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Richard B. Kim
Western University, richard.kim@lhsc.on.ca

Bridget L. Morse
Western University

Ognjenka Djurdjev
The University of British Columbia

Mila Tang
The University of British Columbia

Norman Muirhead
Western University

See next page for additional authors

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Richard B. Kim[1], Bridget L. Morse[1], Ognjenka Djurdjev[2], Mila Tang[2], Norman Muirhead[1], Brendan Barrett[3], Daniel T. Holmes[4], Francois Madore[5], Catherine M. Clase[6], Claudio Rigatto[7] and Adeera Levin[2]; for the CanPREDDICT Investigators[8]

[1]Department of Medicine, Western University, London, Ontario, Canada; [2]Division of Nephrology, University of British Columbia, Vancouver, British Columbia, Canada; [3]Memorial University Newfoundland, St. John's, Newfoundland, Canada; [4]Department of Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada; [5]University of Montreal, Montreal, Quebec, Canada; [6]McMaster University, Hamilton, Ontario, Canada; and [7]University of Manitoba, Winnipeg, Manitoba, Canada

Cardiovascular disease is more common in patients with chronic kidney disease (CKD), and traditional risk factors do not adequately predict those at risk for cardiovascular (CV) events. Recent evidence suggests elevated trimethylamine N-oxide (TMAO), created by gut microflora from dietary L-carnitine and choline, is associated with CV events. We investigated the relationship of TMAO levels in patients with stages 3b and 4 CKD to ischemic CV events using the CanPREDDICT cohort, a Canada-wide observational study with prospective 3-year follow-up of adjudicated CV events. Baseline samples were obtained for 2529 CKD patients. TMAO, choline, and L-carnitine levels were measured using tandem mass spectrometry. Baseline median TMAO level was high for the whole cohort (20.41 *μM;* interquartile range [IQR]: 12.82–32.70 μM). TMAO was independently associated with CV events (hazard ratio 1.23; 95% confidence interval: 1.06–1.42 / 1 SD lnTMAO) after adjusting for all potential CV risk factors. Those in the highest TMAO quartile had significantly higher risk of CV events (adjusted hazard ratio 1.59; 95% confidence interval: 1.04–2.43; $P = 0.0351$) in the analysis of recurring ischemic events. Among those with stage 3b CKD (hazard ratio 1.45; 95% confidence interval: 1.12–1.87 / 1 SD lnTMAO), independent of kidney function, TMAO levels identified those at highest risk for events. Our results suggest that TMAO may represent a new potentially modifiable CV risk factor for CKD patients. Further studies are needed to determine sources of variability and if lowering of TMAO reduces CV risk in CKD.

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Correspondence: *Richard B. Kim, Western University, University Hospital, 339 Windermere Road, London, Ontario N6A 5A5, Canada; or Adeera Levin, University of British Columbia, 1081 Burrard Street, Room 6010A, Vancouver, British Columbia V6Z 1Y8, Canada. E-mail: Richard.Kim@Lhsc.on.ca or alevin@providencehealth.bc.ca*

[8]See Appendix for the members of the CanPREDDICT Investigators.

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Patients with chronic kidney disease (CKD) are at an increased risk for adverse cardiovascular (CV) events, and traditional markers of CV disease do not appear to account for the high CV risk in this population.[1] Multiple interventions using conventional CV risk reduction strategies have had limited success in CKD populations.[2] Recently, a potential novel marker of CV disease, trimethylamine N-oxide (TMAO), was identified in subjects with normal renal function, where increased plasma concentrations of TMAO were associated with an increased risk of experiencing an adverse CV event.[3] Importantly, this association remained even after adjustment for traditional risk factors, suggesting plasma TMAO concentrations to be an independent biomarker of CV risk.[3]

TMAO is the primary metabolite of trimethylamine (TMA), and is formed in the liver via TMA conversion by flavin-containing monooxygenase isoform 3 (FMO3).[4,5] The metabolism and conversion of TMAO has been described in detail.[6] There is an important role of gut flora[7,8] in mediating the metabolism of the precursors, choline and L-carnitine, which are found abundantly in eggs and red meat. TMAO's role in the pathogenesis of CV disease is thought to be via enhanced accumulation of cholesterol in macrophages as well as accumulation of foam cells in artery walls.[7] TMAO is known to be renally excreted;[9] however, urinary excretion in healthy subjects varies nearly 700-fold, which suggests that variability from dietary sources[9] or other mechanisms may be important. It is known that TMAO plasma concentrations are increased in subjects with end-stage renal disease,[10] but TMAO levels have not been characterized in patients with different levels of reduced kidney function, notably those with estimated glomerular filtration rate (eGFR) <45 ml/min per 1.73 m^2.

The use of statins and the use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers are the predominant strategies currently proven to address CV risk in CKD,[11,12] but long-term mortality benefits are diminished when compared to non-CKD patients.[2] New strategies to mitigate or modify CV risk in CKD are needed to address the excess in CV disease in this high-risk population. Given the potential role of diet and drugs in reducing TMAO levels, we were interested in assessing TMAO levels in CKD patients in

the community. Importantly, we hypothesized that TMAO plasma concentrations would be significantly elevated in CKD patients and thereby increase the risk for CV events. Recently, a study by Tang *et al.* demonstrated that TMAO levels are elevated in CKD patients, and that those with higher TMAO levels were associated with higher mortality rate.[13] In the study by Tang *et al.*, higher TMAO levels contribute to renal fibrosis and dysfunction and thereby increase mortality risk from CKD.[13] However, the relationship between TMAO plasma concentrations and ischemic CV events has not been systematically assessed in a cohort of CKD patients, particularly with sufficient sample size to provide robust CV risk–associated outcomes in this group. If TMAO levels in CKD patients can be shown to be independently associated with higher CV risk in the CKD population, then non-pharmacologic interventions that focus on modulation of TMAO levels through diet or alteration of microbial gut flora may prove to be of particular benefit in this population in addition to current CV risk reduction strategies.

