Bumetanide in Autism Medication and Biomarker study

Short title: ***BAMBI***

Bumetanide GABA ASD Treatments

Eudra-CT-Study number 2014-001560-35

Monocenter Randomized Controlled Trial Study

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NL49210.041.15 **BAMBI**

PROTOCOL SIGNATURE SHEET

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABC	**Aberrant Behavior Checklist**
ABC-I	**Aberrant Behavior Checklist-Irritability subscale**
ABR	**ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)**
ADI	**Autism Diagnostic Interview**
ADOS	**Autism Diagnostic Observation Schedule**
AE	**Adverse Event**
AR	**Adverse Reaction**
ASD	**Autism spectrum disorder**
BRIEF	**Behavior Rating Inventory of Executive Function**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CV	**Curriculum Vitae**
DALY	**Disability-adjusted life-years**
DSMB	**Data Safety Monitoring Board**
EEG	**Electroencephalogram**
ERP	**Event Related Potential**
EU	**European Union**
GCP	**Good Clinical Practice**
IC	**Informed Consent**
MCR	**Medical chart reviewing**
METC	**Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)**
NKCC1	**Na-K-Cl cotransporter-1**
RAVLT	**Rey Auditory Verbal Learning Test**
rsEEG	**Resting-state EEG**
(S)AE	**(Serious) Adverse Event**
Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.**
SRS	**Social Responsivenes Scale**
SUSAR	**Suspected Unexpected Serious Adverse Reaction**
Wbp	**Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)**
WISC	**Wechsler Intelligence Scale for Children**
WMO	**1. Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**
YLD	**Years lived with disability**

SUMMARY

Rationale:
Currently no treatments exist for the core symptoms of Autism Spectrum Disorder (ASD). Only two antipsychotics are approved to treat symptoms that often accompany ASD. These drugs are not etiology driven and cause significant adverse effects. In this context, there is an extreme and urgent demand for rational and safe treatments to avert the debilitating consequences of this large group of developmental disorders. Recently an old drug, bumetanide, has been proposed as a first possible etiology driven treatment for ASD. Bumetanide has been used for decades as a diuretic and its safety has been firmly established. Interestingly, recent experimental studies have shown that bumetanide may enhance GABAergic inhibition and ameliorate the symptoms of ASD. In this capacity, bumetanide could become a rational and safe drug for patients with no therapy at present. A first trial (n = 56) has tested bumetanide in a modest sample of children with ASD suggesting a reduction in global symptom severity after 90 days of 0.5mg-1.0mg bumetanide bidaily (BD). Replication is warranted in larger samples with endpoints that have been used in other trials. In addition, prognostic markers are needed to select the most responsive patients.

This study will assess the clinical efficacy of bumetanide using the most common behavioral endpoint scale in ASD trials to establish a first rational treatment concept in ASD that can be administered safely in children and adolescents.

Objectives:

1. Primary aim: to investigate whether thirteen weeks treatment with bumetanide will improve daily life functioning and reduce behavioral symptoms related to hyperexcitability in children with ASD in comparison with usual care.
2. Secondary aim: to identify prognostic biomarkers of bumetanide treatment using cognitive, genetic and neurophysiological assessments.

Study design:
This is a monocenter, randomized, double-blind, parallel-group, placebo-controlled trial.
Study population:
90 children with ASD between 7 and 15 years of age
Main study parameters/endpoints:
Primary endpoint: Social Responsiveness Scale (SRS) at Day 91
Secondary endpoints: resting-state electroencephalogram (rsEEG), event-related potential (ERP) phenotypes, neurocognitive and other behavioral parameters.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness:
The burden and risk of the application of this off-label drug for children with ASD are acceptable while the benefits are expected to be considerable especially when taking into account the total lack of treatments for this highly prevalent and devastating disorder.

Risks
Bumetanide has been used as a diuretic drug for decades. In patients with conditions of fluid overload, its safety and tolerability after short and prolonged treatment has been established in all ages apart from neonates. Experience and safety of bumetanide in patients with ASD is

based upon recent studies, including one randomized controlled trial in a sample of 56 children[1]. These data indicate that bumetanide significantly alleviates behavioral morbidity at dosages ranging from 1 to 2 mg daily. The main adverse events are related to the diuretic activity of the molecule leading to a decrease in electrolytes, notably mild hypokalemia are frequently reported.

Burden

The main burden of this study is posed by the outpatient clinic visits to follow up the safety of the diuretic effects, which requires physical examination and blood and urine tests, which are of negligible and known risks. Additional burden is posed by the measurement of cognitive and EEG tests. These tests are deemed necessary to develop prognostic markers to select the most responsive patients out of the heterogeneous ASD population. We have used the same rsEEG/ERP/cognitive test battery in a previous pilot study and in an ongoing study (METC 15/143, entitled: **"**Sensory processing in autism and childhood epilepsy"). From these studies, we learned that these tests were well-tolerated by children in the intended age groups and did not cause any substantial burden.

Benefit and group-relatedness

Bumetanide could be the first pharmacological treatment for the core symptoms of ASD acting on a key component of the pathophysiology of the disease. Restoring GABAergic inhibition may restore important developmental capacities. As a consequence, we intend to apply bumetanide in children with ASD with the intent to improve the behavioral functioning and cognitive outcome. This trial addresses a very large population of young developing patients for whom no other treatment option currently exists.

1. INTRODUCTION AND RATIONALE

Problem definition and theoretical foundation of the study

Autism spectrum disorder (ASD) is recognized as a neurodevelopmental disorder manifesting within the first 3 years after birth with diverse phenotypic outcomes[2]. Two core behavioral domains clinically characterize ASD according to the 5th version of the diagnostic system manual (DSM-V): deficits in social communication/interaction and restricted/repetitive patterns of behavior. Especially the more severe forms of the disorder are accompanied by emotional, sensory and cognitive distress and can put a tremendous burden on family resources. It is estimated that approximately 1 in 100 children display signs and symptoms that lead to a diagnosis of ASD [3], making it more common than childhood cancer and juvenile diabetes together. Moreover, the prevalence of ASD diagnosis has shown an exponential rise in the last two decades. About half of this rise seems accounted for by factors such as broader diagnostic criteria, lower thresholds for clinical diagnosis or higher parental age, leaving roughly half of the increase unexplained [4]. Bob Wright, co-founder of Autism Speaks, said, "Autism is a global public health crisis. The costs are staggering and will continue to rise as prevalence continues to increase. We know that early diagnosis and treatment are critical, so it is imperative that the U.S. and governments around the world step up their commitment to helping people living with autism today. The investment we make now is essential to reducing the long-term costs of autism."
(https://www.autismspeaks.org/about-us/press-releases/annual-cost-of-autism-triples). The growing awareness of the burden of ASD has fuelled tremendous research efforts, but no etiology-driven treatments are currently available to treat the core symptoms of ASD.

In children under 5 years of age, ASDs have been shown to be the leading cause of disability, in terms of years lived with disability (YLDs), among all mental disorders. ASDs accounted for 7.7 million disability-adjusted life-years (DALYs) in 2010. Globally, ASDs accounted for 170 DALYs per 100 000 males (95% UI 119–237) and 50 DALYs per 100 000 females (95% UI 35–68) [3].
In the UK, mean annual costs for a child with ASD was calculated at €30,000 per year [5]. The largest contributors to these costs were direct nonmedical costs, such as special education (including early intervention services), and indirect costs, such as parental productivity loss. In the Netherlands, the individual costs for ASD have not been published but total annual health care cost for psychiatric disorders in underaged subjects is estimated around €670 million (www.kostenvanziekten.nl). Taken together, there is an extremely urgent need for effective treatments for ASD.

Current treatment options
Only two antipsychotics, aripiprazole and risperidone, marketed as Abilify and Risperdal, respectively, are approved to treat symptoms that often accompany ASD [6]. These drugs were empirically validated and their effect on challenging behaviors has been attributed to sedative effect. However, both medications also cause significant adverse effects including marked weight gain, sedation, and risk of extrapyramidal symptoms [6]. Given the lack of rational drug treatments, behavioral and developmental interventions are currently the predominant treatment approach. In the last decade, there have been therefore major investments to develop etiology driven treatments for ASD, but all of these attempts have failed so far. Here, we aim to show efficacy of bumetanide as the first potential rational drug treatment to reduce ASD morbidity. Bumetanide is off patent due to its application as a diuretic agent for decades. From this experience, it is known that bumetanide is safe, also in newborns and children [7,8].
In this proposal, we aim to show efficacy of bumetanide on ASD behavioral symptoms in children and adolescents with ASD between 7 and 15 years of age, an age range at which ASD is generally characterized by fierce behavioral problems and social burden. In addition, we aim to develop prognostic markers that will enable the selection of most responsive patients out of the heterogeneous population of ASD. It is estimated that around 25000 Dutch children in the intended age group have obtained a diagnosis in the autism spectrum.

GABA, E/I balance and chloride in ASD
In recent years, inhibitory circuit dysfunction has gained increasing attention in ASD research. GABA is the major inhibitory neurotransmitter in the brain, and it is now generally assumed that the imbalance between excitation and inhibition resulting from GABAergic defects might represent a cause for ASD [9,10]. Increased ratio of excitation:inhibition (E:I) in sensory, mnemonic, social, and emotional systems have been proposed as a model underlying at least some forms of autistic disorder. Interestingly, GABA is excitatory in prenatal life and switches to inhibitory action around birth [11]. The GABA switch depends on intracellular chloride levels, which in turn are set by the relative activity of NKCC1 (more Cl^- “in”) and KCC2 (more Cl^- “out”) transporters. NKCC1 is predominantly expressed embryonically and turned off around birth, which enables GABAergic inhibition. Recent findings suggest that elevated chloride levels might persist under pathological conditions and give rise to hyperexcitability and autistic symptomatology, possibly due to persistent NKCC1 expression [12].

