; RTR, renal transplant recipient.
[a]Spearman correlation coefficients comparing FMD and average cIMT with the parameters listed.

was not improved with the inclusion of inflammatory and vascular markers. When CMV lysate antibody data were removed from the analysis, CMV IE-1 antibody became the clearest novel predictor ($P = 0.044$), with age ($P = 0.047$), gender ($P < 0.0001$) and transplant status ($P < 0.0001$) (adjusted $r^2 = 0.281$). With eGFR, the optimal model retained CMV IE-1 antibody ($P = 0.027$).

Healthy adults

In the optimal model without eGFR, only CRP levels ($P = 0.042$) were independently associated with FMD. No associations with CMV antibody levels were retained in the optimal model but CMV IE-1 showed a weak association ($P = 0.042$) when tested individually. CRP retained a

Table 4. Multivariable stepwise regression analyses for the prediction of FMD

	Unstandardised coefficients		Standardised	
	B	SE	β coefficient	P-value
All participants (n = 163; adjusted r^2 for model = 0.281)				
Age	−0.053	0.023	−0.168	0.024
Gender	−2.066	0.555	−0.275	< 0.001
Transplant status	−2.340	0.615	−0.312	< 0.001
BMI	0.061	0.051	0.088	0.238
CMV lysate antibody	−0.001	0.000	−0.184	0.022
All participants controlling for eGFR (n = 163; adjusted r^2 for model = 0.291)				
Age	−0.34	0.025	−0.108	0.170
Gender	−2.074	0.558	−0.276	< 0.001
Transplant status	−1.699	0.785	−0.226	0.032
BMI	0.082	0.052	0.119	0.117
eGFR	0.029	0.017	0.194	0.085
CMV IE-1 antibody	−0.011	0.005	−0.168	0.027
Healthy adults (n = 81; adjusted r^2 for model = 0.052)				
Age	−0.023	0.032	−0.085	0.470
Gender	−0.805	0.811	−0.119	0.324
BMI	−0.021	0.090	−0.028	0.819
CRP	0.585	0.282	0.261	0.042
Healthy adults controlling for eGFR (n = 81; adjusted r^2 for model = 0.069)				
Age	0.036	0.051	0.133	0.477
Gender	−0.755	0.805	−0.112	0.352
BMI	−0.027	0.089	−0.037	0.764
eGFR	0.062	0.041	0.275	0.138
CRP	0.645	0.282	0.288	0.025
RTR (n = 82; adjusted r^2 for model = 0.253)				
Age	−0.086	0.035	−0.268	0.016
Gender	−3.054	0.782	−0.442	< 0.001
BMI	0.046	0.063	0.081	0.466
CMV lysate antibody	−0.001	0.000	−0.293	0.011
RTR controlling for eGFR (n = 82; adjusted r^2 for model = 0.224)				
Age	−0.087	0.035	−0.270	0.017
Gender	−2.379	0.776	−0.344	0.003
BMI	0.115	0.066	0.202	0.087
eGFR	0.041	0.019	0.240	0.040

BMI, body mass index; CMV, cytomegalovirus; eGFR, estimated glomerular filtration rate; FMD, flow-mediated dilation; RTR, renal transplant recipient.

significant association with the inclusion of eGFR in the model.

RTR

When RTR were analysed without adjustment for eGFR, the optimal model showed independent associations between FMD and age (P = 0.016), gender (P < 0.001) and CMV lysate antibody (P = 0.011). With the inclusion of eGFR, CMV lysate antibody was not retained in the optimal

model – probably because antibody levels correlate with eGFR (Table 2).

Multivariable analyses revealed no associations between cIMT and CMV antibody levels or inflammatory and vascular biomarkers

CMV antibody levels, inflammatory and vascular markers were not associated with average cIMT in multivariable analyses when all participants were assessed together or when RTR or healthy adults were evaluated separately (data not shown). The only significant association was with age (P < 0.0005).

DISCUSSION

Evaluations of the effects of CMV require a robust measure of the burden of CMV in an individual which is addressed here in two distinct populations: RTR and healthy adults with a similar age and gender mix. The use of antibodies as a metric of the burden of CMV is complicated by increased levels in older individuals, demonstrated in the Australian population.[21] Here, transplantation overcame the effect of age, but the levels of CMV antibody were elevated in RTR and increased with time post-transplant. This is consistent with episodic subclinical CMV reactivations in patients on antirejection therapy, as evidenced by the detection of CMV DNA in some (not all) RTR. CMV DNA was also sought in ten controls with high levels of CMV antibody – none had detectable CMV DNA. Indeed, reactivations from latency are rare in otherwise healthy individuals of this age group, but more common in the elderly.[22] Overall, our data support a model whereby CMV lysate antibody levels are a useful surrogate marker of cumulative viral burden throughout an individual's lifetime, whereas the detection of CMV DNA is rare and stochastic.[23]

Links between CMV reactivations and inflammation or stress are often cited, but clinical evidence is scant. One study associated CMV antibody levels with TNF-α and IL-6 in sera from young and elderly individuals.[24] We found no correlation between CMV antibody levels and sTNFR1 in healthy controls or patients with nontuberculous mycobacteria,[21] and there was no consistent pattern here in RTR or healthy adults. Our data suggest that CMV antibody is not a surrogate marker of 'inflammaging'.[25] Accordingly,

