NEW RESEARCH

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Bumetanide for Core Symptoms of Autism Spectrum Disorder (BAMBI): A Single Center, Double-Blinded, Participant-Randomized, Placebo-Controlled, Phase-2 Superiority Trial

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Objective: Recent trials have indicated positive effects of bumetanide in autism spectrum disorder (ASD). We tested efficacy of bumetanide on core symptom domains using a single center, parallel-group, participant-randomized, double-blind, placebo-controlled phase-2 superiority trial in a tertiary hospital in the Netherlands.

Method: Unmedicated children aged 7 to 15 years with ASD and IQ ≥55 were block-randomized 1:1 to oral-solution bumetanide versus placebo, titrated to a maximum of 1.0 mg twice daily for 91 days (D91), followed by a 28-day wash-out period. The primary outcome was difference in Social Responsiveness Scale−2 (SRS-2) total score at D91, analyzed by modified intention-to-treat with linear mixed models.

Results: A total of 92 participants (mean age 10.5 [SD 2.4] years) enrolled between June 2016 and December 2018. In all, 47 children were allocated to bumetanide and 45 to placebo. Two participants dropped out per treatment arm. After 91 days, bumetanide was not superior to placebo on the primary outcome, the SRS-2 (mean difference −3.16, 95% CI = −9.68 to 3.37, $p = .338$). A superior effect was found on one of the secondary outcomes, the Repetitive Behavior Scale−Revised (mean difference −4.16, 95% CI = −8.06 to −0.25, $p = .0375$), but not on the Sensory Profile (mean difference 5.64, 95% CI = −11.30 to 22.57, $p = .508$) or the Aberrant Behavior Checklist Irritability Subscale (mean difference −0.65, 95% CI = −2.83 to 1.52, $p = .552$). No significant wash-out effect was observed. Significant adverse effects were predominantly diuretic effects (orthostatic hypotension (17 [36%] versus 5 [11%], $p = .007$); hypokalemia (24 [51%] versus 0 [0%], $p < .0001$), the occurrence of which did not statistically influence treatment outcome.

Conclusion: The trial outcome was negative in terms of no superior effect on the primary outcome. The secondary outcomes suggest efficacy on repetitive behavior symptoms for a subset of patients.

Clinical trial registration information: Bumetanide in Autism Medication and Biomarker Study (BAMBI); https://www.clinicaltrialsregister.eu/; 2014-001560-35.

Key words: ASD, bumetanide, RCT, children, SRS

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Autism spectrum disorder (ASD) diagnoses have grown at a tremendous rate in recent years, due in part to growing awareness of the condition.[1,2] About 1 in 50 to 100 children receives a diagnosis in the spectrum and endures pervasive deficits in social communication and interaction, with restricted patterns of behavior or interests and atypical responses to sensory stimuli.[1,3,4] Children with ASD often exhibit associated symptoms including hyperactivity, seizures, and irritability.[5] Historically, treatment for ASD in children has been most successful in treating these associated symptoms, but at the cost of serious side effects.[1,6] To date, no medication is registered to improve the core defining features of ASD. There is hope, because a concerted effort has identified causal risk factors that have led to the implication of several final common pathways in ASD pathogenesis and have reinvigorated interest in developing rational treatments.[7]

For instance, compelling evidence shows that deficits in GABAergic inhibition can contribute to ASD development.[8] The efficacy of GABAergic inhibition depends on

the regulation of the intracellular neuronal chloride ([Cl^-]i) concentrations.[9,10] Pathologically high [Cl^-]i can reverse the polarity of GABA binding its receptor from inhibition to excitation, a converging mechanism that has been linked to a variety of disorders including ASD.[10] Elevated levels of neuronal chloride and excitatory actions of GABA receptor signaling have been established in animal models of ASD and associated conditions. These observations have raised interest in the development of pharmacological treatments that restore chloride homeostasis and consequently GABAergic inhibition in pathological conditions.[10] The [Cl^-]i is predominantly regulated by the chloride importer NKCC1 and chloride exporter KCC2; the best studied agent is bumetanide, a selective NKCC1 antagonist.[11] Bumetanide has been approved for many decades as a safe loop diuretic to treat conditions of hypervolemia with a mild adverse effect profile, which facilitates its application in neurological disorders.[10,11]

Following a pilot study,[12] Lemonnier *et al.* conducted two consecutive placebo-controlled randomized trials testing bumetanide (1−4 mg/d for 3 months) in 60 and 88 participants, respectively.[13,14] Both trials showed a significant reduction in their primary outcome of broad ASD symptomatology. Further anecdotal evidence supported this evidence through a case study in Fragile X syndrome (FRX)[15] and from studies testing bumetanide on emotion recognition in functional neuroimaging and eye-tracking.[16,17] These results are promising, although several methodological and mechanistic concerns have been raised. Both studies used the Childhood Autistic Rating Scale (CARS), a diagnostic screening questionnaire, as a primary outcome. No prior bumetanide trials included outcomes on the core domain of repetitive behaviors or atypical reactivity to sensory input, or determined levels of cognitive functioning or comorbidities in their participants. Another problem is that most plasma bumetanide is protein bound, and rather low concentrations were found to diffuse into the brain.[11] Therefore, brain penetrance of bumetanide across an intact blood−brain barrier may be limited, and behavioral improvements through peripheral effects have been suggested. Furthermore, the clinical and etiological diversity in ASD may preclude that agents targeting specific elements of GABAergic signaling may only be effective in particular subgroups.[18,19]

The aim of this study, the Bumetanide in Autism Medication and Biomarker (BAMBI) trial, was to test the efficacy of bumetanide on social and the other core behavioral domains of ASD, and to develop stratification biomarkers from electroencephalography (EEG) and neurocognitive measures. In this first report of the trial, we describe the protocol and treatment effects on clinical behavioral outcome measures.