Bumetanide, a chloride lowering agent for the treatment of ASD

Interestingly, bumetanide is a well-tolerated NKCC1 antagonist, e.g. previously used as a diuretic drug that may also normalize chloride levels in neuronal cells and reduce hyperexcitability. The potential application of bumetanide in ASD was boosted last year when a landmark study in Science showed that elevated chloride levels contribute to hyperexcitability in two important animal models of ASD [13] as well as in the model for Down syndrome a genetic condition with high prevalence of ASD[14], (also see http://news.sciencemag.org/brain-behavior/2012/12/diuretic-drug-offers-latest-hope-autism-treatment). In contrast with existing treatments, the application of bumetanide is etiologically driven and will not affect the central nervous system unless chloride homeostasis is affected. Other diuretic agents such as furosemide are not suitable in this context. Furosemide is a less potent diuretic than bumetanide but in addition to NKCC1 also antagonizes the chloride exporter KCC2 and would therefore exert a chloride lowering effect [15] [16]. Bumetanide has a much greater affinity for NKCC1 than for KCC2 [17].

Previous studies with bumetanide in ASD

One previous open label and one randomized placebo controlled trial has tested bumetanide in children with ASD [1]. The RCT conducted by Lemonnier et al. established a modest improvement in global autism symptom severity after 90 days of bumetanide. Although this study was a very important and pioneering study, the sample size in this trial was relatively small and the study has been criticized for using uncommon primary outcome measures. The main reported adverse event was mild hypokalemia due to the diuretic effect of bumetanide. No abnormalities were found on ultrasound and ECG recordings. Furthermore, the treatment was well tolerated. The overall effect of this first trial was considered significant, but it also showed that some patients did not respond to treatment, while others showed remarkable recoveries. Indeed, the widely acknowledged etiological heterogeneity of ASD precludes that all patients will benefit from one particular treatment such as bumetanide [18,19].
Neurochlore, a French biotech company is developing Bumetanide for ASD children and adolescents for which there is presently no efficient pharmacological treatment. This project received a positive opinion from the Pediatric Committee of the European Medicine Agency. Neurochlore is conducting a phase II dose ranging study in 88 ASD children and adolescents. The main objective is to determine the optimal dose for a pivotal phase III study. Neurochlore provides the IB, IMPD and bumatenide and placebo syrup for this study under the agreement of sharing the treatment outcomes and blinded adverse event data.

In summary, larger and more detailed trials are needed to confirm efficacy of bumetanide on ASD morbidity and to deliver markers to select (the most) responsive candidates to the treatment. Indeed, the assumed effect of bumetanide on neuronal hyperexcitability could allow prognostic examinations of neurophysiological markers associated with E:I imbalance. Notably, an fMRI study has indicated that face emotion recognition may designate treatment effect in certain individuals with ASD [20]. Accordingly, testing of neurophysiological (EEG) and neurocognitive traits may offer important insights to select responsive patients and to enhance the rational application of bumetanide [21]. Taken together, bumetanide may become a first etiology driven therapy for ASD but its efficacy needs to be further validated and more restricted patient selection criteria need to be developed.

Case report with bumetanide in a girl with ASD

We treated a 9-yr old girl with bumetanide as an off-label treatment to test the feasibility of our protocol and to confirm the previously suggested effect of bumetanide on behavior and well-being of these patients [1,22,23]. At the age of 8, this girl was referred to our clinic for diagnostic evaluation of increasing learning disability, behavioral inflexibility and emotional disturbance. We observed a delay in language development, rigidity and repetitive behaviors with peculiar interests. Furthermore, she showed evident deficits in long-term memory performance. A diagnosis of ASD was confirmed by diagnostic evaluation and Autism Diagnostic Observation Scale (ADOS) criteria. The Autism Diagnostic Interview- revised (ADI-R)[24] revealed ASD at current age but subclinical scores for the “ever” algorithm. Her Full scale IQ was 71 (Wechsler Intelligence Scale for Children – third version (WISC-III NL)). The patient received 0.5 mg Burinex® (half a 1 mg tablet per dosing) twice a day (bid) (morning and evening) for 180 days under monitoring of behavior, cognition and EEG as proposed in this study. The treatment did not cause adverse effects, hypokalemia or discomfort through diuretic effect. Where previously she initiated limited social activities and struggled with remembering places and interactions, parents noted that she now could recall events in chronological order, with more insight in social context. This improvement was reflected in the parental questionnaires showing that aberrant response to sensory stimuli (Sensory Profile)[25] and behavioral rigidity (Repetitive Behavior Scale-revised)[26] declined from clinical to normal levels. The notion of cognitive improvement was further substantiated in a striking improvement on neuropsychological testing. Assessment before the start of the intervention (T1 baseline) revealed average performance (z-scores > -1) on baseline attention, visual working memory, visual learning, and cognitive flexibility to visual stimuli. In contrast, she showed significant impairments (z-scores < -2) on auditory learning and short-term memory, cognitive flexibility to auditory stimuli and impulse inhibition [27-30].
After 6 months of treatment, her memory skills improved remarkably. Auditory learning and memory skill [29] improved from markedly impaired to within normal ranges (*z*-score = -2,4 at T1; *z* = -1,3 at T2 after 90 days and *z* = - 0,7 at T3 after 180 days of treatments) (Figure 1A). This was unlikely to be due to multiple testing because the three different word-lists of the Rey Auditory Verbal Learning Test (RAVLT) were used on three occasions [29]. The improvements in memory were also clinically significant according to parents (absolute increase in working memory *z*-score = 1.3 on the Behavior Rating Inventory of Executive Function (BRIEF))[31]. Parents however reported significant improvement in flexibility in daily life on the BRIEF. Inhibitory control on a task that required visuospatial processing improved significantly (absolute increase in *z*-score 0.9 at T2 and 1.9 at T3, from abnormal to normal range)[27]. Performance in other tasks of response inhibition and attentional flexibility in response to auditory information remained unchanged through treatment.
To indicate an effect on brain activity, the EEG spectral power was compared to 15 age-matched healthy individuals. A localized (Pz and neighboring channels) progressive increase in power of alpha frequency oscillations (6–12 Hz) through treatment was found. (Figure 1B-1D). Following the bumetanide treatment, a washout period of 28 days was installed. Both parents and patient noticed a recurrence of memory problems and increased distractedness, after which it was decided to reinstall treatment for a longer period.

Embedding of the study
We obtained funding from ZonMw’s Rational Pharmacotherapy programme (in Dutch Goed Gebruik Geneesmiddelen, abbreviation ‘GGG’) to conduct the proposed study. This funding program aims to make the use of available medicines safer, more effective and more

efficient. In accordance with the guidelines of this program, we collaborate with the Health Technology Assessment (HTA) department of the Julius Center in the UMC Utrecht. Health Technology Assessment refers to the interdisciplinary evaluation of new developments in healthcare and medicine. The Health Technology Assessment department performs health technology assessments (HTA) and economic evaluations of medical interventions in the broadest sense (e.g. medication, surgery, diagnostics, public health) in all disease areas. HTA team members Ardine de Wit and Henk van Stel will assist on the data management and analysis. In addition, we have sought consultation from the Dutch Medicines Evaluation Board (CBG) to ensure that the design of this study is maximally embedded in the regulatory framework provided for the implementation of these kind of treatments and to guide the process towards implementation in Dutch clinical standards. In addition, we collaborate with the French biotech company Neurochlore who have obtained EMA approval for a Pediatric Investigation Plan to test bumetanide with a paediatric formulation for the ASD pediatric population. Neurochlore will provide this formulation and subsequent IMPD for this study via a service provider (AmatsiGROUP, France). Neurochlore has performed several pilot studies including a first RCT in ASD as mentioned above. Our study is complementary to this program as their studies do not include prognostic biomarker evaluations and it is mandatory that other groups outside Neurochlore test efficacy of bumetanide.

