Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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Supplementary Appendix

Spironolactone metabolites in TOPCAT: New insights into regional variation

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Methods

TOPCAT design and study population

The design and primary results of the Treatment of Preserved Cardiac Function Heart Failure with an Aldosterone Antagonist (TOPCAT; ClinicalTrials.gov number, NCT00094302) have previously been reported.[1,2] Briefly, TOPCAT was an international, randomized, double-blinded, placebo-controlled trial. This trial, which was sponsored by the National Heart, Lung, and Blood Institute, investigated the impact of spironolactone on the risk of cardiovascular mortality, aborted cardiac arrest or HF hospitalization in 3445 patients aged ≥50 years with symptomatic HF and left ventricular ejection fraction >=45%. Heart failure was defined by the presence of one symptom at screening and one sign in the 12 months prior to randomization, in association with either a hospitalization in the last 12 months for which HF was a major component or an elevated natriuretic peptide level (BNP ≥100 pg/mL or NT-proBNP ≥360 pg/mL) within 60 days of randomization. Randomization was stratified according to whether patients were enrolled by the hospitalization or natriuretic peptide criterion. Major exclusion criteria included a serum potassium level ≥5.0 mmol/L, uncontrolled hypertension or severe renal dysfunction, defined as a serum creatinine ≥2.5 mg/dL or an estimated glomerular filtration rate (eGFR) <30 ml/min/1.73 m^2.

Consenting patients were randomized to receive spironolactone or matching placebo in a 1:1 ratio. Spironolactone (or placebo) was initiated at a daily dose of 15 mg and titrated up to a maximum dose of 45 mg daily (3 tablets) based on tolerance and persistence of HF symptoms. Laboratory values, including serum potassium, were measured at baseline, 1 week after any dose change, and at every study visit. Serum potassium was measured locally at each participating center. The study was approved by local institutional review boards or ethics committees and all patients provided informed consent.

Canrenone substudy

The primary objective of this substudy was to explore the proportion of patients in the repository population who were assigned to and reported taking spironolactone at the 12 month visit, who did not have detectable concentrations of the spironolactone metabolite canrenone (below the limit of detection of the method) in the 12-month sample. Data regarding detectable canrenone were compared between US/Canada and Russia, reflecting the prespecified regions of enrollment from the primary TOPCAT trial.[7] Secondary objectives included testing the relationship between reported spironolactone dose and canrenone concentrations in each region. Finally, we also investigated the relationship between the detection of canrenone concentrations and changes in potassium and aldosterone from baseline to 12 months among patients reporting taking assigned spironolactone.

TOPCAT biorepository

Patients at selected sites in the United States (US), Canada, and Russia were invited to provide separate, written, informed consent to contribute samples of urine, serum, and plasma to a tissue repository. Substudy samples for consenting subjects were collected at baseline and at the 12-month study visit. Samples were initially stored locally at -20 degrees Centigrade or colder, and ultimately transferred to the NHLBI central repository in Gaithersburg, MD (SeraCare BioServices) for long-term storage at -70 degrees centigrade.

Canrenone analyses were restricted to patients with sufficient sample for canrenone evaluation at the 12 month visit.

Canrenone assay

Active metabolites of spironolactone are believed to significantly contribute to its therapeutic effects.[3-6] Canrenone is an active metabolite of spironolactone, and is a more efficient marker of spironolactone use than the parent drug due to its longer half life in the serum (~16.5 hours vs ~1.6 hours for spironolactone).[3] Canrenone, and the related prodrug potassium canrenoate are also mineralocorticoid receptor antagonists (MRAs), and they have been themselves available for clinical use in some countries.[3,4]

Not all sites from the US, Canada, and Russia elected to participate in the repository and many patients at repository-participating site did not participate in the repository. Thus, a total of 366 samples at 12 months were available for canrenone analysis. Of those, 206 were from US/Canada (149 US; 57 Canada) and 160 were from Russia. There were no samples from Argentina, Brazil or Georgia. All analyses were performed blinded to treatment assignment, region and reported dosage.