METHOD

Study Design and Participants

The BAMBI trial was a single center, parallel-group, participant-randomized, double-blind, placebo-controlled, phase-2 superiority trial testing the effect of bumetanide treatment during 91 days, followed by 28-day wash-out. The trial was conducted at the UMC Utrecht, a nationwide tertiary out-patient center, in the Netherlands. Participants had previously sought clinical care or were self-selected through advertisements with the Dutch ASD parent association (NVA) website and magazine. The trial was approved by the medical ethical committee of the UMC Utrecht and conducted in accordance with the provisions of the Declaration of Helsinki and Good Clinical Practice.[20] All participants or their legal representatives signed informed consent. The full trial protocol is available at https://www.umcutrecht.nl/nl/ziekenhuis/wetenschappelijk-onderzoek/bambi-de-resultaten. Participants received no financial compensation.

Eligible participants were children aged 7 to 15 years with an expert confirmed ASD diagnosis according to the *DSM-IV-TR*[21] (ie, autism, Asperger syndrome or PDD-NOS) or the *DSM-5*[22] criteria. Children were enrolled when the expert diagnosis was accompanied by a clinical threshold score on the Autism Diagnostic Observation Schedule (ADOS-2 module 3 or 4 $\geq$ 6)[23] or the Social Responsiveness Scale−2 (SRS-2; *t* score $\geq$60).[24] Given the 85% sensitivity of the ADOS-2,[23] children with an expert diagnosis of ASD and either an ADOS-2-score <6 or an SRS-2 *t* score <60 were evaluated for second opinion by an independent in-house child psychiatrist. When ASD diagnosis was confirmed, children could advance to treatment allocation. Exclusion criteria were an IQ <55; psychoactive medication use <8 weeks prior to screening visit (except chronic melatonin treatment); start of any new therapy for developmental disorder problems (eg, cognitive−behavioral therapy); comorbid neurological disorders; chronic renal disease; unstable serious illness; use of nonsteroidal anti-inflammatory drugs; and/or documented history of hypersensitivity reaction to sulphonamide derivatives. Furthermore, children were allowed to receive care as usual, restricted to stable frequency of supportive care initiated minimally 2 months prior to randomization (eg, physiotherapy, education support) but excluding behavioral therapy, cognitive−behavioral therapy, family therapy, or any

other kind of psychological intervention. No amendments to eligibility criteria were made.

Randomization and Masking

Eligible participants were randomly allocated (1:1) to receive bumetanide or placebo treatment. Sequence generation, concealment, and treatment allocation was overseen by a third-party not involved in the study (ie, Julius Centre, a consultant support agency for clinical research and trials located in the UMC Utrecht). The sequence was generated with restricted randomization using permuted block design with block sizes randomly varying from two to four to six participants. Undistinguishable medication kits were numbered accordingly by Neurochlore, the company who provided the study medication, and were shipped to the local trial pharmacy where a sealed copy of the randomization sequence was stored for emergency unmasking. Treatment allocation was performed through a secure online randomization tool of the Julius Centre using minimization with a probability of 0.75 on subgroups for the participant factors age (7−8/9−10/11−12/13−15 years), intelligence (IQ 55−70/71−85/86−110/ >110) and sex (male/female).[25] The tool allocated a medication kit number to the participant to ensure concealment and masking.

Participants, parents, health care providers, and outcome assessors were masked for randomization. To secure masking of the outcome assessors for possible (diuretic) side effects of bumetanide, medical checks and handling of adverse events during the treatment and wash-out phase were performed by a team at the pediatric nephrology department of the nearby Wilhelmina Children's Hospital who were also masked for randomization. To further mask parents and participants, all subjects irrespective of treatment allocation were instructed to increase fluid intake, and all subjects received identical starting regimens of potassium supplementation. During distribution of the medication, participants were informed that increased diuresis had been observed in placebo-treated subjects in the earlier bumetanide trials, and therefore would not necessarily be indicative of bumetanide treatment.

Procedures

Once participants and/or their legal representatives had consented to take part in the trial, they were scheduled for three baseline screening visits. During the first visit, participants had a consultation with a child psychiatrist for medical screening, clinical observation, and clinical history taking. Medical screening consisted of physical examination, weight, vital signs (including measures for orthostatic hypotension), height, and clinical laboratory tests (Table S1, available online). When the family history was positive for cardiac rhythm abnormalities at young age, a pediatric cardiologist was consulted to evaluate potential cardiac contraindications. Clinical observations included administration of the ADOS-2, and an abbreviated WISC-III intelligence test was conducted (when not tested in the previous 3 or 2 years, respectively). During the first visit, baseline clinical outcomes were assessed. Attention-deficit/hyperactivity disorder (ADHD) comorbidity was defined as the presence of a formally recorded active ADHD diagnosis by a child psychiatrist or psychologist. During the second and third baseline visits, cognitive and EEG measurements were performed.

Within 45 days of the baseline visits, participants were randomized (D0) and received bumetanide liquid formulation (0.5 mg/mL) or placebo formulation matched for taste, smell, and viscosity, albeit without diuretic properties. The formulation was twice-daily administered orally with a dosing syringe and minimally 6 hours between the administrations (eg, typically with breakfast and dinner). Children <30 kg started with twice-daily 0.015 mg/kg bumetanide or an equivalent volume of the placebo formulation. Children ≥30 kg received twice-daily 0.5-mg bumetanide or placebo (ie, 1 mL). When blood analysis showed no abnormalities at D7, the dosage was doubled (ie, twice-daily 0.03 mg/kg or twice-daily 1.0 mg; 2mL). All participating children received supplementation with 0.5 mmol/kg potassium chloride if <30 kg, or twice-daily 8 mmol potassium chloride if ≥30kg. The first 16 participants (17%) received potassium chloride the first 28 days treatment (n = 9 bumetanide), and, after an early amendment to the protocol, potassium chloride was provided during the entire medication phase to reduce venipunctures.