We thus evaluated the relationship between renal function, TMAO plasma concentrations, and ischemic CV risk, due to the proposed pathologic mechanism involving atherosclerotic CV disease, in a cohort of 2529 adult CKD patients who were enrolled across Canada in a multicenter prospective observational study (Supplementary Figure S1). The overall study focused on collection of clinical outcomes and biologic samples, with the goal of identifying more predictive biomarkers of CV disease in CKD subjects (Canadian Study of Prediction of Risk and Evolution to Dialysis, Death and Interim Cardiovascular Events Over Time [CanPREDDICT]).[14]

RESULTS

Baseline characteristics and TMAO levels

Table 1 describes the baseline characteristics for the overall CKD population, and in those patients who did and did not have a primary outcome, that is, an ischemic CV event over 3 years of follow-up. The mean age of the participants was 68 years; over 60% were men, and the prevalence of diabetes was high. One-third of study participants had a history of ischemic heart disease, while 46% of these patients also had a history of congestive heart failure. The mean (SD) eGFR at the baseline visit was 28.0 ml/min per 1.73 m^2 (9.0 ml/min per 1.73 m^2); 39% of patients had stage 3b CKD (eGFR 30–45 ml/min per 1.73 m^2) and 61% had stage 4 (eGFR <30 ml/min per 1.73 m^2).

Table 1 | Baseline characteristics, overall and according to ischemic cardiovascular events during 3-year follow-up

Characteristic	All participants $N = 2529$	Participants without event $N = 2265$	Participants with events $N = 264$	*P* value
Age	68.2 (12.7)	67.8 (12.9)	71.4 (10.2)	<0.0001
Male	1580 (62.5%)	1404 (62.0%)	176 (66.7%)	0.14
Caucasian	2243 (88.7%)	2007 (88.6%)	236 (89.4%)	0.70
Diabetes	1218 (48.2%)	1053 (46.5%)	165 (62.5%)	<0.0001
Ischemic heart disease	848 (33.5%)	701 (30.9%)	147 (55.9%)	<0.0001
Congestive heart failure	681 (26.9%)	584 (25.8%)	97 (36.7%)	0.0001
Systolic blood pressure, mm Hg	133.8 (20.0)	133.3 (19.6)	137.5 (22.8)	0.0054
Diastolic blood pressure, mm Hg	70.9 (11.9)	71.1 (12.0)	69.0 (10.8)	0.0034
Weight, kg	83.5 (19.7)	83.5 (19.8)	83.6 (19.6)	0.94
BMI, kg/m^2	29.5 (6.4)	29.5 (6.5)	29.5 (6.1)	0.98
TMAO, μM	20.41 [12.82–32.70]	19.71 [12.44–31.84]	26.21 [16.56–40.89]	<0.0001
Carnitine, μM	23.7 (6.3)	23.6 (6.3)	24.7 (6.4)	0.0084
Choline, μM	21.2 (7.3)	21.0 (7.3)	22.7 (6.9)	0.0003
Creatinine, mg/dl	2.31 (0.79)	2.3 (0.9)	2.52 (0.79)	<0.0001
eGFR, ml/min/1.73 m^2	28.0 (9.0)	28.2 (9.0)	25.9 (8.35)	<0.0001
eGFR ≥ 30 ml/min/1.73 m^2	989 (39.1%)	904 (39.9%)	85 (32.2%)	0.015
eGFR <30 ml/min/1.73 m^2	1540 (60.9%)	1361 (60.1%)	179 (67.8%)	
Urine ACR, mg/mmol	16.3 [3.0–86.6]	15.75 [2.90–80.30]	31.20 [4.40–159.30]	0.0003
Albumin, g/l	40.4 (4.3)	40.5 (4.2)	39.3 (4.7)	<0.0001
Hemoglobin, g/l	123.2 (15.6)	123.6 (15.6)	119.6 (15.5)	<0.0001
Calcium, mmol/l	2.31 (0.14)	2.31 (0.14)	2.28 (0.15)	0.0055
Phosphate, mmol/l	1.21 (0.25)	1.21 (0.24)	1.27 (0.27)	0.0006
1,84-Parathyroid hormone, pg/ml	44.2 [26.1–75.6]	15.75 [2.90–80.30]	31.20 [4.40–159.30]	0.0002
Bicarbonate, mmol/l	25. 5 (3.4)	25.5 (3.4)	25.5 (3.5)	0.96
Sodium, mmol/l	140.2 (4.9)	140.2 (5.0)	139.7 (3.3)	0.018
Total cholesterol, mmol/l	4.25 (1.17)	4.26 (1.12)	4.21 (1.58)	0.70
HDL, mmol/l	1.18 (0.44)	1.18 (0.44)	1.12 (0.48)	0.11
LDL, mmol/l	2.22 (0.87)	2.23 (0.87)	2.08 (0.80)	0.041
Triglycerides, mmol/l	1.94 (1.29)	1.91 (1.20)	2.21 (1.90)	0.057
Aspirin	1351 (53.4%)	1191 (52.6%)	160 (60.6%)	0.013
ACE inhibitors and/or ARBs	1823 (72.1%)	1639 (72.4%)	184 (69.7%)	0.36
β-Blockers	1155 (45.7%)	1003 (44.3%)	152 (57.6%)	<0.0001
Statins	1697 (67.1%)	1501 (66.3%)	196 (74.2%)	0.0091

ACE, angiotensin-converting enzyme; ACR, albumin-to-creatinine ratio; ARB, angiotensin receptor blocker; BMI, body mass index; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TMAO, trimethylamine N-oxide.

The overall TMAO level was high (median 20.41 μM). The distribution of TMAO levels is presented in the Supplementary Material online (Supplementary Table S1, Supplementary Figure S2). Stage 4 patients with severe CKD had higher TMAO levels (median 25.15; IQR: 16.64–38.56 μM) than stage 3b patients with moderate CKD (median 14.32; IQR: 9.14–22.69 μM; $P < 0.001$). Relationships of TMAO, expressed in quartiles, and baseline characteristics are presented in Supplementary Table S1. TMAO level showed slightly fair correlation with measures of kidney function: cystatin C ($r = 0.42$; $P < 0.001$), eGFR ($r = -0.40$; $P < 0.001$), and serum creatinine ($r = 0.40$; $P < 0.001$).