Serum samples (40 µL) were submitted to protein precipitation using a solution of testosterone in acetonitrile as the internal standard (800 ng/mL, 80 µL). The supernatant (75 µL) was diluted with an aqueous ammonium formate solution (5 mM, pH 5, 50 µL). Prepared samples (20 µL) were analyzed by high pressure liquid chromatography (HPLC) coupled to electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode. Sample concentrations were determined by calculating the ratio of the peak response of the analyte over the peak response of the internal standard.

The limit of detection (LOD) of the canrenone analytical method was 0.2 ng/mL, while the lower limit of quantification (LLOQ) was 0.75 ng/mL. Linearity was confirmed over the range of 0.75 to 200 ng/mL. The back-calculated mean standard concentrations were between 85 and 115% of the nominal values for all concentrations, except LLOQ

where it was between 80 and 120%. Mean assay precision was not more than 15% RSD and mean assay accuracy was between 85 and 115% of the nominal value. Extraction recovery was 96%.

The validation demonstrated that the method was selective, and all interfering peaks were not more than 25% of the LLOQ at the retention time of canrenone. Endogenous levels of testosterone in the blank serum were not more than 1% of the peak height of testosterone in the internal standard.

Canrenone was stable in serum at room temperature for at least 120 min as demonstrated by a recovery of 104% of the initial concentration. Freeze/thaw stability (3 cycles) was also determined, and canrenone was stable after three freeze/thaw cycles as demonstrated by a recovery of 100% of the initial concentration. Aliquoting from the main serum sample was performed on ice. To avoid degradation, samples were also thawed on ice for less than 30 minutes for the canrenone measurement, and immediately extracted.

Aldosterone measurement

Aldosterone was measured in serum collected at baseline and the 12 month visits using the ALPCO Aldosterone ELISA kit (American Laboratory Products Company [ALPCO], ISalem, NH).

Statistical analyses

Continuous data were summarized as mean±SD or median [IQR], as appropriate. Binary data were summarized using counts and percentages. Two-sample comparisons of binary data were performed using Fisher's exact test. Relationships between detectable canrenone concentrations and reported study drug dose were assessed using Spearman correlation. Unadjusted and adjusted analyses of potassium and aldosterone changes from baseline were performed using t-tests and linear regression models, respectively. P-values <0.05 were considered statistically significant. No adjustments were made for multiple comparisons. All analyses were performed using STATA 14.0 (College Station, TX).

Results

Consistent with our previous report, participants in the repository who were included in US/Canada differed significantly than those included in Russia (Table S1). Nevertheless, individuals from this substudy were representative of those from their respective regions. The only consistent difference in both regions was a lower proportion of substudy participants who were from the hospitalization randomization stratum and a lower heart rate compared to non-participants.

Limitations

Only a subset of the overall TOPCAT population was enrolled in the repository, and thus, we cannot completely exclude the possibility of selection bias. Furthermore, no subjects from Georgia, Brazil, and Argentina were represented. However, as shown in Supplementary Table S3, patients from Argentina and Brazil had similar event rates and responses to spironolactone to those from the US and Canada, while patients from Georgia exhibited low event rates and lack of spironolactone effect comparable to those from Russia. Unfortunately, in TOPCAT, no consistent data regarding pill counts to assess adherence was available. An additional potential limitation is that differences in canrenone levels as a result of differences in collection and storage of blood samples cannot be excluded. However, because the detection of canrenone was associated with changes in serum potassium in both regions, it seems unlikely that storage conditions are sufficient to explain these results. Another potential limitation is that, given its long half-life, detection of extremely low concentrations of canrenone may have overestimated adherence to therapy. Indeed, although measuring the concentrations of a given drug or metabolite has the advantage of providing actual physical evidence that a patient has taken his medication, they do not account for long-term adherence.[8] Finally, we cannot exclude some spironolactone use prior to the 12 month visit in patients with undetectable canrenone concentrations. Nevertheless, in individuals presenting steady-state concentrations of canrenone of 15 to 20 ng/ml, and with an estimated canrenone half-life of 16.5 hours, one would expect to detect canrenone for at least 2 to 3 days after its chronic use.

Supplementary Tables

Supplementary Table S1. Baseline characteristics of repository participants vs non participants in the current sub-study.