After an early amendment to the protocol, safety visits were scheduled at D4, D7, D14, D28, D56, D91, and D119 at the department of pediatric nephrology of the Wilhelmina Children's Hospital. The initial protocol involved extra visits at D35 and blood analysis at D91 and D119. At all visits, adverse events, weight, height (monthly), and vital signs were checked. At D4, D7, D14, D28, and D56, blood was obtained and analyzed for adverse events (Figure S1, available online). Adverse events were documented according to severity, duration, attribution and outcome with the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) rating scale and classified in Medical Dictionary for Regulatory Activities (MedDRA) categories. Hypokalemia was the main adverse event to be expected; hence a treatment protocol was formulated beforehand (Table S2, available online).

Participants returned for outcome evaluations at the end of the 91-day medication phase and at the end of the 28-day wash-out period. After having completed the trial, parents returned for an interview about their experiences (ie, treatment and wash-out evaluations) and were asked to predict which treatment their child had received.

Outcomes

The primary outcome was symptom severity of social communication and social interaction, the first core domain of ASD described in the *DSM-5* and measured by the SRS-2 total score after 91 days of treatment (range 0−195; higher score indicates more affected). Secondary outcomes were severity of restricted and repetitive behaviors (core domain of ASD), measured by the Repetitive Behavior Scale−Revised (RBS-R; range 0−129, higher score indicates more affected) and severity of behavioral responses to sensory stimuli measured by the Sensory Profile (SP-NL; range 125−625, lower score is more affected) total score at D91. In addition, the Aberrant Behavior Checklist (ABC) was administered predominantly to analyze effects on the ABC-irritability subscale (range 0−75, higher score is more affected), which has been used to register antipsychotics for ASD. Adverse events were collected passively (spontaneous report) and actively (evaluation of known side effects). Incomplete individual clinical questionnaires were imputed as no change when answers to fewer than four questions were missing (n = 3, all SP-NL). When answers to four or more questions were missing, the outcome measures were excluded from analysis (n = 5). To develop potential future stratification biomarkers, cognitive (including neurocognitive tests and Behavior Rating Inventory of Executive Functioning [BRIEF]) and EEG measures were administered at all time points in the trial and will be reported in a separate dedicated publication.

Statistical Analysis

This study was powered at 85% to detect an effect size of 10 points with a standard deviation of 16 points on the primary outcome measure,[13] assuming a two-sided alpha level of 0.05. Allowing for a 10% attrition rate, 100 participants had to be randomized.

We analyzed outcomes by modified intention-to-treat allocated participants (see Results for details).[26,27] Screening differences between randomized and eligible nonrandomized participants were analyzed with appropriate *t* statistics or Fisher exact tests for dichotomized variables.

Primary and secondary outcomes at all available time points were analyzed with a linear mixed model. A random intercept was included to correct for multiple follow-up measurements per participant. Treatment and treatment by time interaction were included to assess the difference between placebo and bumetanide. In a second step, sex, age, and baseline measurement of the corresponding outcome measures were included to correct for potential confounding and to optimize the statistical analysis for power.[27,28] Statistical assumptions of the models (ie, distributional assumptions, homoscedasticity) were assessed by examining residuals.[29] From these models, we derived estimated means for each treatment arm as well as a mean difference between treatment arms at 91 days with 95% CI and p values. Additional analyses were performed for treatment interactions with sex, age, total IQ, ADHD comorbidity, and prior medication use (ie, psychoactive medication used before participation in the study) and were evaluated with likelihood ratio tests (LRT). Safety was analyzed in all allocated subjects. Differences in adverse events were analyzed with Fisher exacts tests. Agreement of predictions by parents of the allocated treatment arm versus the actual treatment allocated to children was analyzed with Cohen's kappa. All analysis were performed with SPSS v25 (IBM Corp., Armonk, NY) and SAS v9.4 (SAS Institute, Cary, NC).

Study safety was overseen twice a year by the Data Safety Monitoring Board (DSMB) of the UMC Utrecht. This study was registered with the EudraCT trial registry (EudraCT 2014-001560-35).