Incidence of ischemic events

During 3-year follow up (median 35 months), 264 participants had ischemic events representing 41.6 events per 1000 person-years (Table 2). Of those who had ischemic events, 72 also had episodes of congestive heart failure and 9 had other CV events. Table 1 describes the differences in baseline risk profiles between those who developed new ischemic events and those who did not. Note that lipid profiles were not different between the 2 groups; however, all traditional CV risk factors and CKD-specific risk factors (eGFR, urine albumin-to-creatinine ratio, hemoglobin, albumin, and cystatin C) were significantly different in the univariate analyses.

TMAO levels and ischemic events in the CKD cohort

Despite the very high levels of TMAO in the entire CKD cohort, with the highest levels in those with most severe CKD, those patients who had an ischemic event had significantly higher baseline TMAO levels as compared to those who remained event-free in the entire cohort (Table 1) and within each CKD stratum. Figure 1 presents the distributions of natural log–transformed TMAO (due to skewed TMAO distribution) by level of kidney function for patients with and without events. In untransformed values, the median TMAO levels were significantly higher in participants with events (19.59 [IQR 11.09–28.43] μM) than in participants without events (14.10 [IQR 8.94–21.95] μM; $P = 0.0004$) in stage 3b patients; as was also the case among stage 4 patients: the median TMAO for those with events was 29.47 μM (IQR 19.68–45.18 μM) and for those without events was 24.38 μM (IQR 16.34–37.45 μM; $P = 0.0002$).

The Kaplan–Meier analysis demonstrated a graded increase in ischemic event risk with higher TMAO levels among CKD patients (log-rank $P < 0.0001$; Figure 2). Participants in the highest quartile of TMAO levels, as compared with those in the lowest quartile, had a significantly increased risk of an event (unadjusted hazard ratio [HR] 2.33; 95% confidence interval [CI]: 1.63–3.33; $P < 0.001$).

TMAO is independently associated with ischemic CV events

Unadjusted and multivariable adjusted HRs for ischemic events according to baseline TMAO are expressed as a continuous variable and in quartiles in Table 2. The relationship between elevated TMAO levels and risk of ischemic events observed in unadjusted analyses was not altered after adjusting for demographic characteristics and history of CV events. Participants in the highest versus the lowest quartile demonstrated an almost 2-fold greater risk of ischemic events (model 1, $P < 0.0001$).

After adjustment for other traditional CV risk factors and for use of cardioprotective and renoprotective medications, elevated TMAO levels remained a significant predictor of events (model 2, $P = 0.0055$). Among traditional CV risk factors, only systolic blood pressure level was a statistically significant predictor of events; the use of cardioprotective and renoprotective medications was not statistically significantly associated with events in this cohort. Further adjustments for eGFR and other CKD-specific risk factors slightly attenuated the graded relationship between TMAO and ischemic event risk (model 3, $P = 0.065$), while the adjusted HR of TMAO expressed as a continuous variable remained statistically significant ($P = 0.0081$). In model 4, after adjusting for all significant traditional CV and CKD-specific predictors of ischemic events, increased TMAO levels remain an independent predictor of ischemic events (adjusted HR 1.23; 95% CI 1.06–1.42 / 1 SD lnTMAO; $P = 0.0059$; see Supplementary Table S2 for the full model).

Improved risk classification with TMAO above traditional and CKD-specific risk factors

The inclusion of TMAO as a predictor resulted in a significant improvement in risk classification over traditional and

Table 2 | Risks of ischemic event by natural log–transformed TMAO and ascending quartiles of TMAO

	N events	Rate per 1000 person-years	Hazard ratio (95% confidence interval)				
			Unadjusted	Model 1	Model 2	Model 3	Model 4
Per 1 SD lnTMAO	264	41.6 (36.9–46.9)	1.45 (1.28–1.64)	1.38 (1.21–1.57)	1.39 (1.22–1.59)	1.24 (1.07–1.43)	1.23 (1.06–1.42)
TMAO quartile							
1	44	26.8 (19.9–36.0)	1 (Reference)	1 (Reference)	1 (Reference)	1 (Reference)	1 (Reference)
2	48	29.5 (22.2–39.1)	1.10 (0.73–1.66)	0.93 (0.62–1.41)	0.93 (0.61–1.41)	0.85 (0.56–1.29)	0.93 (0.61–1.42)
3	78	49.6 (39.7–61.9)	1.85 (1.28–2.67)	1.50 (1.03–2.18)	1.50 (1.02–2.19)	1.23 (0.83–1.83)	1.31 (0.88–1.96)
4	94	62.6 (51.2–76.7)	2.33 (1.63–3.33)	1.89 (1.32–2.73)	1.86 (1.28–2.69)	1.37 (0.91–2.06)	1.42 (0.94–1.15)

Model 1: Adjusted for age, sex, race, and presence or absence of diabetes and cardiovascular comorbidities at baseline. Model 2: Adjusted for covariates in model 1 plus traditional cardiovascular risk factors: systolic blood pressure, total cholesterol and high-density lipoprotein cholesterol levels, smoking status; and use of aspirin, β-blockers, statins, and angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers or both. Model 3: Adjusted for covariates in model 2 plus chronic kidney disease–specific risk factors: eGFR, lnACR, albumin, and hemoglobin. Model 4: Adjusted for all covariates with statically significant association with ischemic events: age, diabetes and cardiovascular comorbidities at baseline, systolic blood pressure, eGFR, albumin, hemoglobin, phosphate, and sodium.