	US/Canada			Russia		
	Non-participant (n=1271)	**Participant** (n=206)	**p-value**	**Non-participant** (n=906)	**Participant** (n=160)	**p-value**
Age (years), y	71.6 ± 9.9	72.4 ± 9.4	0.28	65.5 ± 8.5	65.6 ± 8.5	0.90
Female, n (%)	619 (48.7%)	89 (43.2%)	0.14	467 (51.5%)	77 (48.1%)	0.42
Race, n (%)			0.05			n/a
White	975 (76.7%)	173 (84.0%)		906 (100%)	160 (100%)	
Black	243 (19.1%)	25 (12.1%)				
Other	53 (4.2%)	8 (3.9%)				
Hispanic	51 (4.0%)	6 (2.9%)	0.45	1 (0.1%)	0 (0.0%)	0.67
Country, n (%)			0.037			n/a
US	1002 (78.8%)	149 (72.3%)		n/a	n/a	
Canada	269 (21.2%)	57 (27.7%)		n/a	n/a	
Russia	n/a	n/a		906 (100%)	160 (100%)	
Ejection fraction, %	57.8 ± 7.5	58.3 ± 7.6	0.35	57.1 ± 7.6	57.9 ± 7.7	0.23
NYHA class			0.57			0.10
1	71 (5.6%)	7 (3.4%)		10 (1.1%)	0 (0.0%)	
2	716 (56.6%)	119 (57.8%)		508 (56.1%)	104 (65.0%)	
3	471 (37.2%)	78 (37.9%)		382 (42.2%)	56 (35.0%)	
4	8 (0.6%)	2 (1.0%)		5 (0.6%)	0 (0.0%)	
Heart Rate	69.1 ± 11.2	67.1 ± 10.2	0.019	69.3 ± 10.2	67.6 ± 8.2	0.048
Systolic blood pressure, mmHg	127.2 ± 16.0	125.1 ± 14.2	0.08	130.4 ± 11.7	127.9 ± 10.9	0.011
Body mass index, kg/m^2	34.4 ± 8.9	33.7 ± 7.3	0.25	31.1 ± 5.6	30.9 ± 4.9	0.71
Hospitalization stratum, n (%)	698 (54.9%)	87 (42.2%)	<0.001	846 (93.4%)	112 (70.0%)	<0.001
Medical History, n (%)						
Any cardiovascular disease history	632 (49.7%)	114 (55.3%)	0.13	758 (83.7%)	130 (81.2%)	0.45
Myocardial infarction	277 (21.8%)	47 (22.8%)	0.75	365 (40.3%)	75 (46.9%)	0.12
Hypertension	1138 (89.7%)	194 (94.2%)	0.043	867 (95.8%)	152 (95.0%)	0.65
Atrial fibrillation	556 (43.8%)	106 (51.5%)	0.041	323 (35.7%)	51 (31.9%)	0.35
Diabetes	601 (47.4%)	96 (46.6%)	0.84	213 (23.5%)	21 (13.1%)	0.003
Stroke	125 (9.9%)	16 (7.8%)	0.35	81 (9.0%)	15 (9.4%)	0.86
Coronary bypass graft surgery	262 (20.6%)	54 (26.2%)	0.07	81 (9.0%)	16 (10.0%)	0.67
Percutaneous coronary intervention	276 (21.7%)	53 (25.7%)	0.20	105 (11.6%)	17 (10.6%)	0.72
Angina	376 (29.6%)	71 (34.5%)	0.16	728 (80.4%)	117 (73.1%)	0.035
Implantable cardioverter defibrillator	33 (2.6%)	9 (4.4%)	0.16	2 (0.2%)	0 (0.0%)	0.55
Pacemaker	209 (16.5%)	20 (9.7%)	0.013	26 (2.9%)	1 (0.6%)	0.10
Peripheral artery disease	166 (13.1%)	26 (12.6%)	0.86	83 (9.2%)	11 (6.9%)	0.35
Dyslipidemia	952 (75.0%)	163 (79.1%)	0.20	584 (64.5%)	92 (57.5%)	0.09
Chronic obstructive pulmonary disease	245 (19.3%)	23 (11.2%)	0.005	88 (9.7%)	21 (13.1%)	0.19
Asthma	161 (12.7%)	22 (10.7%)	0.42	19 (2.1%)	7 (4.4%)	0.09
Smoking Status			0.06			0.47
Current	89 (7.0%)	8 (3.9%)		137 (15.1%)	24 (15.0%)	
Former	669 (52.8%)	125 (60.7%)		193 (21.3%)	41 (25.6%)	
Never	510 (40.2%)	73 (35.4%)		575 (63.5%)	95 (59.4%)	
Sodium, mmol/L	139.7 ± 3.0	139.3 ± 3.0	0.13	143.1 ± 4.5	140.8 ± 4.9	<0.001
Potassium, mmol/L	4.2 ± 0.4	4.2 ± 0.4	0.80	4.4 ± 0.4	4.1 ± 0.5	<0.001
Estimated glomerular filtration rate, ml/min per 1.73 m^2	64.0 ± 21.8	64.3 ± 19.0	0.86	69.9 ± 17.5	69.6 ± 17.1	0.85
Blood urea nitrogen, mg/dL	24.7 ± 12.3	24.9 ± 10.9	0.82	18.9 ± 5.8	19.2 ± 5.4	0.72
Medications, n (%)						