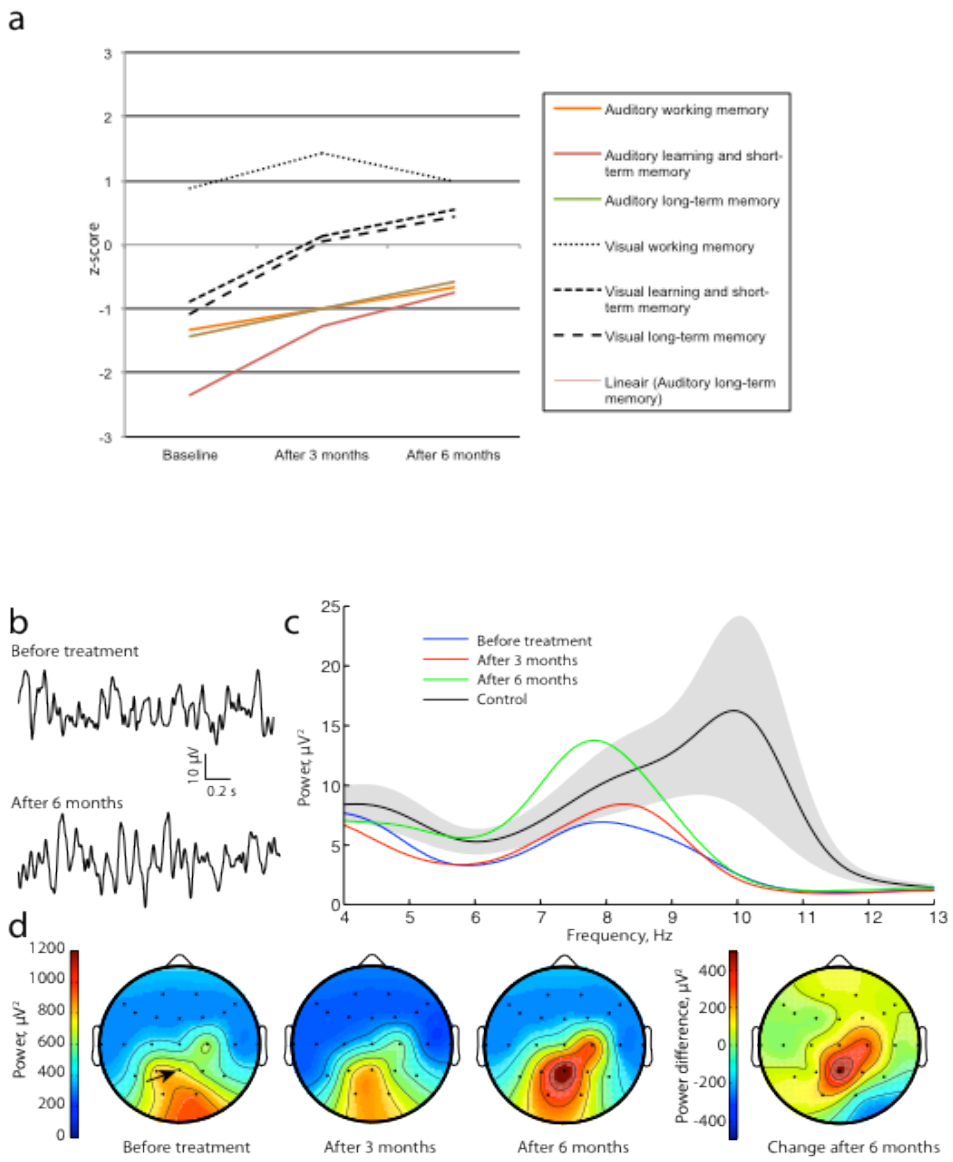

Figure 1: Effects of bumetanide treatment on memory and resting-state brain activity.
a) Change in memory skills (*z*-score based on age-match Dutch population norm scores).
b) Example EEG trace in Pz channel (black arrow in d) before and after 6 months of treatment.
c) Power spectrum density plot of Pz channel showing the increase of power in the alpha frequency band during bumetanide treatment compared to healthy control children (n=15, age 10-11 yrs). Control data shown as mean power (black) with SEM (grey).
d) Power of the alpha frequency band (6-12 Hz) in 23 EEG channels of the patient before and during treatment; the difference in power after 6 months of treatment is shown right.
Median power in the 6–12 Hz range went from 3.9 ± [3.5:4.5] □V² median ± [95% confidence interval of power density estimate] before treatment, 4.2 ± [3.7:4.8] □V² after 90 days, to 6.1 ± [5.4:7.0] □V² after 6 months. Control subjects had a median power (6–12 Hz) of 4.2 ± [2.1:13.4] □V²

2. OBJECTIVES

Primary aim: to investigate whether thirteen weeks treatment with bumetanide will improve daily life functioning and reduce behavioral symptoms related to hyperexcitability in children with ASD in comparison with usual care.

Secondary aim: to identify prognostic biomarkers of bumetanide treatment using cognitive, genetic and neurophysiological assessments.

3. STUDY DESIGN

This is a monocenter, randomized, double-blind, parallel-group, placebo-controlled trial testing the efficacy of bumetanide treatment during three months with the primary end point of change in SRS at Day 91 in 90 children with ASD between 7 and 15 years of age. Usual care + bumetanide will be compared with usual care consisting of a family component including parent training and incorporation of a high degree of structure through elements such as predictable routine and visual activity schedules. In addition, we will test a selected number of cognitive and neurophysiological markers to establish effects on brain activity and functioning and to generate predictive markers of treatment response.

4. STUDY POPULATION

4.1 Population (base)

Ninety children with ASD between 7 and 15 years of age will be included, either male or female, with an IQ> 55. These children will be recruited from the patient population referred to the department of developmental disorders (DDD) of the UMC Utrecht. Yearly 350 new patients visit the DDD, of whom per year approximately 150 children will be eligible for the current study. In addition, we have a prevalent population of 400 children with ASD, of who around 150 are eligible. As the treatment is well-tolerated and patients and their parents have no alternative treatment options, we expect that parents and children will be eager to participate given the absolute lack of similar treatment options for ASD and that at least 25% will consent would be a sufficiently conservative estimate. After an additional drop out of 10% this ensures the participation 100 children ((0.25*(150+150+150))-10%) to participate. If unexpectedly we fail to recruit sufficient patients, we can approach colleagues in large regional centers in our vicinity to refer eligible patients to our center. In addition, we will advertise the study via the website of the Dutch Autism Association / Balans (parent organization) (text and layout are in preparation), the website of the Division Brain of the UMCU, the website of Rhino (a training institute for psychologists and other professionals involved in the care for children with ASD) and via the LinkedIn group of Dutch Child and Adolescent Psychiatry.

4.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

1. Males or females aged ≥7 years to ≤15 years;
2. Criteria met for autism on DSM-IV or V and Social Responsiveness Scale (SRS) (24).
3. Written informed consent.

4.3 Exclusion criteria

1. Total IQ < 55 (WISC) and/or inability to comply with the protocol-specified procedures for the duration of the study, including treatment and blood sampling to control diuretic effects.
2. Serious, unstable illnesses including, gastroenterologic, respiratory, cardiovascular (arythmias, QT interval lengthening), endocrinologic, immunologic, hematologic disease, dehydration or hypotension, electrolyte disturbances (Na <133 mmol/L, K <3.5 mmol/L or Ca < 2.17 mmol/L (<13y) or <2.2 mmol/L (>13y))
3. Renal insufficiency (CKD st2-5; estimated glomerular filtration rate < 90 ml/min/1.73m2), congential or acquired renal disease with decreased concentration capacity (tubulopathy, diabetes insipidus) and liverinsufficiency interfering with excretion or metabolism of Bumetanide;
4. Neurological disorders such as epilepsy, seizures and microcephaly;
5. Start of behavioral treatment during study
6. Treatment with psychoactive medications, (antipsychotics, antidepressants, anxiolytic drugs, psychostimulant drugs or other medication with effect on the central nervous system, including anti-epileptic drugs), in the last 8 weeks prior to start of the study, except melatonin; no use of other psychoactive substances is allowed from 8 weeks prior to the pre-study evaluation until the endpoint measurements at the end of the washout period. If clinically feasible and desired by the patients and/or parents, then it

is allowed to stop psychoactive medication to allow enrollment in the study after a 8 week washout period of their psychoactive medication.

7. Treatment with NSAIDS, aminoglycosides, digitalis, antihypertensive agents, indomethacin, probenecid, Lithium, other diuretics (e.g. furosemide, hydrochlorothiazide), drugs known to have a nephrotoxic potential
8. Documented history of hypersensitivity reaction to sulfonamide derivatives.

4.4 Sample size calculation

We plan to randomly assign 100 patients to obtain at least 90 assessable patients (45 per group), allowing for a 10% attrition rate. The sample size calculation based upon the SRS (the primary outcome scale of the study) showed that 2x47 subjects are needed to provide at least 85% power to test the primary hypothesis for the SRS using a two-tailed two-sample t test with $\alpha = 0.05$ and assuming that the true difference in average change (reduction following treatment) in SRS was 10 units with a SD of 16 units [32]. In addition, we calculated based upon ABC irritability subscale an important secondary endpoint of this study as this is the most common outcome measure used in autism trials[33]. The sample size of 90 patients will provide 93% power to differentiate between placebo and bumetanide when the true difference in mean change from baseline in ABC irritability subscale score is 7.0. This assumed an SD of 9.3 [33] and a 2-sided test at the 0.05 level of significance.

5. TREATMENT OF SUBJECTS

5.1 Investigational product/treatment

The investigational products consist of bumetanide or placebo. Patients with a body weight ≥ 30 kg will be given a dose between 0.5mg and 1.0mg twice a day (before breakfast and at the end of the afternoon, at least 2.5 hours before bedtime) for 91 days. The IP will be administered in the formulation of a solution (1 mL per intake = 0.5 mg bumetanide or placebo) and will be administered directly into the mouth by means of a graduated dosing syringe. Starting dosage will be 0.5 mg twice a day. Subsequently, the dose will be increased to 1.0 mg twice a day, when blood electrolytes are normal and no signs of dehydration are present in the outpatient clinic visit at day 7. For patients with a body weight of <30 kg, the bumetanide (0.5 mg/mL solution) or placebo dose will be calculated based on body weight. For these children, the starting dose will be 0.03 mg/kg divided over 2 dosages per day and increased to 0.06mg/day in 2 dosages per day under the same physical conditions as described above. Parents and patients (as applicable) will be provided with user instructions to favor the correct and full administration of the IP solution. Dose reductions to manage side effects will be allowed at any time. The treatment period will be followed by a wash-out period to evaluate return of symptomatology and reversibility of treatment effect. The duration of the wash-out will be 28 days in concordance with the study by Lemonnier et al.[1]

5.2 Use of co-intervention

Due to the expected chance of frequent mild to moderate hypokalemia in the first phase of bumetanide application, and the experience in the previous bumetanide studies in ASD patients, all subjects will receive standard potassium supplementation during the 91 days of treatment in the trial. This supplementation will be administered either in the form of potassium chloride 0.5mmol/ml or potassiumchloride 600mga tablets, at an approxiamte dosage of 0.5 mmol/kg body weight divided over 2 gifts. Children below 30kg receive 0.5mmol/kg KCl solution, whereas children ≥30kg will receive KCl tablets MGA 600mg (equivalent to 8mmol) (see table 1). The child nephrologist member (Mandy Keijzer) of the research team will oversee and monitor the use of this co-intervention. Supplementation of potassium chloride to placebo subjects and/or subjects without hypokalemia due to bumetanide is safe and without any adverse effect in patients with normal kidney function.