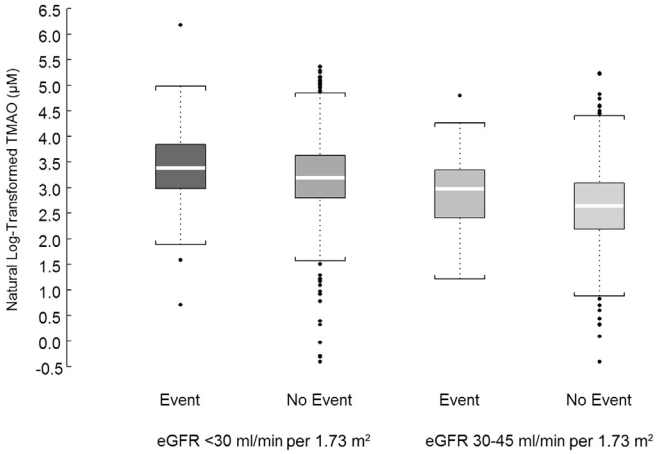

Figure 1 | TMAO levels for patients with and without ischemic cardiac events by kidney function (eGFR). The trimethylamine N-oxide (TMAO) levels are statistically significantly higher in patients with events, both in the estimated glomerular filtration rate (eGFR) <30 ml/min per 1.73 m^2 ($P = 0.0004$) and in the eGFR 30 to 45 ml/min per 1.73 m^2 ($P = 0.0005$) subgroup.

CKD-specific risk factors (net reclassification improvement 10.4%; 95% CI: 3.1%–24.7%). Integrated discrimination improvement and *c*-statistics also showed improvements to various degrees (Supplementary Table S2).

Recurring events

During the 3-year follow-up, of 264 patients with any ischemic CV event, 43 (16%) had recurrent events as follows: 2 had 5 events, 3 had 4 events, 9 had 3 events, and 29 had 2 events. Unadjusted and multivariable adjusted HRs for recurring ischemic events according to baseline TMAO, modeled using the Andersen–Gill approach, are presented in Supplementary Table S3. The results of recurrent events modeling are consistent with the results of time-to-the-first-event modeling, except that TMAO levels, when included as a categorical variable, were also statistically significantly associated with recurring ischemic events after adjusting for traditional CV and CKD-specific predictors (adjusted HR 1.55; 95% CI: 1.01–2.40; $P = 0.0481$; and adjusted HR 1.59; 95% CI: 1.04–2.43; $P = 0.0351$ for model 3 and model 4, respectively).

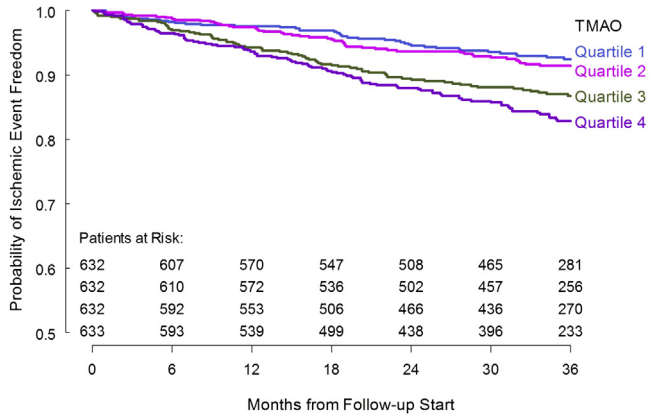

Figure 2 | Kaplan–Meier estimates for ischemic events by the TMAO quartiles. The log-rank test (32.44) by trimethylamine N-oxide (TMAO) quartiles is statistically significant ($P < 0.0001$).

Stratified and sensitivity analyses

Elevated TMAO discriminates ischemic events better at higher levels of kidney function. The risk of ischemic event according to TMAO was not homogenous across categories of eGFR. Although TMAO levels were higher in more advanced CKD, elevated levels of TMAO were independently associated with greater risk of ischemic event (adjusted HR 1.45; 95% CI: 1.12–1.87; $P = 0.0044$) in participants with baseline eGFR between 30 and 45 ml/min per 1.73 m^2 than in those with eGFR lower than 30 ml/min per 1.73 m^2 (adjusted HR 1.14; 95% CI: 0.96–1.37; $P = 0.13$; Figure 3).

Stratified analysis. Figure 4 describes the multivariable-adjusted stratified analyses, by confounder level, in which elevated TMAO levels are consistently associated with greater risk of ischemic events. In all subgroups, the association of the elevated levels of TMAO and the risk of ischemic events was significant irrespective of baseline risk of CV events. This was true even in those subgroups with lower overall risk as follows: females, those without known history of ischemic events, and those with normal systolic blood pressure, albumin, and phosphate levels. It thus appears that TMAO may predict future ischemic events in both high- and low-risk groups with CKD.

Competing risk analysis. Analyses using the competing risk approach showed no significant differences between the reported Cox proportional hazards method results and the results of regression modeling accounting for competing risk of death (Supplementary Table S4). The risk ratio for the entire CKD cohort, per 1 SD of natural log (ln) TMAO, was 1.21 (95% CI: 1.05–1.39; $P = 0.008$) after adjustments for all traditional and CKD factors. The fully adjusted risk ratio for stage 4 (eGFR <30 ml/min per 1.73 m^2) patients was 1.14 (95% CI: 0.95–1.36; $P = 0.15$), while the fully adjusted risk ratio for stage 3b (eGFR 30–45 ml/min per 1.73 m^2) patients was 1.42 (95% CI: 1.12–1.80; $P = 0.003$).

DISCUSSION

Patients with established CKD are known to be at a much higher risk for CV disease, not explained by conventional Framingham risk factors.[15] Since TMAO is known to be renally cleared, we examined the relationship of baseline TMAO levels to CV events in a well-characterized national cohort of CKD patients under the care of nephrologists. We describe measured plasma levels of TMAO that are approximately 5 times those described in the general population, and 4 times higher than those described in patients with CV events.[3,16] Interestingly, TMAO levels display marked inter-patient variability even within the range of eGFR between 15 and 45 ml/min per 1.73 m^2. These elevations and variation may not be simply due to lower eGFR. Furthermore, the observation that the association of increased risk of ischemic CV events with highest values of TMAO within each eGFR stratum is in keeping with the proposed biologic role of