Angiotensin-converting enzyme inhibitor	629 (49.5%)	98 (47.6%)	0.60	711 (78.6%)	113 (70.6%)	0.027
Angiotensin receptor blocker	398 (31.3%)	68 (33.0%)	0.63	98 (10.8%)	21 (13.1%)	0.40
Beta-blocker	1016 (80.0%)	180 (87.4%)	0.012	685 (75.7%)	126 (78.8%)	0.40
Calcium channel blocker	508 (40.0%)	86 (41.7%)	0.64	302 (33.4%)	54 (33.8%)	0.93
Diuretics	1139 (89.7%)	187 (90.8%)	0.63	647 (71.5%)	100 (62.5%)	0.022
Aspirin	753 (59.3%)	125 (60.7%)	0.71	664 (73.4%)	112 (70.0%)	0.38
Nitrate	239 (18.8%)	42 (20.4%)	0.59	145 (16.0%)	24 (15.0%)	0.74
Statin	885 (69.7%)	156 (75.7%)	0.08	433 (47.8%)	85 (53.1%)	0.22
Warfarin	452 (35.6%)	90 (43.7%)	0.025	141 (15.6%)	23 (14.4%)	0.70

NYHA: New York Heart association

Supplementary Table S2. Reported study drug dosing at 12 months.

	US/Canada			Russia		
Reported daily dose at 12 month, mg	**Spironolactone**	**Placebo**	**p-value**	**Spironolactone**	**Placebo**	**p-value**
0, n (%)	25 (25)	14 (13)		4 (6)	8 (9)	
15, n (%)	25 (25)	11 (10)		3 (4)	5 (6)	
30, n (%)	35 (35)	50 (48)		37 (53)	47 (52)	
45, n (%)	16 (16)	30 (29)		26 (37)	30 (33)	
Average dose, mg	21.2±15.5	28.7±14.4	<0.001	33.2±11.7	31.5±12.9	0.39

Supplementary Table S3. Regional differences in response to spironolactone in the overall TOPCAT population.

End point	North America	South America	Russia	Georgia
Primary endpoint, relative risk	0.84 (0.70, 1.01)	0.70 (0.43, 1.16)	1.12 (0.78, 1.60)	1.05 (0.51, 2.17)
Hyperkalemia, relative risk	3.5 (2.6, 4.8)	2.6 (1.4, 4.9)	1.4 (1.1, 2.0)	0.6 (0.2, 2.3)
Change in potassium, mmol/L[1]	+0.31 (0.03)	+0.33 (0.07)	+0.08 (0.04)	+0.08 (0.04)
Change in systolic blood pressure, mmHg[1]	-3.9 (1.1)	-5.1 (2.4)	-2.3 (0.9)	+1.6 (0.9)
Change in serum creatinine, mg/dL[1]	+0.11 (0.02)	+0.17 (0.04)	+0.02 (0.01)	+0.05 (0.01)

[1] Difference in change from baseline between spironolactone and placebo group

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