Weight	<30kg	>30kg
Dose	2dd 0.25mmol/kg KCl solution	2dd 600mga tablet

Table 1: KCl suppletion protocol at start of study

Escape medication (if applicable)
n/a

6. INVESTIGATIONAL PRODUCT

6.1 Name and description of investigational product(s)

Bumetanide will be applied in the formulation of an oral solution containing a bumetanide concentration of 0.5mg/mL. Bumetanide is a selective inhibitor of the NKCC1 chloride transporter. It has been used since several decades as a loop diuretic used in the treatment of edema, including that associated with congestive heart failure or hepatic or renal disease, and hypertension. It belongs to the same family as Furosemide. Pharmacological and clinical studies have shown that 1 mg Bumetanide has a diuretic potency equivalent to approximately 40 mg furosemide. The major site of Bumetanide action is the ascending limb of the loop of Henle. Recent studies have shown Bumetanide also blocks NKCC1 in the brain, and thus decreases internal chloride concentration in neurons. This concentration change makes the action of GABA more hyperpolarizing, which may be useful for treatment of ASD.

Placebo product will be administered as comparator of the Bumetanide 0.5 mg/mL oral solution. The qualitative and quantitative composition in excipients of the Placebo product is comparable to that of Bumetanide 0.5 mg/mL oral solution.

6.2 Summary of findings from non-clinical studies

Refer to section 4 of the IB.

6.3 Summary of findings from clinical studies

Refer to section 5.1-5.5 of the IB.

6.4 Summary of known and potential risks and benefits

Refer to section 5.6 of the IB.

6.5 Description and justification of route of administration and dosage

Description and justification of route of administration:

The oral liquid formulation of Bumetanide has been developed by Neurochlore (Neurochlore, Marseille, France) for use in the paediatric population and has been applied in the first RCT in children with ASD [1]. This formulation is currently used in a dose ranging phase II study conducted by Neurochlore in France.

Liquid formulations remain the most suitable formulation for young children but adolescents may be rebellious and reject medicinal products they have previously taken. They are developing independence from adults and will usually be responsible for their own medicinal products administration. Oral liquid dosage forms are normally considered acceptable for children from full term birth. It should be noted that many children with ASD suffer from sensory processing difficulties, which may interfere with the tolerance of solid pills and who may favor a liquid formulation [34]. In summary, there are important advantages with liquid formulations:

- Permit a precise dose delivery,
- Titration possible by varying the volume,
- Rapid absorption
- Suitable for young children

Dose justification:
It is reported that oral or intravenous Bumetanide (0.5–2 mg) produced a dose-related increase in sodium, chloride and water excretion leading to loss of extracellular volume and therefore activation of renin angiotensin aldosterone system and secondary potassium loss in the collecting ducts of the distal nephron [35]. On a mg/kg basis, the daily dosages of Bumetanide according to the SpC in the different groups are the following: 0.02 to 0.2 mg/kg in adults with fluid retention, 0.005 to 0.2 mg in newborns (intravenous), toddlers and children with fluid retention, 0.1 to 0.3 mg/kg (IV) in newborns with seizures and 0.017 to 0.33 mg/kg bid in children with ASD. The proposed dosage for children and adolescents with ASD is therefore at the low end of the standard range of Bumetanide. Indeed, the Dutch pediatric formulary of medications recommends a starting dose between 0.01-0.1mg/kg/dose and 0.2 mg/kg/dose with a maximum dosage of 10 mg/day. Our proposed dosage is well within these ranges.
Available clinical data with Bumetanide in patients with ASD were obtained with daily oral doses ranging from 1 to 2 mg Bumetanide in several studies: the exploratory study in 5 ASD children[36], in RCT study in 56 children by Lemonnier et al. [1] and in our previous case study[22]. In the Lemonnier RCT study the average body weight of the ASD children was 24.1 ± 6.1 kg corresponding to 0.04 mg/kg of Bumetanide. The NeuroClin02 study conducted by Neurochlore in 88 autistic children and adolescents confirms that dosages of 0.5 and 1.0 mg twice a day are well tolerated (Neurochlore, non published data).The duration of treatment of 91 days has been chosen as a significant improvement of the cognitive function was seen after a 90 day treatment period in the Lemonnier study and our own experience[1,22].

With regards to the potassium suppletion, the normal average potassium requirement is approximately 1-2 mmol/kg/d which amounts to a couple of glasses of apple or orange juice (200 ml apple juice contains approximately 4 mmol K and 200 mlorange juice nearly 8 mmol). The proposed dosage of potassium comedication (i.e. 0,5mmol/kg) during 91 days of treatment is lower than generally supplied in addition to diuretics (1-2 mmol/kg) and is not expected to cause any complications in either the bumetanide group or placebo group. Potassium suppletion is only harmful when supplied via rapid intravenous infusion and/or with persons with a renal insufficiency who can not properly excrete potassium. Such patients are excluded through the exlusion criteria in the study.

6.6 Dosages, dosage modifications and method of administration

Dosage:

<u>For patients with a body weight of ≥30 kg</u>: The investigational products consist of bumetanide or placebo and will be given at a dosage between 0.5mg and 1.0mg twice a day (before breakfast and at the end of the afternoon, at least 2.5 hours before bedtime) for 91 days. The IP will be administered in the formulation of a solution (1 mL per intake = 0.5 mg bumetanide or placebo) will be administered directly into the mouth by means of a graduated dosing syringe. Starting dosage will be 0.5 mg twice a day, then the dose will be increased to 1.0 mg twice a day, if blood electrolytes are normal and no signs of dehydration are present after the outpatient clinic visit at day 7.

For patients with a body weight of <30 kg: Bumetanide (0.5 mg/mL solution) or placebo dose will be calculated based on body weight. For these children, the starting dose will be 0.03 mg/kg divided over 2 dosages per day and increased to 0.06mg/day in 2 dosages per day under the same physical conditions as described above. Parents and patients (as applicable) will be provided with user instructions to favor the correct and full administration of the IP solution.

Dosage and dosage modifications:
Patients will be assigned to receive Bumetanide or placebo for a 91 days double-blind treatment period. If the subject demonstrates no safety or tolerability concerns at D7 at the starting dosage of 0.5mg twice daily then the dose will be increased to 1 mg twice daily (see also section 5.1) by Team 2 (see section 8.3.1). In case of hypokalaemia or dehydration, dose modifications between 0.5-1.0mg twice daily to manage side effects are allowed at any time in this study (see section 8.3). On day 7, Team 2 members (see section 8.3) will evaluate the dose based upon the assessment of safety and tolerability.

Method of administration
Bumetanide or placebo oral solution 0.5 mg/mL will be administered twice daily; in the morning and at the end of afternoon. The patient's parent(s) will receive a box containing 6 bottles of 60 mL 0.5mg/mL placebo or Bumetanide for the 91 day treatment period. The box will be provided with a notice indicating the volume of Bumetanide to be administered, the schedule of treatments, the instructions for use, safety recommendations and the dosing syringe. Parents and patients (as applicable) will be provided with user instructions (see APPENDIX 1:Instructions for Use of the Bumetanide Solution 0.5 mg/ml) to aid the correct and full administration of the IP solution. The IP solution will be administered directly into the mouth by means of a graduated dosing 2 mL syringe by the patient's parent BID (before breakfast and at the end of the afternoon, at least 2.5 hours before bedtime) (Figure 2). A minimum of 6 hours is required between doses and the second dosage should not be administered after 17hrs to avoid disruption of sleep through increased frequency of diuresis. The IP solution must not be mixed with food or beverages. It is, however, allowed to drink a beverage (water) following the administration of the IP dose with the dosing syringe. The first (Day 0) and the last dose (Day 91) of IP will be administered at the study center by Team 2 (Section 8.3.1) assigned to this task. In addition, the patients will receive a calendar where they are asked to write down details concerning dose administration (e.g. sickness, time of dose in case it was forgotten, etc.), for drug accountability. (see appendix 3)

As shown on the pictures below (Figure 2), the syringe is connected to a plastic insert which avoids immersing the syringe into the solution, limiting the risk of contamination.

For retrieving the solution, the bottle is turned upside down.

Figure removed to protect privacy

For administration, the syringe is introduced into the mouth of the patient.

Figure 2: Preparation of the Investigational Product (IP)

6.7 Preparation and labeling of Investigational Medicinal Product

The IMPs for this study will be manufactured and labeled in Dutch for this study according to GMP guidelines by Amatsigroupi (Saint Gely du Fesc, France). Bumetanide will be supplied as a 0.5 mg/mL oral liquid formulation in 60 mL Type III amber glass bottles with a polypropylene/low density polyethylene child-resistant and tamper-evident cap. A 2 mL dosing syringe, consisting of a polystyrene plunger and a low-density polyethylene barrel and piston, will be also supplied (see also Figure 2). A 91 day's supply of Bumetanide will consist of 6 bottles packaged in a box. The box will be provided with a notice indicating the volume of Bumetanide to be administered, the schedule of treatments, the instructions for use, safety recommendations and the 2 ml dosing syringe. Medicine bottles will be labeled with the randomization numbers, as described in Section 8.2. Labeling will be prepared to meet the local regulatory requirements (GCP).

For the composition of bumetanide refer to table 1 in section P1 of the IMPD for bumetanide.

The placebo is visually indistinguishable from the IP formulation. The physical appearance, pH value and composition of the placebo formulation (same excipients, same concentrations) are the same as for the Bumetanide solution. The placebo will be presented in identical 60 mL Type III amber glass bottles, labeled with the randomization number, packaged up and provided to the patient as described for Bumetanide.