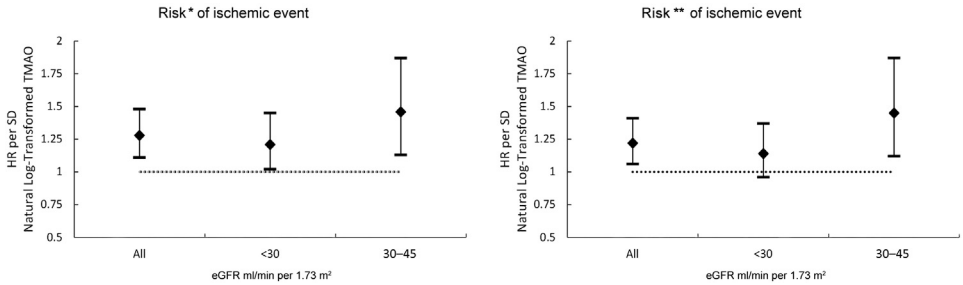

Figure 3 | Adjusted risk of ischemic events per 1 SD of natural log–transformed TMAO, overall and by kidney function level. Multivariable adjusted risks of ischemic events per unit increment in SD of natural log–transformed TMAO: *Adjusted for age, history of CV events, diabetes, and eGFR. Statistically significant adjusted hazard ratios (HRs) were noted for all patients ($P < 0.0009$), or when subgrouped by eGFR <30 ml/min per 1.73 m^2 ($P < 0.0340$), or eGFR 30 to 45 ml/min ($P < 0.0034$). **Adjusted for age, history of CV events, diabetes, eGFR, systolic blood pressure, albumin, hemoglobin, phosphate, and sodium. The adjusted HRs for all patients and patients with eGFR 30 to 45 ml/min per 1.73 m^2 are statistically significant ($P = 0.0059$ and $P = 0.0044$, respectively), while the HR for patients with eGFR <30 ml/min per 1.73 m^2 is not statistically significant ($P = 0.1318$). CV, cardiovascular; eGFR, estimated glomerular filtration rate; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

TMAO in atherogenesis.[7] The relationship of TMAO and ischemic CV events is consistently demonstrated after adjustment for known baseline risk factors and level of kidney function and after adjustment for other CV risk factors associated with CKD. Indeed, even among those with similar degree of eGFR (Figure 3), those who exhibit higher TMAO levels are more likely to have CV events. The reported nearly 700-fold variation in urinary TMAO levels in healthy subjects with no renal dysfunction suggests that irrespective of the fact that baseline clearance of TMAO is renally mediated, the marked interpatient variation is likely due to dietary differences.[9] Since there is no *a priori* reason to suggest renal handling of TMAO is different among patients with similar eGFR, then it is plausible that the markedly higher TMAO levels seen in many CKD patients likely reflect gut microbiome–mediated synthesis of TMA in response to diet rich in choline and carnitine and subsequent conversion in the liver to TMAO (Figure 5). Some of the variability may also relate to clearance of TMAO by transporters expressed in the intestine, liver, and kidney. Clearance studies of L-carnitine and TMAO provide strong evidence of the involvement of carrier-mediated transport processes in the kidney, which appear to be saturable, suggesting that tubular secretion as well as reabsorption, which is not predicted by eGFR, may play an important role.[10] Further studies are needed to evaluate the role of renal transporters capable of TMAO uptake and tubular secretion. This emphasis is consistent with recent attention to tubular function in addition to eGFR to understand CKD more fully.

Others have noted that subjects with ESRD on dialysis harbor markedly different gut flora than healthy subjects,[17] and hypothesized that this difference in gut flora changes the pool of circulating bile acids in subjects with renal disease.[18] However, variation in gut flora in the setting of non-dialysis CKD has not been delineated to our knowledge. Since bile acids are regulators of the nuclear receptor FXR, which regulates expression of FMO3, changes in renal function might not only modulate gut flora–mediated TMA production, but also alter FXR activation. Therefore, modulation of FMO3 expression could alter the rate of TMAO synthesis from TMA. In addition, loss-of-function mutations in FMO3 are known to exist.[4,5] The association between TMAO and ischemic events was stronger within patients with stage 3b than within patients with stage 4 CKD. The strength of the association may be weakened in more advanced CKD due to survival bias; those with severe CVD may not survive to stage 4. Also, in stage 4 patients, decreasing kidney function may have stronger association both with higher TMAO levels and with ischemic events and hence obscure the relationship of TMAO and events.The strength of our data is in the fact that they represent a national cohort population, well characterized, and reflective of those under the care of nephrologists. All CV events are adjudicated, and the TMAO assays were run in a single laboratory under highly regulated conditions. Using robust statistical techniques we were able to demonstrate the statistically independent association of TMAO values and ischemic CV events in this advanced CKD cohort. The known biologic actions of this molecule help to add credibility to the potential importance of TMAO. Furthermore, we purposefully examined only adjudicated ischemic events given the known role of TMAO in atherogenesis. The relationship of TMAO to other CV events requires further exploration.

We should also note that analytical methodology used to measure TMAO levels, that based on liquid chromatography–tandem mass spectrometry (LC-MS/MS), is considered to be the gold standard in terms of accuracy and absolute quantification of TMAO. Indeed, some older studies of TMAO levels have shown plasma concentrations of TMAO, even among healthy volunteers, to be far higher than those currently described when measured using LC-MS/MS.[10] A systematic validation of LC-MS/MS methodology as well as normal population distribution of TMAO levels in various age categories has recently been published by Wang *et al.*[16] Importantly, in the general population, even among those 60 years of age or older, median TMAO level is less than 6 μM.[16]

Limitations of this analysis are relative; this is a cohort with a limited range of eGFR. Whether these results are generalizable