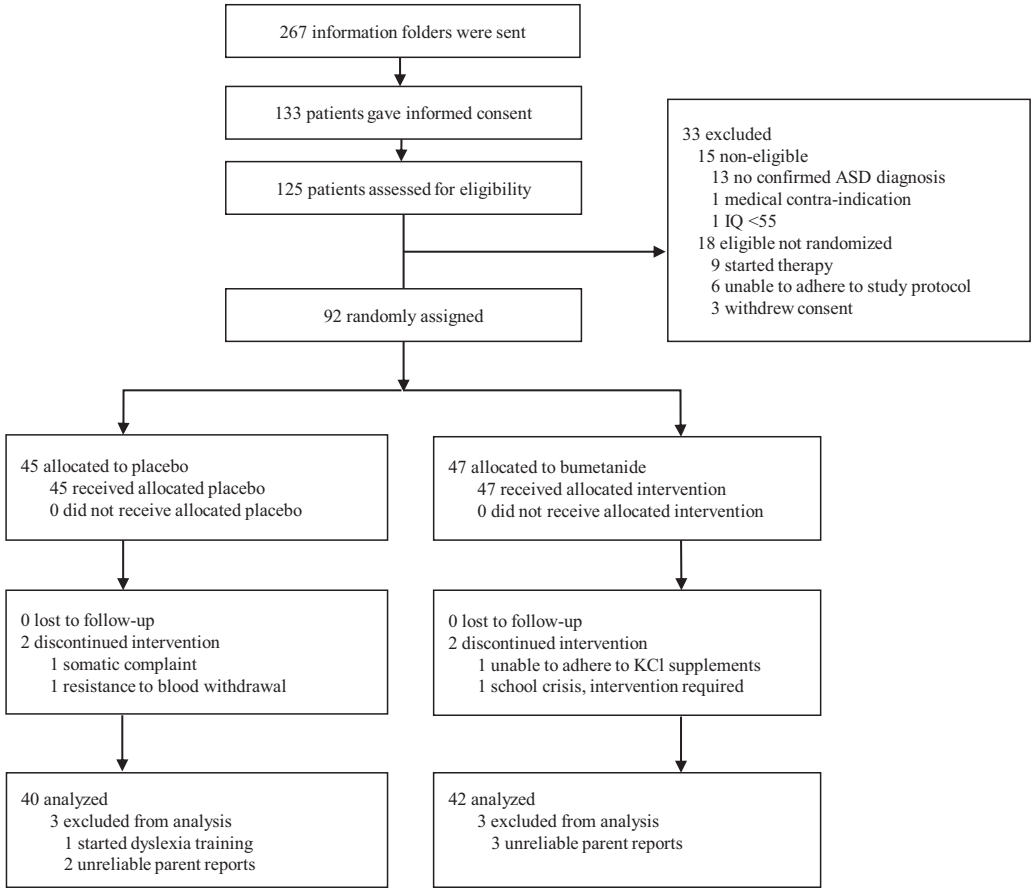
RESULTS

Participant Characteristics

Participants were enrolled between June 21, 2016, and December 6, 2018, the end of planned recruitment. A total of 267 caregivers contacted the research team to obtain the study information folder (Figure 1). Of these potential participants, 133 gave informed consent and 125 were assessed for eligibility. In all, 32 of these participants did not advance to randomization for reasons of noneligibility (n = 13), requirement of immediate psychiatric intervention (n = 9), inability to adhere to study protocol (n = 6), or withdrawal of consent (n = 3). Finally, 92 participants were randomly allocated to treatment (Table 1). There was no difference in baseline characteristics in eligible participants (n = 110) who did and who did not advance to randomization ($p \geq 0.163$).

Of the 92 participants who were randomized, all started allocated treatment and were included in the modified intention-to-treat analysis, excluding 10 allocated participants (Figure 1). A total of 47 children were allocated to bumetanide (15 girls and 32 boys) and 45 to placebo (14 girls and 31 boys). Four participants discontinued treatment

FIGURE 1 CONSORT Diagram of the Trial



Note: *ASD = autism spectrum disorder.*

prior to collecting outcomes, two in each treatment arm. One participant in the placebo arm stopped because of nonspecific somatic complaints and another because of intractable resistance to venipunctures. The two discontinued treatments in the bumetanide arm were because of inability to adhere to potassium supplementation and one because of a school crisis requiring immediate psychiatric intervention. During the trial, nobody had to be unmasked. No further participants were lost to follow-up and all completed the trial, although some participants did not complete all behavioral outcome measures at different time points (D91 n = 3 placebo and n = 4 bumetanide; D119 n = 6 placebo, n = 4 bumetanide).

After completion of the trial and before unmasking, outcome measures of six participants had to be excluded from analysis. One participant appeared to have started extensive dyslexia training during the medication phase. The outcomes of the other five participants were excluded because parents explicitly mentioned unreliable reporting on outcome measures due to stress of, for example, pending divorce lawsuits or conflicts to obtain access to health care provisions. Unmasking revealed that three of these six had been allocated to bumetanide and three to placebo treatment.

Treatment adherence was monitored through interview, drug diary, and inspection of returned medication bottles. There was no evidence of unreliable adherence of the participants in any of the treatment arms. The mean administered bumetanide dose was 0.0482 mg/kg/d (range 0.0264−0.0648). The treatment dose was increased at D7 in all 92 children, although eventually the target dose had to be reduced in four children. In two cases, the dose remained halved throughout the study because of nonspecific somatic complaints (n = 1 placebo) and persistent hypokalemia (n = 1 bumetanide), and in two other children the dose was temporarily halved for 7 and 14 days because of hypokalemia (n = 2, bumetanide).

After the last study visit of a participant, we inventoried the parent predictions of the treatment that their child had received. In the bumetanide arm (n = 47), 30 parents thought to have been allocated to bumetanide, 11 parents

TABLE 1 Baseline Characteristics of the Intention-to-Treat Population

	Placebo arm (n = 45)	Bumetanide arm (n = 47)	Total (n = 92)
Age, y; mean (SD)	10.25 (2.4)	10.5 (2.5)	10.5 (2.4)
Sex, n (%)			
Male	31 (69)	32 (68)	63 (68)
Female	14 (31)	15 (32)	29 (32)
IQ, mean (SD)	103.1 (19.7)	99.4 (21.1)	101.0 (20.4)
ADOS-2, mean (SD)	8.96 (3.7)	9.36 (4.3)	9.16 (4.0)
SRS-2, mean (SD)	88.3 (19.0)	90.7 (21.3)	89.5 (20.1)
Prior medication use, n (%)			
Medication naive	24 (53)	24 (51)	48 (52)
AP	5 (11)	3 (6)	8 (9)
STM	11 (24)	14 (30)	25 (27)
AP and STM	5 (11)	6 (13)	11 (12)
Comorbidities, n (%)			
ADHD	7 (16)	10 (21)	17 (18)
Learning disorder	6 (13)	4 (9)	10 (11)
Anxiety disorder	3 (7)	0 (0)	3 (3)