6.8 Drug accountability

AmatsiGROUP will prepare, package and label IPs for administration to and will send the IP within 2 weeks to the UMC Utrecht pharmacy for storage and dispensing. AmatsiGROUP will be sending a pack list, a QP release document and receipt of acknowledgement. Each

medication kit contains sufficient medication for 1 patient for 91 days of treatment. With each medication kit, 1 unblinding envelope is printed which will be sent with the medication kit to the site. In addition, the delegated person at Amatsi is in principle able to unblind study medication by means of the randomisation list (see below). Bumetanide and placebo flasks and kits will be packed in the exact same way in order to preserve blinding for the investigators as well as the subjects.

Upon receipt of the IP, the pharmacist in the UMCU will (1) verify accurate delivery, (2) acknowledge receipt by signing/initialing and dating the documentation provided by Amatsi. A copy of each of these documentations will be retained for the Investigator file. The IP should be stored in a secure location at room temperature by the pharmacy.

The boxes containing the IP bottles should not be opened upon receipt at the site. Only the clinical researcher assigned to administering the first dose of IP on Day 1 is allowed to open the box with the patient/patient's parent(s). The dispensing of the blinded IP to each patient will be recorded on appropriate drug accountability forms (these will be provided by Amatsi). Accurate drug accountability records will be available for verification by the study and pharmacy following database lock installed by the Julius center.
IP accountability records will include:

- Confirmation of delivery of the IP to the trial site;
- Inventory at the site;
- Dispensation of the product;
- A calendar for tracking details of bumetanide administration (See appendix 3)
- Return to the Sponsor or alternative disposition of unused product(s) (following database lock).

Records should include dates, quantities, batch numbers, expiration dates and any randomization numbers assigned to the patients. Investigators will maintain records, which document adequately that:

- All patients will be provided with the correct IP box as per the assigned randomization number (refer to appendix 1 on solution)
- All IP provided by the Sponsor will be fully reconciled.

Unused IP must not be discarded or used for any purpose other than the present study. Patients are instructed to return both used and unused IPs, in their original packaging. IP that has been dispensed to a patient and returned unused must not be re-dispensed to a different patient.

During the study, drug accountability will document the date the IP was dispensed to the patient. At the end of treatment, the site will open the blinded IP boxes containing the returned bottles of IP and update the accountability as required.
Following database lock, unused bottles will be destructed by the study site. The used bottles will be checked and destructed by the study site at the end of each individual treatment.

The investigator will perform a final review of the drug accountability forms and check all IP returns (both unused and used bottles) prior to authorizing their destruction by the study site.

7. NON-INVESTIGATIONAL PRODUCT

n/a

8. METHODS

8.1 Study parameters/endpoints

All endpoint measurements will be taken:

- at baseline: between D -45 and D0
- end of treatment: D91 (- 6 days)
- end of the wash-out period: D119 (+/- 4 days) except neurocognitive measures as these tests cannot be repeated within 4 weeks without test-retest confounding effects.

The 6 day window is necessary to perform the EEG and cognition measurements.

8.1.1 Main study parameter/endpoint

Primary endpoint:

To investigate whether core features of ASD improve over time in children taking bumetanide, we will examine if parental report of a positive effect of bumetanide over the course of the study is associated with a decline in symptom burden, as measured serially using the Social Responsiveness Scale (SRS), a quantitative measure of autistic symptoms. The SRS is an extensively validated quantitative trait measure of social-communicative and restricted/repetitive behavior deficits referable to core ASD symptoms, in which informants base their ratings on cumulative observations of the subject in the subject's natural social settings[37,38]. Scores are continuously distributed across the entire population, exhibit a unitary factor structure in cross-sectional research [39,40], and are largely independent of intelligence quotient (IQ) among verbal ASD subjects [41], such that total scores index quantitative variations in the severity of autistic impairment. Higher scores correspond to greater impairment, and distinguish children with ASD from those with other psychiatric conditions. Subscale scores on the SRS 1) describe domains of core autistic symptoms (social awareness, social cognition, social communication, social motivation, and mannerisms) that may be differentially affected by a given treatment; and 2) relate to the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) criterion domains for a diagnosis of ASD [40,42] [37,43,44] See also section 8.3.2.

8.1.2 Secondary study parameters/endpoints (if applicable)

Secondary endpoints:

Quality of life and societal improvement

- The PedsQL Measurement Model is a modular approach to measuring health-related quality of life (HRQOL) in healthy children and adolescents and those with acute and chronic health conditions. It is the most appropriate quality-of-life measure for use in children and youth with ASD [45].
- Quality of life (QoL) (World Health Organization QoL [WHOQOL - BREF]) and EQ5D-5L to assess the parent's quality of life assessing the individual's perceptions in the context of their culture and value systems, and their personal goals, standards and concerns.
- EQ-5D-5L and the EQ5D-Youth version to assess health related quality of life in parents and children. This questionnaire enables the calculation of quality adjusted

life years for the economic evaluation, either to be completed by parents (younger patients) or children (older patients).
- PCQ and TiC-P to assess health care costs for the cost-effectiveness analysis
- Teacher Report Form (TRF) and BRIEF to evaluate performance, behavior and integration of the child in his/her educational settings.

Behavioral psychometric measures
- The Aberrant Behavior Checklist (ABC) irritability subscale is an important secondary endpoint, because 1) this scale is the most commonly used outcome scale in ASD trials[6] and 2) a clear effect on this scale was noticed in our pilot study, 3) the irritability subscale measures behavior related to hyperexcitability. However, we did not choose to take this scale as the primary endpoint as it does not measure the core symptoms in social interaction and communication. A recent meta-analysis has supported the convergent and divergent validity of the ABC as a measure of behavior problems in ASD [33]. The ABC is a parent interview including five subscales: (1) Irritability, Agitation, Crying; (2) Lethargy, Social Withdrawal; (3) Stereotypic Behavior; (4) Hyperactivity, Noncompliance; and (5) Inappropriate Speech [33]. Factor analysis indicates that these subscales are statistically separate [33,46] and therefore the appropriate approach is to select a relevant subscale. The ABC has been validated for the Dutch pediatric population. Age, sex, and IQ are largely unrelated to ABC subscale scores [33].
- ABC subscales other than irritability (hyperactivity, stereotypy, inappropriate speech, and lethargy/social withdrawal). Repetitive Behavior Scale (RBS-V) to measure repetitive and restricted behaviors [26] and Highly Sensitive Child/Parent Scale, Sensory Profile questionnaire (SP-NL)[25] and school companion (SP-SC) to measure sensory behaviors; (all parent administered).

Neurocognitive measurements
- verbal and visual short-term learning and memory [28-30]
- inhibition and attentional flexibility in response to auditory and visual information [27].

Electroencephalography (EEG)
- rsEEG/ERP assessments will be performed to assess sensory and sensorimotor gating (P50 suppression and PPI), and mismatch negativity abilities (odd-ball paradigm) and resting-state EEG to assess basic power spectra and mean frequencies.

Genetic analysis
- Genetic analysis will be conducted to test for pharmacogenetic responses

8.1.3 Other study parameters

n/a

8.2 Randomisation, blinding and treatment allocation

A randomisation list will be set up using permuted blocks by the responsible person at the Julius Center (JC). Randomisation will be done using the secure online randomisation tool of the Julius Center. We will be using minimization on age subgroups (7-8, 9-10, 11-12, 13-15

years), intelligence (IQ 55-70, 71-85, 86-110, >110) and gender (M/F)[48] in order to have similar distributions of these patient characteristics in each treatment group. The full randomization list will contain 100 patient study numbers, 50 for bumetanide treatment and 50 for placebo treatment. Amatsi label the kits with flasks containing the IPs according to the number on the list provided by the Julius center. .Nobody else at JC, Amatsi and the UMC Utrecht study members will see the randomisation list to avoid unintentional unblinding, and these persons will remain blinded in the study until database lock. At enrolment, the responsible person at the Julius Center will send requested information (patient study number and initials, date of consent) and the randomization number with allocated treatment code to the pharmacy at UMC Utrecht. The delegated person at this pharmacy will dispense the IP kit allocated to this code.

EMERGENCY UNBLINDING
There should always be a way to obtain an individual subject's treatment assignment during an emergency in blinded trials. The trial coordinator (Hilgo Bruining) is responsible for emergeny unblinding: he makes the decision to unblind and is responsible for following the procedures. If the principal investigator delegates this responsibility to another member of the research team, this should be documented in the Authorisation Log.

Emergency unblinding is indicated in the following situations ONLY:
1. unblinding is necessary for the subject's emergency treatment at the investigators discretion
2. unblinding is required by local laws or regulations (in case of SUSAR)
3. the Data Monitoring Safety Board decides that unblinding is necessary for proper study management of the subjects and the overall safety of the other subjects in the study

The BAMBI study will use a secured PDF document for emergency unblinding situations containing the name of the treatment in each specific kit. This list will be secured with a password and will be accessible only by the unblinded pharmacist at the UMCU pharmacy. Outside normal working hours, an on-call pharmacist always has access to this document. If the person who is required to perform the unblinding is not the investigator or a delegated study team member, he/she needs to notify the investigator or study team member that unblinding is required. Each patient and his/her parents will receive emergency instructions via a letter and a study identification card stating their name and study identification number and a telephone number to request information in case of emergency. In addition, this card will state the name and acronym of the study and the product (bumetanide or placebo) under investigation.