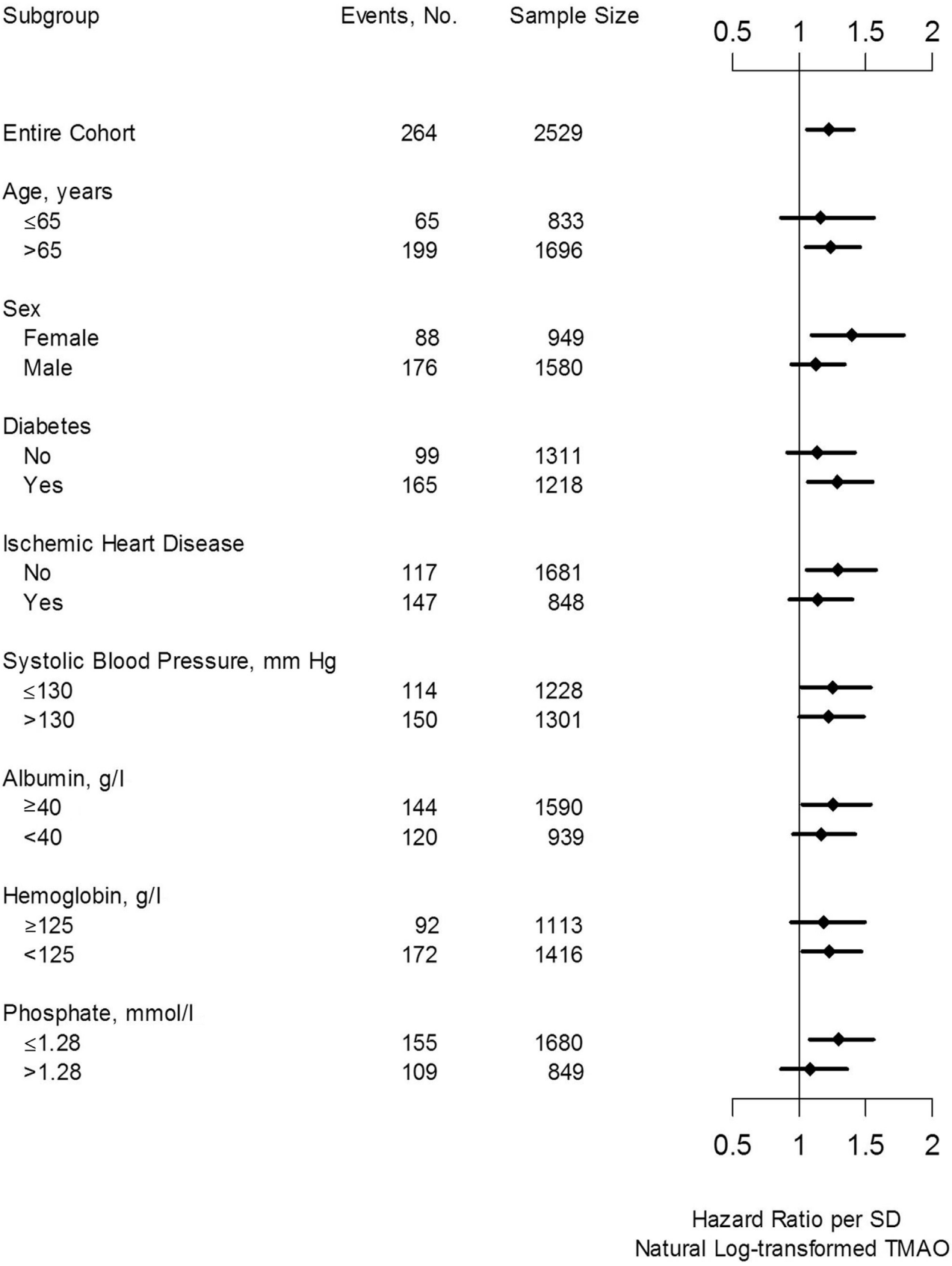

Figure 4 | Multivariable adjusted stratified analysis of risk of ischemic event by TMAO levels. Stratified analysis of multivariable adjusted risks of ischemic events per unit increment in SD of natural log–transformed TMAO adjusted for age, history of CV events, diabetes, eGFR, systolic blood pressure, albumin, hemoglobin, phosphate, and sodium. CV, cardiovascular; eGFR, estimated glomerular filtration rate; TMAO, trimethylamine N-oxide.

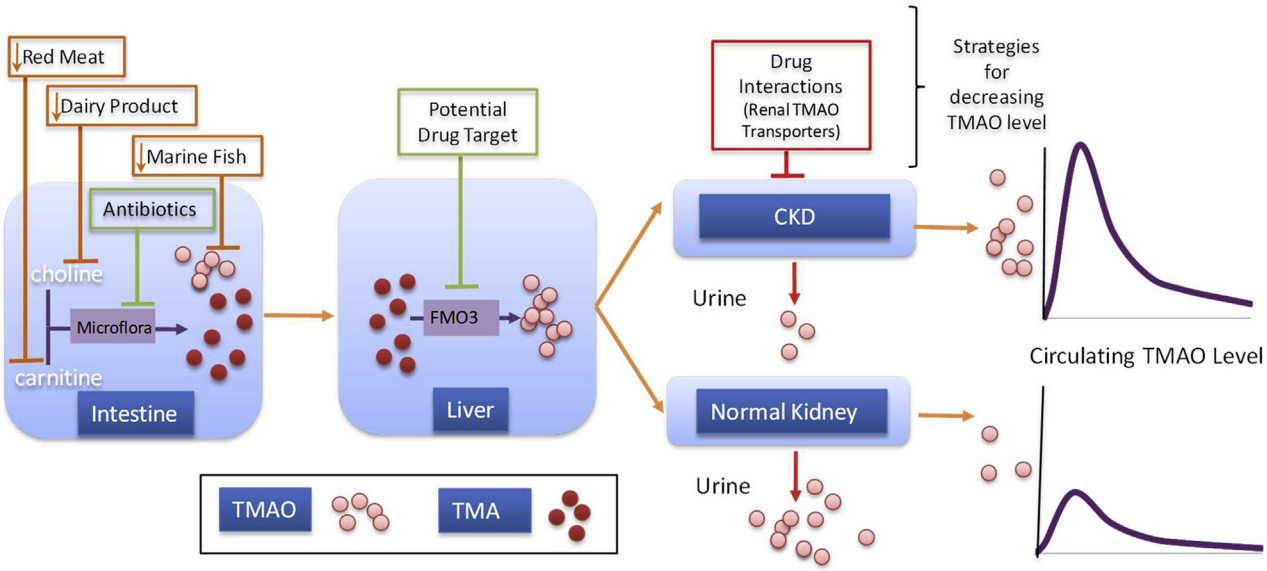

Figure 5 | Overview of TMAO production relative to renal function and attained TMAO levels. CKD, chronic kidney disease; FMO3, flavin-containing monooxygenase isoform 3; TMAO, trimethylamine N-oxide.

to those patients with higher eGFR or those not under the care of nephrologists is unknown. We have not measured kidney function, and while some variability may exist from estimated GFR values relative to "true GFR," the variability seen in TMAO levels exceeds that reported between estimated and measured GFR at this level of kidney function, and would not likely be an explanation for the findings. Another limitation of our current study is the inability to characterize or define gut microbiome of the study participants, which would have allowed for the linkage of gut microbiome signatures with elevated TMAO levels. Detailed history of diet and antibiotic utilization is another variable that is not adequately addressed in our current study; thus effects of antibiotic use in modulating gut microbiome–mediated TMAO synthesis will need to be incorporated in future studies in this field.