Note: ADHD = attention-deficit/hyperactivity disorder; ADOS = Autism Diagnostic Observation Scale; AP = antipsychotics; SRS = Social Responsiveness Scale (range 0–195; higher score is more affected); STM = stimulants.

expected to have been allocated to placebo, and 6 parents were uncertain. In the placebo arm (n = 45), 18 parents thought to have been allocated to bumetanide, 25 parents expected to have been allocated to placebo, and 2 parents were uncertain. There was fair agreement between expected and actual treatment allocation ($\kappa = 0.312$ [0 indicating effective masking, 1 indicating failure of masking], $p = .004$).

Outcomes

Analysis of treatment effects revealed that bumetanide was not superior to placebo in SRS-2 total scores (mean difference −3.18, 95% CI = −9.49 to 3.14, $p = .319$), the primary outcome of the study (Table 2, Figure 2). A significant superior effect of bumetanide was found on the secondary outcome measure RBS-R, indicating a positive effect of bumetanide on repetitive behavior, a core symptom domain of ASD (model adjusted for heteroscedasticity, mean difference −4.16, 95% CI = −8.06 to −0.25, $p = .0375$). No effect was found on atypical responses to sensory stimuli with the SP-NL (mean difference 5.64, 95% CI = −11.30 to 22.57, $p = .508$). Finally, no effect of bumetanide was observed on the irritable behavior measure ABC-I (mean difference −0.65, 95% CI = −2.83 to 1.52, $p = .552$). The study was not sufficiently powered to test subscales (data not shown) in any of the endpoint measures. Descriptive results of the subscales are presented in Table S3, available online.

The subanalyses on treatment interaction of sex, age, total IQ, ADHD comorbidity, or prior medication use showed a marginally significant treatment-by-age effect on the SRS-2 (mean difference −2.54, 95% CI = −5.06 to −0.02, LRT = 3.4, $p = .065$), indicating that younger participants may tend to show more improvement on SRS-2 with bumetanide in this small study population. Furthermore, a marginally significant treatment-by-sex effect was revealed on RBS-R (mean difference −7.89, 95% CI = −17.95 to 2.17, LRT = 3.7, $p = .054$), indicating that female participants may tend to show better treatment response than male participants.

Individual changes in SRS-2 and RBS-R showed a conspicuous distribution (Figure 2). For both outcome measures, the nine participants with the largest improvement had been allocated to bumetanide treatment, which alludes to a responsive subset. Nevertheless, only two participants overlapped indicating limited phenotypic similarity, and, accordingly, a larger correlation between change in SRS-2 and RBS-R D91-D0 scores was found in the placebo-treated group ($r = 0.529$, $p = .001$) than in the bumetanide-treated group ($r = 0.294$, $p = .074$) (Figure S2, available online). No dose-dependent relationship was found. Mean treatment dose showed no correlation with change in SRS-2 or RBS-R score in the bumetanide arm (respectively, $r = 0.191$, $p = .251$; $r = 0.016$, $p = .926$).

Tolerability and Adverse Effects

Bumetanide was generally well tolerated. Adverse events occurring in more than 4% of the participants are listed in Table 3. All events were mild to moderate in intensity according to the CTCAE rating scale and resolved. Three serious adverse events (SAEs) occurred: syncope after venipuncture requiring a short period of clinical observation (bumetanide arm), extended hospitalization after a Kieselbach coagulation (placebo arm), and the occurrence of acute appendicitis requiring appendectomy (bumetanide arm). The SAEs were determined to be probably unrelated to treatment with the study medication, except for syncope, which was possibly related. Hypokalemia, orthostatic hypotension, dehydration, and diuresis were the most frequent and expected adverse events despite being treated with the preventive measures in the protocol. Diuresis and hypokalemia also occurred independently. A total of 51% of the participants receiving bumetanide treatment developed hypokalemia against none in the placebo arm ($p < .0001$). Hypokalemia did not occur before D14, and potassium levels did not drop below 3.0 mmol/L (Table S4, available online); 36% of the participants in the bumetanide arm

TABLE 2 Changes in Primary and Secondary Outcome Measures After Treatment and Wash-Out

	Placebo arm			Bumetanide arm			Treatment effect	*p*
	Baseline	D91	D119	Baseline	D91	D119		
SRS-2 total								
N	37	37	33	38	38	36		
Mean (SD)	90.8 (19.0)	85.7 (22.2)	86.2 (22.0)	91.4 (23.0)	81.5 (28.4)	82.6 (26.8)		.319
Mean difference with 95% CI							−3.18 (−9.49 to 3.14)	
RBS-R total								
N	37	37	31	38	38	36		
Mean (SD)	19.6 (12.4)	17.7 (14.0)	18.8 (15.7)	21.3 (13.9)	14.5 (9.9)	14.7 (10.1)		.038
Mean difference with 95% CI							−4.16 (−8.1 to −0.25)	
SP-NL total								
n	36	36	28	37	37	32		
Mean (SD)	457.2 (50.1)	463.6 (59.6)	459.0 (51.8)	446.2 (50.9)	460.2 (57.2)	462.3 (58.7)		.508
Mean difference with 95% CI							5.64 (−11.30 to 22.57)	
ABC-I subscale								
n	37	37	32	37	38	36		
Mean (SD)	14.5 (7.9)	11.2 (7.2)	12.6 (7.0)	14.3 (8.2)	10.4 (8.5)	10.4 (7.5)		.552
Mean difference with 95% CI							−0.65 (−2.83 to 1.52)	