The date and reason that the blind was broken must be recorded in the eCRF of the unblinded patient. The trial coordinator of the study must be notified before breaking the blind, unless identification of the study drug is required for emergency therapeutic measures. The investigator needs to inform the trial coordinators or project manager of the study on the following information:
- Name and title of the person who requested the unblinding
- Patient number
- Reason for unblinding
- Date and time of unblinding
- Name of person authorizing the procedure

- Signature of person performing the unblinding

8.3 Study procedures

8.3.1 Study phases and procedures

Following completion of the pre-study evaluations, included subjects will undergo the following phases:

- Phase I: pretreatment and screening
  - between D -45 to Day -1
    - Screening for eligibility
    - Baseline outcome measurements
    - Randomization

  Phase II: treatment
  - between D0 and D91
    - Blood analysis at D4, D7, D14, D28, D56, (+/- 2 days)
    - End of treatment outcome measurements D91 (- 6 days)

  Phase III: washout
  - Between D91 and 119
    - End of washout outcome measurements D119 (+/- 4 days)

An essential element of the study procedures is that the research team involved in outcome measurements cannot be involved in the check-ups for adverse effects during the treatment. This separation of tasks is crucial to avoid suspicion of treatment allocation: symptoms related to diuretic treatment such as increased diuresis or hypokalemia will raise suspicion of bumetanide treatment arm allocation and conversely, the lack of these symptoms may be interpreted as an indication of placebo allocation. Therefore, the trial procedure enforces a strict separation of tasks related to either the measurement of endpoints, or the check-ups for adverse effects during treatment. This separation is embodied in two teams:

- Team 1: responsible for screening and outcome measurements (Phase I,II and III). These tasks will be performed by the research team members of the department of psychiatry (including HB, JS, CV, BO) involved in behavioral EEG and cognitive measurements, as well as in the screening procedures.
- Team 2: This team consists of an experienced pediatric nephrologist (Mandy Keijzer) and a pediatric nurse of the department of pediatric nephrology who will be responsible to oversee the management of the patient's condition, including safety and blood analysis, intercurrent illnesses, tolerability and prescription of medication and follow up on the other protocol-related procedures, including the adjustment of dosage as described under section 6.6. Accordingly, these check-ups will take place not in sight of team 1 (to avoid unbinding due to observation of adverse effects), at the department of pediatric nephrology at the Wilhelmina Children's Hospital of the UMC Utrecht. These research team members will also be responsible for the administration of the first dosage of IP on day 0, the dispensation of the potassium chloride co-medication, the increase in dosage at D7 (if allowed) and the last dosage on day 91. During the treatment phase, patients will report to this team with possible

AEs including signs of dehydrations and/or electrolyte disturbance expected to have arisen from diuretic effects.

Table 1 provides an overview of the trial flowchart with all the visits and procedures of the study.

Phase	**Screening Phase**			**Treatment Phase**							**Post-treatment Phase**
Visit	Screening Visit	Start of Treatment								End of Treatment	End of Study Visit
Day	**Days -45 to -1**	**Day 0**	**Day 4**	**Day 7**	**Day 14**	**Day 28**		**Day 56**	**[1]Day 91**	**Day 119**	
Allowed time windows	none	None	+/- 2 days	+/- 2 days	+/- 2 days	+/- 2 days		+/- 2 days	- 6 days	+/- 4 days	
Informed consent	X										
ASD diagnosis	X										
Demographics	X										
Medical history	X										
SRS	X										
IQ (WISC)[@]	X										
Study eligibility confirmed	X										
Physical examination	X								X	X	
Weight	X	X	X	X	X	X		X	X	X	
Vital signs	X	X	X	X	X	X		X	X	X	
Blood analysis	X		X	X	X	X		X			
Urine analysis	X								X		
Concomitant therapy checking	X	X	X	X	X	X		X	X	X	
Primary and secondary outcome measures	X								X	X	

[@]Previous IQ measurement by WISC < 2 years old is also acceptable
AE = adverse effects

If the patient is potentially eligible to take part in the study and written informed consent has been provided, a Screening visit will be conducted within 5 weeks of the Day 0 visit (Start of Treatment).

A short screening eCRF and screening form will be completed for all patients who signed the informed consent but who did not subsequently enter the study. Patients will be identified by their code assigned through the randomization procedure (section 8.2). In addition, their race, sex and reasons for exclusion from the study will be recorded.

Phase I: evaluations and procedures for screening (D-45 to -1):
Team I will perform the following tasks:

- Date of signed informed consent;
- Confirmation of study eligibility (inclusion and exclusion criteria);
- ASD diagnosis based on the DSM V criteria and ADOS or SRS
- Demographics and medical history (including family history)

- IQ (short)WISC, if available < 2 years, it is not necessary to repeat it;
- Baseline outcome measurements
- Physical examination; vital signs; concomitant therapy
- Blood analysis
- Urine analysis
- eGFR analysis (modified Schwartz formule (36.5*length (cm) / serum creatinine (umol/L)).
- Serum or urine pregnancy test for females of child-bearing potential;
- Cardiac risk screening (see section 8.3.3)

Completion and outcome of all the Phase I procedures must be reviewed by the Principal Investigator prior to patient enrolment in Phase II.

Phase II: Treatment Phase

Treatment will commence once the principal investigator confirms that the patient is eligible for inclusion, and that Phase I screenings and baseline outcome measurements have been completed within the correct timeframe. When participants fail eligibility during phase I, the assessments of phase I are completed and discussed with parents as outpatient clinical care.

Day 0: Start of Treatment Visit at UMCU

Team II will carry out the following tasks:

- concomitant therapy check
- Administration of first dose of IP by the research nurse assigned to this task
- Dispensing of IP kit
- Dispensing of potassium chloride suppletion for 91 days (see section 5.2)

Days 4,7,14, 28,56: Safety checks at UMCU (+/- 2 days)

Team II will carry out the following tasks:

- Weight; vital signs; concomitant therapy
- Blood analysis
- AEs

Day 91: end of treatment visit (- 6 days):

Team I will carry out the following tasks:

- outcome measurements (must be scheduled before end of treatment)

Team II will carry out the following tasks:

- Physical examination; vital signs; concomitant therapy
-
- AEs

Phase III: Washout phase

Day 119: end of study visit (+/- 4 days):

Team I will carry out the following tasks:

- outcome measurements

Team II will carry out the following tasks:

- Physical examination; vital signs; concomitant therapy
- AEs

8.3.2 Study Investigations:

Behavioral parent questionnaires

Parents will be asked to fill in multiple questionnaires about the behavioral and psychological functioning of their child at day -45/-1, day 91 and day 119. Filling in all the questionnaires can take up to 60 minutes per episode, and can be filled in at home. Questionnaires include:

ABC
Refer to section 8.1.2

Quality of life measurements
Refer to section 8.1.2

Sensory Profile and Sensory Profile School Companion
The Sensory Profile, developed by Dunn et al. [25], is a caregiver questionnaire that measures a child's sensory processing abilities and their impact on daily functioning. It consists of a 125-item assessment on which parents report the frequency their child responds to items in eight categories: Auditory, Visual, Taste/Smell, Movement, Body Position, Touch, Activity Level, and Emotional/Social. The Sensory Profile has been translated in Dutch and has been validated with norm-scores for Dutch normal developing children of different age ranges [1,49]. A version for children between 3-12 and a version for children aged 11 and older exist. The questionnaire consists of 125 items leading to 14 sensory processing category scores and a total score. The Sensory Profile has been translated in Dutch and has been validated with norm-scores for Dutch normal developing children of different age ranges. The Sensory Profile takes about 28 min to complete and a computer-based scoring program is available.

The Sensory Profile can further be used to classify children into one of the four general sensory processing "*quadrants*", e.g. patterns of sensory processing derived on the basis of

SRS
The Social Responsiveness Scale (SRS) [43] is a caregiver questionnaire that distinguishes autism spectrum conditions from other child psychiatric conditions by identifying presence and extent of autistic social impairment. This 65-item rating scale measures the severity of autism spectrum symptoms as they occur in natural social settings. The SRS provides a clear picture of a child's social impairments, assessing social awareness, social information processing, capacity for reciprocal social communication, social anxiety/avoidance, and autistic preoccupations and traits.

HSP
The highly sensitive child or parent scale is a self-administered 12 item child or caregiver questionnaire that determines sensory sensitivity. The questions are scored on a 7 point Likert-scale.

Neurocognitive investigations

Neurocognitive assessment focuses on verbal and visual learning and memory, inhibition and attentional flexibility in response to auditory and visual information. Total duration of these tests is 120 minutes.

Memory tests included digit span of the WISC-III and spatial span of the Wechsler Nonverbal Scale of Ability (WNV) to assess working memory for respectively auditory information and visual information; the Rey Auditory Verbal Learning Test [29] and the Rey Visual Design Learning Test [50] to examine verbal and visual learning and memory and the "recalling sentences" subtest of the Clinical Evaluation of Language Fundamentals to examine semantic memory as was performed previously [51]. When available different versions of a task were used in an ABA design to avoid practice effects.

The Amsterdam Neuropsychological Tasks battery (ANT) will be used to examine baseline response speed, prepotent response inhibition (go-nogo task and condition two of the auditory and the visual shifting set tasks), attentional flexibility (condition three of the auditory and the visual shifting set tasks). [27].
All neuropsychological measurements will be converted to age-specific z-scores by standardizing to the population based reference data, to harmonize the data and to facilitate interpretation.

EEG investigations
Electro encephalogram (EEG) recordings will be performed at baseline and at all assessments of treatment effects. The measurements will be used to detect changes in brain state and sensory processing through resting–state EEG and sensory evoked EEG measurements. EEG will be recorded from 64 scalp electrode locations. The EEG will be recorded using a BioSemi system at a sample frequency 512 Hz. The total duration of the EEG/ERP battery is 60 minutes, while the preparation and set-up takes 60-90 minutes.