A recent study that carried out a non-biased transcriptional and metabolic profiling demonstrated that FMO3, the key enzyme that generates TMAO, is a target of insulin, and knockdown of FMO3 appeared to prevent atherosclerosis in liver insulin receptor knockout (LIRKO) mice, through suppression of a key gene associated with metabolic syndrome, *FoxO1*.[19] Another study demonstrated that FMO3 knockdown stimulated liver X receptor (LXR)–mediated macrophage reverse cholesterol transport.[20] Taken together, these findings suggest that FMO3 activity, as well as the overall amount of TMAO generated through the actions of gut microflora, provides a strong mechanistic basis for TMAO-associated CV risk. Moreover, TMAO appears to be directly toxic to renal tissues, and thereby contribute to kidney injury.[13] Therefore conversion of dietary carnitine and choline to TMA through gut microbiome, FMO3 activity, and clearance of TMAO through GFR and renal secretion, likely through uptake and efflux transporters, all contribute to overall risk for elevated TMAO levels and CVD.

In summary, we describe the strong independent association between plasma TMAO and risk of ischemic events in CKD. Given the association with CV events, strategies that target reduction in plasma TMAO may be important to prevent CV disease in this population. Modification of gut flora with antibiotics may be 1 potential treatment for those with high TMAO levels, although antibiotic resistance would need to be considered as an obstacle to this potential therapeutic option. A more sustainable strategy of ensuring a lower TMAO level may be to encourage diets that avoid red meat or eggs, such as the widely described "Mediterranean diet."[21] Further studies are required to evaluate whether dietary modification can provide a sustained change in TMAO levels and whether such changes result in a decrease in CV events. Interestingly, diet modification in CKD has met with variable success in reducing increased morbidity and mortality. Diets with reduced animal proteins, or vegetable proteins, those with reduced phosphate and potassium, or those supplemented with L-carnitine have all been demonstrated to offer some benefit,[22,23] so future exploration of the impact of these diet modifications on TMAO levels in CKD populations is warranted. Given the high burden of CV disease in CKD populations, identification of modifiable risk factors is imperative to improve the outcomes of these patients. TMAO offers the potential to be an important focus of future studies.

MATERIALS AND METHODS

Study design

CanPREDDICT is a prospective observational cohort study of CKD patients with eGFR of 15 to 45 ml/min per 1.73 m^2, under nephrology care, in urban and rural centers across Canada. Approval was obtained from the University of British Columbia Research Ethics Board. All participants provided written informed consent. Demographics, clinical status, conventional laboratory values, and

serum and plasma specimens were collected every 6 months for 3 years (Supplementary Figure S1).

Outcomes and adjudication

The primary outcome of interest in this analysis was time to ischemic CV events. Ischemic CV events were adjudicated and defined as myocardial infarction, unstable angina, ischemic stroke, coronary revascularization, new onset of coronary heart disease (proven by cardiac catheterization), amputation due to peripheral vascular disease, peripheral artery bypass, and gangrene. Deaths were reported with source documentation and adjudicated for ischemic or congestive CV death, other CV death, non-CV death, and unknown cause of death. The adjudication team consisted of a cardiologist, a nephrologist, and a neurologist (ClinicalTrials.gov identifier: NCT00826319).[14]

Laboratory testing

Plasma concentrations of TMAO were determined from whole blood aliquots collected in ethylenediamine tetraacetic acid tubes and centrifuged; plasma was stored at –80 °C until analysis. TMAO was quantified by LC–MS/MS using a stable-isotope dilution assay, similar to that previously described.[24] d9-TMAO served as the internal standard. A quantitative mass transition (76→58) was used for determination of TMAO plasma concentrations and compared with a second qualitative mass transition (76→59) to ensure lack of assay interference by other endogenous compounds. Human interleukin-6 (Quantikine Immunoassays, R&D Systems, Minneapolis, MN) was analyzed at the University of British Columbia, James Hogg Research Centre, with 10% random duplicate. High-sensitivity C-reactive protein (Siemens BNII Nephelometric Immunoassay, Malvern, PA) was tested at Providence Health Care Clinical Laboratory, an accredited central laboratory.

Statistical analysis

Summary statistics were expressed as mean (SD), median (IQR), or *n* (percent) as appropriate. Univariate comparisons between participants who had ischemic events versus participants who did not were performed using Student's *t*-test or the Wilcoxon rank-sum test for continuous variables, and χ^2 tests for categorical variables. We transformed TMAO and urine albumin-to-creatinine ratio, markers with highly skewed distributions, to the natural logarithmic scale. Spearman correlations were used to describe correlations between TMAO, eGFR, and other laboratory values.

The event rate per 1000 person-years, with a 95% confidence interval, is calculated using the Poisson regression. The overall group rate and rates by TMAO quartiles are presented. Patient free-of-ischemic-event probability was estimated using the Kaplan–Meier method. Survival curves by TMAO quartiles were compared using the log-rank test.