Note: Data are shown for participants who completed D91. Treatment effects are measured with linear mixed models and are shown with 95% CIs. Significance level is p < .05. *ABC-I = Aberrant Behavior Checklist–Irritability (range 0–75; higher score is more affected; RBS-R = Repetitive Behaviors Scale–Revised (range 0–129; higher score is more affected). SP-NL = Sensory Profile–Dutch version (range 125–625; lower score is more affected); SRS-2 = Social Responsiveness Scale–2 (range 0–195; higher score is more affected).*

developed orthostatic hypotension (bumetanide 17 [36%], placebo 5 [11%], $p = .007$). No paradoxical response or deterioration of irritability was observed.

In an attempt to account for potential unmasking through diuretic effects, we performed linear mixed model analysis comparing treatment effects in three groups: placebo, bumetanide with hypokalemia, and bumetanide without hypokalemia. A larger treatment effect in the bumetanide with hypokalemia group may be expected when masking would be compromised. No treatment effect difference was suggested between participants with and without hypokalemia for all outcome measures (SRS-2 mean difference −0.65, 95% CI = −9.52 to 8.22, $p = .884$; RBS-R mean difference 2.04, 95% CI = −2.64 to 6.72, $p = .387$; ABC-I mean difference 1.54, 95% CI = −1.49 to 4.57, $p = .342$; and SP-NL mean difference 4.92, 95% CI = 18.70 to 28.54, $p = .679$). The presence of diuresis did not show a difference in treatment effect (SRS-2 mean difference −5.77, 95% CI = −15.43 to 3.90, $p = .238$; RBS-R mean difference 1.80, 95% CI = −4.06 to 7.67, $p = .541$; ABC-I mean difference −2.65, 95% CI = −6.29 to 0.98, $p = .149$; and SP-NL mean difference 0.55, 95% CI = −25.54 to 26.63, $p = .967$).

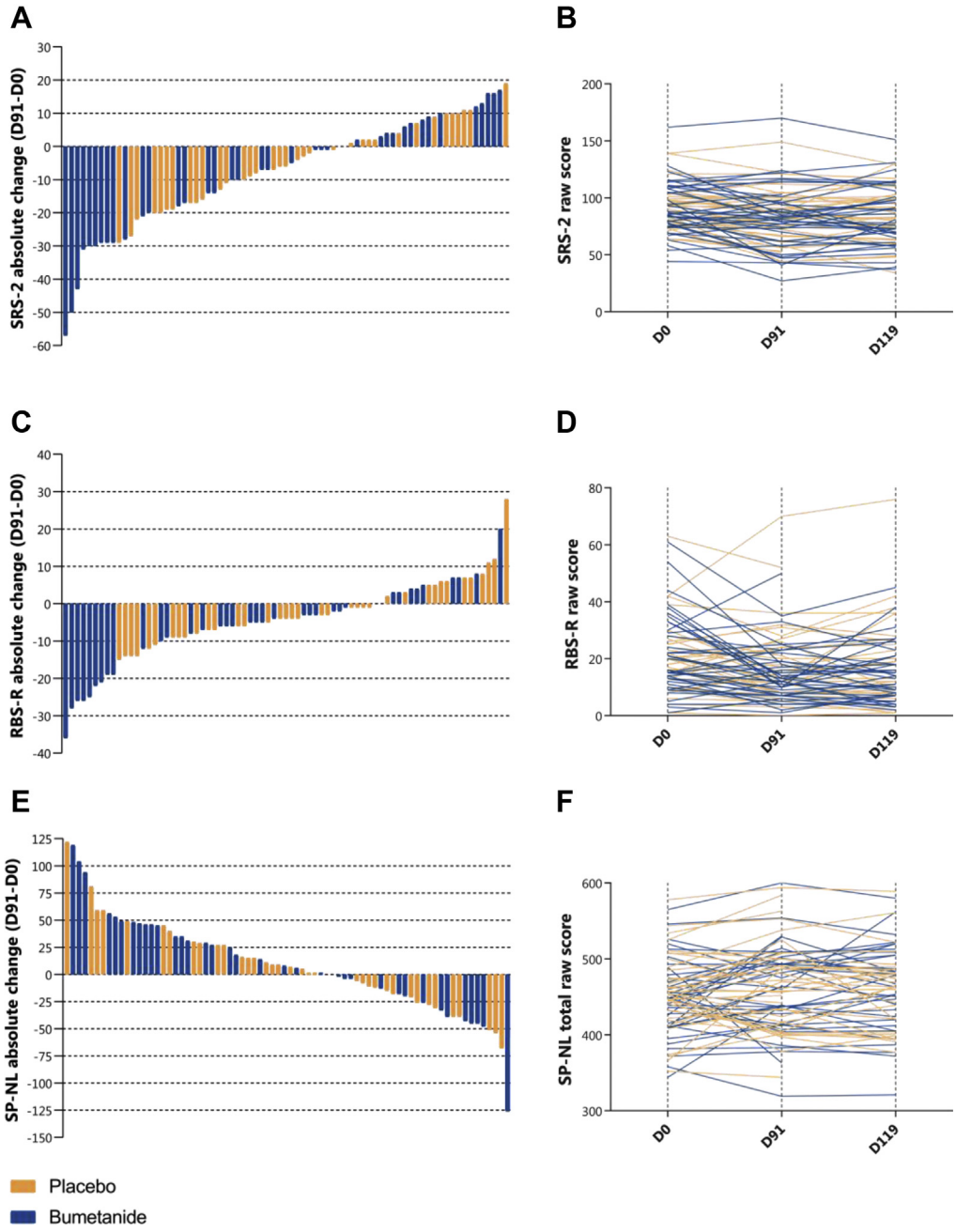
DISCUSSION

These results from the BAMBI trial did not show a superior effect of bumetanide over placebo on the primary outcome of a broad scale of ASD symptomatology, and indicated a nominal significant superior effect on a secondary outcome measure of repetitive behaviors. In contrast with the earlier trials, our findings do not support broad applicability in ASD, but may indicate effectiveness in subgroups on a specific symptom domain.