'inflammaging' is characterised by age-dependent increases in plasma pro-inflammatory markers (IL-6, TNF-α and CRP) that are also seen in CMV-seronegative individuals.[26]

The key finding of our study is evidence that the levels of CMV antibody, but not inflammatory or vascular markers, were a significant independent marker of reduced FMD values in RTR, after adjustment for age, BMI and gender. FMD assesses response of the endothelium to shear stress and nitric oxide release, and impairment is considered to be an early marker of poor endothelial health, atherosclerosis and a predictor of future cardiovascular events.[20] Amongst healthy adults, the clearest determinant of FMD was CRP, and the optimal statistical models were weak. No significant models were obtained for cIMT. Although cIMT correlated with CMV antibodies in bivariate analyses, this may reflect parallel rises in both parameters with age.

High levels of CD14, sIFNαR2, sTNFR1, CRP, ICAM-1 and VCAM-1 correlated with poor kidney function (low eGFR). Accordingly, plasma levels of sCD14 were increased in patients with reduced kidney function and associated with cardiovascular disease in patients with chronic kidney disease.[27] We observed negative associations between CMV antibody levels and eGFR (Table 2), as previously reported in elderly individuals.[28] Persistent CMV infection may have a significant impact on renal vasculature via direct and indirect mechanisms. CMV DNA has been detected in renal arteries from seropositive donors, and these tissues can be infected *in vitro* by several CMV strains.[29] Furthermore, the presence of CMV in the graft was associated with arteriosclerotic changes in the small arterioles.[30] The relationship between CMV and renal function warrants further investigation in larger cohorts.

Whilst the small size of our cohort precludes Bonferroni corrections for the large numbers of bivariate comparisons, our multivariable analyses provide evidence that CMV associates with the elevated risk of vascular pathology seen in RTR. The association was not evident in healthy adults of a similar demographic mix. The association was marked by the levels of antibody reactive with a lysate of CMV-infected fibroblasts, which may provide a more stable measure of the burden of CMV than the detection of CMV DNA. Indeed CMV antibody was a clearer marker of declining FMD in RTR than other well-documented inflammatory and vascular biomarkers. In contrast, a marker of 'inflammaging' more effectively marked low FMD values in healthy adults, and cIMT values increased with age with no other significant influences identified. This does not refute a link between CMV and cardiovascular change in the general population, but shows that it is not evident in a cohort of this size assessed using CMV antibody levels or plasma CMV DNA.

METHODS

Study cohorts

Renal transplant recipients were recruited from the renal clinics at Royal Perth Hospital, Western Australia. All participants were clinically stable on standard immunosuppressive regimens, > 2 years after transplantation, with no CMV disease within 6 months of blood collection and no ongoing antiviral treatment. Healthy adults were recruited through local advertisements. Venous blood samples were collected, and plasma was aliquoted and stored at −80°C. Plasma creatinine was measured at PathWest Laboratory Medicine (Western Australia) and estimated glomerular filtration rate (eGFR) was calculated using Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula.[31] Written informed consent was obtained from each participant. The project was approved by Human Ethics Committees of Royal Perth Hospital, University of Western Australia and Curtin University.

DNA, CMV antibodies and plasma biomarkers

Plasma was screened for CMV DNA in the Department of Microbiology (Royal Perth Hospital) using a commercial kit (Abbott Diagnostics, Lake Bluff, IL, USA) detecting > 20 copies per mL. Plasma antibody titres were determined using three antigen preparations [CMV lysate, glycoprotein B (gB) antigen and IE-1 antigen].[17] Coefficients of variance were 6.1% (CMV lysate), 2.9% (gB) and 4.8% (IE-1). CMV seropositivity was defined as > 3 standard deviations above mean antibody levels from 10 seronegative individuals assessed using ARCHITECT CMV IgG assays (Abbott Diagnostics). Results are presented as arbitrary units (AU) per mL based on a standard plasma pool. Plasma levels of vascular biomarkers (P-selectin, ICAM-1 and VCAM-1) and inflammatory biomarkers (sTNFR1, sCD14 and CRP) were quantified by ELISA using antibody pairs (R&D Systems, Minneapolis, MN, USA). Plasma sIFNαR2 was quantified using precoated ELISA kits kindly donated by PBL Assay Science (Piscataway, NJ, USA).

Noninvasive assessments of vascular pathology

cIMT (mm; measuring carotid artery wall thickness and detecting atherosclerotic plaques) was based on guidelines from the American Society of Echocardiography.[19] Brachial artery diameter was determined before and after occlusion of the artery, and FMD was calculated as a percentage

difference between the resting and inflated diameters.[20] Systolic blood pressure (BP) and diastolic blood pressure were assessed after 10 minutes of rest.

Statistical analyses

Bivariate analyses utilised GraphPad Prism (La Jolla, CA, USA). Data are presented as median (range) and analysed using nonparametric Mann–Whitney rank sum tests (unpaired data), Wilcoxon tests (paired data) and Spearman's correlations. Multivariable regression analyses were run with Stata (SE 14.2, StataCorp LP, College Station, TX, USA) to identify factors independently associated with vascular health. Significance was defined as $P \leq 0.05$.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.