We used time-to-event analyses to examine risk of ischemic events according to baseline TMAO levels, which were expressed as a continuous variable with HRs calculated per SD increment of natural log–transformed TMAO, and in quartiles, with the lowest quartile defined as the reference group. We used Cox proportional hazards regression to examine unadjusted and multivariable adjusted relationships between TMAO and CV events. We hierarchically adjusted for (i) demographic and comorbidity factors (age, sex, race, and presence or absence of diabetes and CV comorbidities at baseline); (ii) traditional CV risk factors (systolic blood pressure, total and low-density lipoprotein cholesterol, smoking status) and the use of cardioprotective and renoprotective medications (aspirin, β-blockers, statins, and angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers); (iii) CKD-specific risk factors: estimated GFR (based on the modified Modification of Diet in Renal Disease [MDRD] study equation) and natural log–transformed urinary albumin-to-creatinine ratio, hemoglobin, and serum albumin; and (iv) all statistically significant "traditional" factors associated with ischemic events. Schoenfeld residuals were examined to confirm the proportionality assumption.

The overall fit of models was validated using the Akaike information criterion.[25] Improvement in model classification due to the inclusion of TMAO was quantified using the net reclassification improvement statistic for survival data.[26] The concordance statistic (*c*-statistic) and integrated discrimination improvement[26–28] were used as measures of improvement in models' discrimination with the addition of TMAO levels. The differences in statistics between the model based on traditional factors and the model including TMAO were calculated using 1000 bootstrap repetitions to generate the corresponding CIs for these methods.

Recurring events. Repeated ischemic events during the 3-year follow-up are modeled using the Andersen–Gill intensity model.[29]

Stratified and sensitivity analyses. Because reduced kidney function is an independent risk factor for CV events, we performed stratified analysis by baseline eGFR and tested for interaction with TMAO. We also performed stratified analyses and tests for interaction with TMAO for the individual, statistically significant, CV risk factors. Because death precludes the occurrence of CV events, we used a competing risk approach in a sensitivity analysis of ischemic events. To evaluate the effect of the competing risk, we used the regression analysis approach proposed by Fine and Gray[30] for the direct regression modeling of the effect of covariates on the cumulative incidence function for competing risks.

The analyses were performed using SAS software, version 9.3 (SAS Institute Inc., Cary, NC) and R software, version 3.1.0. Two-sided *P* values less than 0.05 were considered significant.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Cohort flow diagram (screening, eligibility, enrollment, missing information), based on STROBE guidelines.
Figure S2. Histogram of TMAO distribution. (2a) Without transformation. (2b) Natural log–transformed TMAO.
Table S1. Baseline characteristics by quartiles of TMAO.
Table S2. TMAO and risk of ischemic events during 3-year follow-up adjusted for traditional cardiovascular and chronic kidney disease–specific risk factors.
Table S3. Risks of recurring ischemic events by natural log–transformed TMAO and ascending quartiles of TMAO using Andersen–Gill intensity models.

Table S4. Risks of ischemic events by natural log–transformed TMAO and ascending quartiles of TMAO in presence of competing risk of death, using Fine and Gray approach.[30]

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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APPENDIX

The members of the CanPREDDICT investigators and coordinators are Mohsen Agharazii, MD, Joanne Blouin, France Samson, Centre hospitalier universitaire de Québec: L'Hôtel-Dieu de Québec; Ayub Akbarii, MD, Judy Cheesman, Jennilea Courtney, Sabrina Hamer, Edita Delic, Valerie Cronin, University of Ottawa; Paul Barré, MD, Jeffrey Golden, Royal Victoria Hospital; Brendan Barrett, MD, Elizabeth Langille, Sandra Adams, Janet Morgan, Eastern Regional Health Authority, Health Sciences Centre; Catherine Clase, MD, Cathy Moreau, St Joseph's Hospital; Susan Cooper, MD, Brian Forzley, MD, Susan Caron, Shauna Granger, Susan Valley, Helen Sather, Penticton Regional Hospital; Serge Cournoyer, MD, Lorraine Menard, Michèle Roy, Hélène Skidmore, Dolores Beaudry, Charles Le Moyne Hospital; Janis Dionne, MD, Josephine Chow, Valla Sahraei, British Columbia Children's Hospital; Sandra Donnelly, MD, Niki Dacouris, Rosa Marticorena, St. Michael's Hospital; Brenda Hemmelgarn, MD, Sharon Gulewich, Troy Hamilton, Foothills Hospital; Paul Keown, MD, Nadia Zalunardo, MD, Daniel Rogers, Reena Tut, Matthew Paquette, Rossitta Yung, Vancouver General Hospital; Adeera Levin, MD, Nancy Ferguson, Mila Tang, Helen Chiu, Kathleen Carlson, Lina Sioson, Taylor Perry, Zainab Sheriff, Naama Rozen, St. Paul's Hospital; Charmaine Lok, MD, Michelle Cross, Cathy Forrester, Alexandra Cotoi, University Health Network; François Madore, MD, Manon Maltais, Hôpital du Sacré-Cœur; Louise Moist, MD, Kerri Gallo, Sarah Langford, Leah Slamen, Danielle Cram, London Health Science Centre–Victoria Campus; Norman Muirhead, MD, Mary Jeanne Edgar, Taylor Gray, Cameron Edgar, Karen Groeneweg, Eileen McKinnon, Erin McRae, Kyla Blackie, London Health Science Centre–University Campus; Bharat Nathoo, MD, Kimmy Lau, York Central; Malvinder Parmar, MD, Sylvie Gelinas, Timmins & District Hospital; Martine Leblanc, MD, Lucie Lépine, Maisonneuve-Rosemont Hôpital; Claudio Rigatto, MD, Dolores Friesen, Marla Penner, St. Boniface Hospital; Steven Soroka, MD, Susan Fleet, Jeanette Squires, QEII Health Sciences Centre; Siva Thanamayooran, MD, Michael Binder, Christine Hines, Brenda McNeil, Patrice McDougall, Joy Howard, Deborah Gillis, Kathleen Hines, Cape Breton District Health Authority; Sheldon Tobe, MD, Mary Chessman, Nancy Perkins, Martha Agelopoulos, Stacey Knox, Tiffany Richards, Sunnybrook Hospital; Marcello Tonelli, Susan Szigety, Dawn Opgenorth, University of Alberta; Karen Yeates, MD, Karen Mahoney, Kingston General Hospital.