There is an ongoing debate on selecting outcome measures for randomized controlled trials (RCTs) in ASD. Previous RCTs testing bumetanide showed effect on the CARS as a primary outcome, a scale that has been developed as a screening measure for ASD but with unknown ability to measure change.[30] The primary outcome of this study, the SRS-2, measures ASD symptomatology as a single quantitative trait and has been regarded as a potential reliable outcome measure for ASD trials.[30] Nonetheless, the lack of a proven accepted measure for change in core symptoms remains a problem.[30] We also chose the SRS-2 for comparability to other recent trials and found similar effect sizes.[31-34] In comparison with the most recent bumetanide RCT that included the SRS-2 as a secondary outcome,[35] we found a comparable mean SRS-2 change in the bumetanide arm (9.9 versus 13.2 points); however, in our study, a greater placebo effect was obtained (5.1 versus 1.5 points). Other ASD trials showed effect sizes on SRS-2 similar to those in this study.[31-33,36]

It is important to note that the previous bumetanide RCT[14] included, on average, younger and more severely affected children (112.3 SRS-2 score versus 89.7 in our study) without characterization of IQ or comorbidity. We used a different statistical analysis to test superiority and to

FIGURE 2 Individual Treatment Effects on the Autism Spectrum Disorder Core Domain Outcomes



Note: *(A) Individual SRS-2 total score changes between D91 and D0. A negative score indicates improvement. (B) Individual SRS-2 total scores over the different time points. (C) Individual RBS-R total score changes between D91 and D0. A negative score indicates improvement. (D) Individual RBS-R total scores over the different time points. (E) Individual SP-NL total score change between D91 and D0. A positive score indicates improvement. (F) Individual SP-NL total scores over the different time points. RBS-R = Repetitive Behavior Scale–Revised; SP-NL = Sensory Profile–Dutch edition; SRS = Social Responsiveness Scale. Please note color figures are available online.*

include baseline measurement of outcomes to correct for potential confounding and to optimize the statistical analysis for power.[27-29] We noted that in the BAMBI trial, younger children showed marginal significance toward more improvement on SRS-2, which may be consistent with better efficacy in younger children with ASD. Indeed, there is a suggestion that efficacy of treatments targeting GABAergic inhibition is related to so-called windows of

TABLE 3 Adverse Events Occurring in More Than 4% of Participants Classified in Medical Dictionary for Regulatory Activities (MedDRA) Categories

	Bumetanide arm				Placebo arm			
Symptom	**No. of AEs**	**No. of part.**	**Severity**	**IR[a]**	**No. of AEs**	**No. of part.**	**Severity**	***p***
Total AE	276	46			161	43		
Metabolism and nutrition disorders								
Hypokalemia	31	24	Moderate	1	0	0	Moderate	<.0001
Dehydration	8	8	Moderate	1	1	1	Moderate	.031
Hypoglycemia	1	1	Mild	3	3	3	Mild	.617
Gastrointestinal disorders								
Vomiting	14	11	Mild	2	5	4	Mild	.089
Nausea	13	10	Mild	2	8	7	Mild	.594
Abdominal pain	17	13	Mild	3	14	11	Mild	.814
Diarrhea	3	3	Mild	3	5	5	Mild	.481
Obstipation	6	5	Moderate	2	1	1	Moderate	.204
Dyspepsia	4	4	Mild	2	1	1	Mild	.362
Gastroenteritis	3	3	Mild	3	2	1	Mild	.617
Vascular disorder								
Orthostatic hypotension	22	17	Mild	1	7	5	Mild	.007
Epistaxis	3	3	Moderate	3	2	2	Moderate	1.000
Syncope	3	3	Mild	2	3	1	Mild	.617
Infections and infestations								
Common cold	21	19	Mild	3	16	13	Mild	.279
Otitis media	4	4	Moderate	3	2	2	Moderate	.677
Musculoskeletal and connective tissue disorders								
Myalgia	12	12	Mild	2	10	8	Mild	.452
Muscle cramp	5	4	Mild	2	5	5	Mild	.737
Renal and urinary disorders								
Dysuria	5	4	Mild	2	4	4	Mild	1.000
Enuresis[b]	2	2	Mild	1	1	1	Mild	1.000
Diuresis	14	14	Mild	1	4	4	Mild	.017
Nervous system disorders								
Headache	12	10	Mild	3	19	15	Moderate	.244
Dizziness	8	6	Mild	3	5	5	Mild	1.000
Blurred vision	2	2	Mild	3	3	3	Mild	.674
Psychiatric disorders								
Insomnia	10	9	Mild	3	6	6	Mild	.575
General disorders and administration site conditions								
Fatigue	6	6	Mild	2	5	5	Mild	1.000
Skin and subcutaneous tissue disorders								
Dermal abnormalities	14	12	Moderate	3	8	7	Moderate	.306
Injury, poisoning and procedural complications								
Injury	7	6	Moderate	3	3	3	Mild	.486