Resting state EEG
Neural oscillatory anomalies in ASD suggest that resting-state EEG markers may relate to the putative excitatory/inhibitory imbalances that may be attenuated through Bum treatment. Across studies, an U-shaped profile of electrophysiological power alterations, with excessive power in low-frequency and high-frequency bands [52]. To further delineate markers that predict efficacy of bumetanide and may be used to monitor treatment effect, we will assess these basic EEG properties before and after treatment to assess changes in spectral content of EEG. EEG data will be processed offline using the Neurophysiological Biomarker Toolbox (http://www.nbtwiki.net/), an open source Matlab toolbox for the computation and integration of neurophysiological biomarkers. With this toolbox, a wide array of resting state parameters can be evaluated including power spectra and coherence and has been applied to neurological clinical studies [53]. The data processing will be performed using MATLAB 7.12.0 software (The MathWorks Inc., Natick, MA, 2011a).

ERP investigations
During EEG, hearing test, a P50 suppression task and a PPI task subjects will be tested for their selective attention and mismatch negativity abilities. During the tasks, the subject is requested to maintain his gaze at a fixation dot on the opposite wall (approximately 2.5 meters away).

PPI paradigm
Directly following a 5 minutes acclimatization period to background noise (70 dB white noise), three blocks of stimuli are presented, against this same background noise. Block 1 and 3 will be identical, and used for assessment of habituation. Block 2 will be used to assess percentage PPI. Specification:
- Blocks 1 and 3: habituation will be assessed by presenting a series of 8 pulse alone trials (White noise burst with an intensity of 115 dB and a duration of 20 ms). Intertrial intervals are randomized between 10 and 20 s.
- Block2: PPI will be assessed by randomly presenting pulse alone trials and combinations of prepulse-pulse trials. Pulse stimuli are identical to those as presented in the habituation trials. Prepulses will consist of white noise bursts with an intensity of either 6 dB above background (76 dB) or 15 dB above background (85 dB), with a duration of 20 ms. The stimulus onset asynchrony (SOA) in prepulse-pulse trials is either 60 or 120 ms, the intertrial intervals are randomized between 10 and 20 s. Ten trials of each prepulse-pulse combination will be presented, adding up to a total of 50 trials in block 2.
The maximum time involved in this PPI paradigm is 20 min.

P50 paradigm
A standard P50 paradigm will be used: sounds will be white noise bursts (duration:1.4 ms, intensity: 90 dB), presented in click pairs with ISI 500 ms and fixed intertrial intervals of 10 s. To prevent drowsiness, stimuli are presented in three blocks of 40 click pairs each (total of 120 trials). The total time that is involved in this P50 suppression paradigm is 21 min.

Mismatch negativity paradigm
The mismatch negativity (MMN) paradigm consists of 1800 stimuli that are presented binaurally. Four types are presented: standard tones with a frequency of 1000 Hz and duration of 50 ms (presented in 83% of all cases), deviant tones with a frequency of 1200 Hz and duration of 50 ms (presented in 6% of the cases), deviant tones with a frequency of 1000 Hz and duration of 100 ms (presented in 6% of the cases), and finally deviant tones with a frequency of 1200 Hz and duration of 100 ms (also presented in 6% of the cases). All stimuli are 75 db. The stimuli are presented in 1 run, with an ISI randomized between 300 and 500 ms. The duration of the task is approximately 12 minutes. Subjects are requested to ignore the stimuli, and are therefore presented a (soundless) video with neutral content (animal documentary).

Selective attention paradigm
The auditory selective attention task that is used in this test battery consists of 400 stimuli, presented randomly in either the right or the left ear. Two types of stimuli are presented: standard tones, which appear in 80% of the cases, and deviant tones, which appear in the remaining 20% of the cases. The stimuli are evenly presented to the left or right ear (attended deviants are never presented immediately following each other). The subject is instructed to push a button as fast as possible if the deviant tone occurs in a previously designated ear. Ear designation is balanced randomly across the subjects. After this initial task the subjects are presented the next auditory selective attention task in which they have to monitor the other ear for deviant stimuli. Standard and deviant stimuli differ in pitch only (either 1000 and 1200 Hz respectively). The intensity of each stimulus is 75 dB and they are 50 ms in duration, presented with an ISI between 700 and 900 ms. This means that the total of two tasks will last approximately 11 minutes. The selective attention task will provide data

on P300 amplitude, processing negativity (PN) as well as behavioral data (hits/misses/false alarms and reaction time).

Genetic analysis:
Participants and parents are asked to consent to obtain blood samples for genome analysis. The blood will be collected during the blood withdrawals required for safety analysis. Blood will be collected and stored at the laboratory of the UMC Utrecht. After blood of all consenting participants is collected genomic analysis will be performed over the entire batch. Rest material will be discarded. The genetic analysis could be performed outside the UMC Utrecht. To this end it may be necessary to send encoded whole blood samples to a research center other than the UMC Utrecht.

8.3.3 Safety investigations

Assessments of safety will be performed by team 2 under supervision of a pediatric nephrologist (MK) as described under section 8.3. This person will be responsible for the management of the patient's condition, including safety assessments, intercurrent illnesses, tolerability and prescription of medication and follow up on the other protocol-related AE procedures.

The following safety parameters will be assessed:

- AEs;
- Physical examination and vital signs;
- Blood laboratory tests: blood electrolytes (ionogram) and extended blood tests including liver functions and full blood count);
- Urinalysis;
- eGFR;

Adverse Events (AEs)
All AEs which occur from the date of written informed consent to the last day of IP administration or at the final study visit (whichever is the longer), will be recorded in the eCRF. AEs will be documented with respect to severity, duration, relationship to the IP, management, and outcome. Patients (and their parent[s]) should be specifically questioned about dehydration, orthostatic hypotension, hypersensitivity reactions, cramps, asthenia, diarrhea, myalgia, arthralgia, dizziness and nausea. Investigators should also evaluate the patient for hypokalemia, health problems including cardiovascular and renal functions, and intercurrent illnesses.

Physical Examination and Vital Signs
The Investigator will observe the patient's appearance, general health, and behavior, along with measuring height (at the Screening visit only) and weight.
With the patient in the seated position, the following systems will be assessed:

- Skin: The exposed areas of the skin are observed; the size and shape of any lesions are noted;
- Mouth and pharynx: The lips, gums, teeth, roof of the mouth, tongue, and pharynx are inspected;

Normal results of a physical examination correspond to the healthy appearance and normal functioning of the body. Abnormal results of a physical examination include any findings that indicated the presence of a disorder, disease, or underlying condition.
Body weight will be assessed in kg with patients as lightly dressed as possible. Height will be measured in cm. Vital signs will include sitting BP (mmHg) (systolic/diastolic), pulse rate (PR) (beats/min) (supine after 5 min rest) and body temperature. Additional vital sign measurements should be recorded as clinically indicated.

Blood analysis (D4, D7, D14, D28, D56)
The blood analysis will be performed by the UMC Utrecht clinical laboratory. Blood parameters to be analyzed at all planned time points are sodium, potassium, chloride, uric acid, urea, creatinine, glucose; estimated glomerular filtration rate (eGFR), hematocrit, hemoglobin, erythrocytes, leukocytes, thrombocytes and total protein. At the screening and D91 blood analysis additional measurements will be included: see below. Results will be sent to Team 2 (see section 8.3.1) only.

Hypokalaemia is the predominant AE expected to occur (see section 1), Management of hypokalemia will depend on the current study medication dose the participants receive:

Twice daily 0.5mg

1. Hypokalaemia between 3.0-3.5 mmol/ml:
   - Increase of KCl suppletion to 1 mmol/kg/day
   - Repeat the blood analysis in 3-4 days. When K+ concentration is stable or increased, raise the study medication to twice daily 1mg and repeat blood analysis in 3-4 days. Maintain KCl suppletion at 1 mmol/kg/day

2. Hypokalaemia between 2.5-3.0 mmol/ml:
   - Stop study medication
   - Increase KCl with 0.5 mmol/kg/day
   - Blood analysis in 3-4 days. When K+ recovers start study medication at twice daily 0.5mg and repeat blood analysis in 3-4 days. Maintain KCl dosage

3. Hypokalaemia < 2.5 mmol/ml: Magnesium (Mg) analysis in blood, ECG analysis, discontinuation of study medication.
   a. If no abnormalities on ECG, Mg normal (>0.70 mmol/l) and no clinical symptoms:
      - No hospital admittance required
      - Increase potassium suppletion to 2 mmol/kg/day
      - Revision of blood analysis and clinical symptoms after 2 days at the outpatient department
      - Withdrawal from study
   b. Abnormalities present on ECG or symptomatic hypokalemia, and/or Mg < 0.70 mmol/L:
      - Immediate hospital admission with ECG monitor surveillance
      - Immediate extra oral dosage of potassium chloride (1 mmol/kg)
      - Checking of Mg levels in addition to blood analysis
      - Intravenous potassium chloride suppletion according to the protocol of the Wilhelmina Children's hospital (WKZ)
      - Withdrawal from study

Twice daily 1mg

1. Hypokalemia between 3.0-3.5 mmol/ml:
   - Increase KCl supplementation with 0.5mg/kg/day
   - Repeat blood analysis in 3-4 days. When K+ is stable or increased maintain KCl supplementation at current dose

2. Hypokalemia between 2.5-3.0 mmol/ml:
   - Stop study medication
   - Increase KCl supplementation with 0.5mg/kg/day
   - Blood analysis in 3-4 days
      a. When K+ increases >3.0 mmol/ml:
         - Start study medication at twice daily 0.5mg. After two consecutive K+ levels of >3.5 increase study medication to 1.0mg twice daily. KCl dosage remains at increased dosage
      b. When K+ remains <3.0 mmol/ml withdrawal from study.