Note: Data are numbers (n). Differences were tested with Fisher exact tests. Significance level is $p < .05$. *AE = adverse event; IR = intervention relationship; Part = participants.*

[a] *1 = definitely related; 2 = possibly related; 3 = not related.*

[b] *Occurring in <4% of participants but listed as important expected AE.*

plasticity in which excitation−inhibition balance is expected to be crucial for functional brain development.[37] Our observed potential age effect seems consistent with the increasing notion that ASD trial drugs should not be abandoned purely on the basis of effects in adults, but always should also be tested in younger children. The previous RCT tested different dosages including a higher dosage regimen (2 mg twice daily) that contributed to a higher

drop-out rate.[14] We therefore aimed for the suggested optimal dosage of 1.0 mg twice daily, and observed no dose-dependent effect in the currently applied range. The previous bumetanide studies have been criticized for potential unmasking through diuretic effects, which we tried to reduce through increased fluid intake instruction and supplementation of potassium in both treatment arms as well as organizing treatment surveillance through an independent team. We could therefore better analyze potential influences of diuretic effects on treatment outcome, and found no statistical indication that bumetanide treatment results were substantially influenced by their occurrence.

We analyzed additional scales of core symptomatology and the ABC-I to assess potential outcome measures for more targeted future studies. A potential superior improvement of bumetanide versus placebo on the RBS-R, a measure of repetitive behaviors, was found. Other recent ASD trials also incorporated this measure. To our knowledge, this is the first trial to report a potential effect on this scale, although we note that type I error may account for the marginally significant finding. Our baseline RBS scores were similar to those of the large EU-AIMS LEAP study, which showed a mean RBS-R score of 16.75 and an SD of 13.85 points ($n = 346$).[38] The previous bumetanide RCTs did not test this scale,[13,14] although an effect on the repetitive behavior subscales of the ADOS-2 and SRS-2 in the first and second bumetanide RCT, respectively were described. Together, efficacy for repetitive behavior may be suggested by our findings, which needs replication in a follow-up trial. Such a study may take into account that our observed effect on RBS-R seemed to be more explicit in female participants. It is important to mention that repetitive behaviors are not always perceived as challenging by patients and caregivers, and can have a function to cope with stress and anxiety. Future bumetanide studies may include measures of stress and anxiety to gain more understanding of how a reduction in repetitive behaviors may be mediated. No apparent effects on sensory reactivity (SP-NL) or irritable behavior (ABC-I) were observed. The SP-NL was included because of the recent addition of sensory reactivity to the second core domain of ASD in the *DSM-5*. The SP-NL has not been developed as an outcome scale and is generally used to characterize sensory behavior profiles, which limits its usefulness in RCTs.[39] The second measure, the ABC-I, has been used to validate the use of antipsychotic drugs for irritability in ASD.[1] These studies used as an inclusion criterion a high threshold for baseline ABC-I scores that would have led to the exclusion of the majority of individuals in the current study population.

There are several important limitations to the present study. Recruitment was stopped at the end of the scheduled 2 years, and the intended 100 participants were not reached. However, because of a lower attrition rate, we nearly reached our inclusion target, with 88 instead of 90 participants finishing the trial. The presented analyses were nonetheless best suited for power limitations, and the study appeared to be sufficiently powered to detect changes in RBS-R with adjustment for heteroscedasticity, albeit uncorrected for multiple testing; therefore, this result should be replicated and interpreted with caution. We chose to follow a modified ITT analysis to add to existing evidence and to optimize generalizability to real-world treatment effects, and excluded unreliable data from the analysis before unmasking. Our sample may not have been representative of the whole ASD population because of the exclusion of concomitant medication use, comorbidities such as seizures, and severe intellectual disability. Our sample had a greater prevalence of female participants (3:1) than encountered in most studies (4:1), allowing for subgroup analysis by sex. The average IQ was higher, possibly because of the exclusion of children at the lower end of the IQ spectrum. ADHD comorbidity seems lower (18%), although history of stimulant prescription (39%) suggests that this is equal to other descriptive studies.[40] We have presented the largest bumetanide trial to date, but the size of the population and the observation period still preclude conclusions of the best responders in terms of age, severity, and clinical characteristics. The nine greatest SRS-2 and RBS-R responders all had been treated with bumetanide, but we found a limited correlation between the changes in these outcomes. It has been questioned in this respect whether medication can be expected to directly improve social communication.[30] Perhaps changes in repetitive behavior can be more readily observed in 3-month treatment, whereas social behavioral change is a more complex phenotype requiring longer treatment duration or additional behavioral training.[30] For instance, SRS-2 score improvements manifested only after a 12-week treatment duration in a recent extensive trial testing memantine.[31] The study shows a high placebo effect, which affected the estimation of the treatment effect. Other designs such as placebo run-in may be considered for future studies. Another unresolved issue is whether different symptoms across different individuals can be caused by the same pathway.[18] An evident problem here is that chloride regulation in the brain cannot yet be tested in humans, and elevated chloride in neuronal cells has been established only in animal models. Furthermore, limited penetrance of bumetanide across the blood–brain barrier has been indicated, implying that systemic non-neuronal effects of treatment should also be considered.[11]

Our results did not show an effect on the primary outcome of broad autism symptomatology, but suggest efficacy of

bumetanide on repetitive behaviors in yet-to-be-defined subgroups. The findings highlight the complexity of ASD heterogeneity in trial research,[41] and the necessity of inclusion of functional brain measures to understand treatment effect variability and to develop stratification markers.[42] For now, we conclude that random off-label prescription of bumetanide for children with ASD is not recommended by our findings.

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