3. Hypokalemia < 2.5 mmol/ml: Mg analysis in blood, ECG analysis, discontinuation of study medication.
   a. If no abnormalities on ECG, Mg normal (>0.70 mmol/l) and no clinical symptoms:
      - No hospital admittance required
      - Increase potassium suppletion to 2 mmol/kg/day
      - Revision of blood analysis and clinical symptoms after 2 days at the outpatient department. When potassium is recovered, restart study medication at 2dd0.5mg
   b. Abnormalities present on ECG or symptomatic hypokalemia, and/or Mg < 0.70 mmol/L:
      - Immediate hospital admission with ECG monitor surveillance
      - Immediate extra oral dosage of potassium chloride (1 mmol/kg)
      - Intravenous potassium chloride suppletion according to the protocol of the WKZ
      - Checking of Mg concentration in addition to blood analysis,
      - withdrawal from study

In case a patient is withdrawn due to hypokalemia, he/she will not be replaced.
Behavioural adverse events are at first evaluated by team two. If further psychiatric evaluation of behavioural adverse events is warranted, then participants are referred to the independent expert. The independent expert will consult parents and decide on further treatment, un-blinding and study termination.

Extended Blood Tests (Screening)
Laboratory tests will be performed by the local laboratory and will include the following assessments:

- Alanine Transaminase (ALAT);
- Aspartate Transaminase (ASAT),
- Gamma-glutamyltransferase (γ-GT);
- Alkaline phosphatase;
- Sodium;
- Potassium;
- Chloride;
- Calcium;
- Uric acid;
- Urea;
- Creatinine;
- Glucose;
- Haematocrit;
- Hemoglobin
- Erythrocytes
- Leukocytes
- Thrombocytes
- Total protein;
- Plasma renin and aldosterone activities (Screening visit).

* In children, the estimated eGFR is calculated by the modified Schwartz formula (Schwartz, 2009), which uses serum creatinine (µmol/L), the child's height (cm) and a constant to estimate the glomerular filtration rate (36.5):

- eGFR=36.5*(height [cm]/serum creatinine [µmol/L])

Urinalysis (Screening)
Urinalysis (portion) will include the following assessments:

- Sodium
- Potassium
- Chloride
- Calcium
- Protein;
- Creatinine;
- Uric acid;
- Micro-albumineria;

Patients will be provided with bottles in which to collect a urine sample before the center visit.

Cardiac risk screening
During the medical check at the screening visit a cardiac risk screening will be performed, in which family history and cardiac auscultations are examined. Evidence of cardiac complaints, a positive family history suspect for arrhythmia's, or abnormal auscultative findings will lead to a referral to the pediatric cardiologist in the WKZ. This procedure of cardiological surveillance has been formally agreed with the cardiologists of the WKZ and a pharmacist at

the UMC Utrecht. These experts confirmed that.there is no evidence that bumetanide increases QT-interval or causes arrhythmias. The main effects of bumetanide on the heart are mediated by electrolyte disturbances, which are controlled for by the laboratory check-ups (correspondence between the study team and the cardiologist and pharmacist in this regard are to be found in appendix 5)

Checking for concomitant therapy/medical management of adverse events
Any medications, except those listed below, which are considered necessary for the patient's welfare, and which will not interfere with the study medication, may be given at the discretion of the Investigator.
Concomitant therapy should be recorded from the date of written informed consent to the last day of IP administration or at the final study visit (whichever is the longer).
Two types of medications should be used with caution or excluded from the clinical studies with Bumetanide: drugs whose therapeutic activity may affect potassium levels and drugs associated with ototoxicity.
Administration of all concomitant drugs must be reported in the appropriate section of the eCRF along with dosage information, dates of administration and reasons for use.
Additionally, any unplanned diagnostic, therapeutic or surgical procedure performed during the study period must be recorded in the appropriate section of the eCRF.
Special care should be taken in questioning of the patients (or patient's parent[s]) on any self-medication taken by the patient.

Medications that may NOT be administered
The patient must not take any of the following medications during the course of this study (during the treatment period, and prior to the start of treatment, as specified below and also in the exclusion criteria under section 4.3). Should any of these medications be required for the treatment of the patient, the patient must be withdrawn from the study:

- All psychoactive medications (including antipsychotic, psychostimulant, antidepressant, anxiolytic, mood stabilizer, antiepileptic/convulsive and neuroleptic agents) will be prohibited and discontinued. If feasible, psychoactive medications can be stopped to participate in the study but should have been discontinued at least 4 weeks before the screening visit;
- Aminoglycosides.
  Despite potentiation, ototoxicity has not been tested for Bumetanide. The ototoxic effects of aminoglycosides may be increased by the concomitant administration of diuretics such as Bumetanide.
  Aminoglycosides are a group of that are used to combat infections due to aerobic, Gram-negative bacteria. Neomycin, kanamycin, gentamycin and amikacin are the most likely to cause problems with hearing. They produce cochleotoxicity through a poorly understood mechanism. Aminoglycosides also cause nephrotoxicity by inhibiting protein synthesis in renal cells, particularly in repeated dose treatments. This mechanism specifically causes necrosis of cells in the proximal tubule, resulting in acute tubular necrosis which can lead to acute renal failure
- Non-steroidal anti-inflammatory drugs (NSAIDs) are prohibited. NSAID inhibit the effect of Bumetanide. Diuretics may enhance the nephrotoxicity of NSAIDs. NSAIDs constitute a heterogeneous group of compounds that share similar therapeutic effects

as well as side effects. Since these drugs can be obtained without a prescription, they are potentially available for long-term use. The dominant ototoxic effect of salicylates appears to be the production of tinnitus as well a reversible mild to moderate symmetric sensorineural hearing loss. Patients who may require NSAIDs or Aminoglycosides will be withdrawn from the study and replaced.

- Other drugs to be avoided include digoxin, antihypertensive agents, indomethacin, probenecid, Lithium, drugs known to have a nephrotoxic potential.
- Patients may not start concurrent psychotherapy, social skills training, or behavioural interventions (e.g., applied behaviour analysis) during the study. If the patient had been receiving these services prior to the study, they will have the option of continuing such services during the study.
- The use of any herbal/natural products or other "folk remedies" should be discouraged, but use of those products, as well as the use of vitamins, nutritional supplements, and all other concomitant medications must be recorded in the eCRF.
- Use of other diuretics (e.g. furosemide, hydrochlorothiazide)

Guidance on medications to be administered/patient management for expected Adverse Events
Based on the inclusion/exclusion criteria, it is anticipated that the need for concomitant medications will be limited. Melatonin could be administered at the Investigator's discretion for sleep aid. Administration of Vitamin D is also accepted. Long-term or regular use of salicylates or paracetamol should be avoided.

Extended laboratory tests: Alanine transaminase (ALAT), aspartate transaminase (ASAT), gamma-glutamyltransferase (γ-GT), alkaline, phosphatase, sodium, potassium, chloride, calcemia, uric acid, urea, creatinine, glucose, haematocrit, hemoglobin, erythrocytes, leukocytes, thrombocytes and total protein.
Urinalysis (portion): Sodium, Potassium, Calcium, protein, creatinine, uric acid and micro-albuminuria.

8.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.
Patients (and their parent[s]) will be informed that they have the right to withdraw from the study at any time, without prejudice to their medical care, and are not obliged to state their reasons. Any withdrawals must be fully documented in the eCRF and should be followed up by the Investigator.
Additionally, the Investigator may withdraw a patient at any time if they consider this to be in the patient's best interest.

Patients **MUST** be discontinued for any of the following reasons:

- Hypokalemia <2.5 mmol/L;
- Prescription of antimicrobial aminoglycosides

- Prescription of non-steroidal anti-inflammatory drugs (NSAIDs)
- Serious intercurrent illness or significant worsening of intercurrent illness;
- Withdrawal of patient consent;
- If in the Investigator's opinion continuation in the study would be detrimental to the well-being of the patient;
- At the specific request of the Sponsor.

Patients **MAY** be discontinued for any of the following reasons:

- Protocol violations, including non-compliance with study procedures, patient lost to follow-up and patient refusal;
- Intolerable or persistent AEs;

If a patient fails to return for a scheduled visit/follow up, attempts should be made to contact the patient (the parent[s]) to ensure that the reason for not returning is not an AE. Likewise, if a patient (and/or the patient's parent[s]) declares his/her wish to discontinue from the study e.g. for personal reasons, an attempt should be made to establish that the true reason is not an AE (bearing in mind the patient is not obliged to state his/her reasons).
If the investigational product (IP) therapy is prematurely discontinued, the primary reason for discontinuation must be recorded in the appropriate section of the eCRF and all efforts will be made to complete and report the observations as thoroughly as possible.
A complete final evaluation following the patient's withdrawal should be made in the eCRF and any AEs followed up until resolution or a period of 30 days from the last dose of IP has elapsed, whichever is the longer.

The study will be terminated if in the opinion of the sponsor significant safety concerns arise during the conduct of the study.

8.4.1 Specific criteria for withdrawal

n/a

8.5 Replacement of individual subjects after withdrawal

All patients leaving the study early, regardless of the reason, will be requested to return to the site for an Early Termination visit to finalize participation. If the patient is not willing to complete all measures, priority will be given to the SRS, the primary outcome measure. There are no consequences if a patient also refuses this.

Recruitment will continue until at least 90 patients are evaluable. An evaluable patient is defined as a patient who has:

- Met all of the inclusion and none of the exclusion criteria specified in Sections 4.1 and 4.2 within the specified time-frame;

- Received the allotted course of treatment and is assessable for the primary efficacy endpoint;
- Had their eCRF completed.

Should a patient drop out or be withdrawn from the study, his/her randomization number will not be reallocated. A new patient will be enrolled and assigned the next number available. If eligible they will be randomized. Patients experiencing hypokalemia <3 mEq/L will be not replaced. A patient should be replaced if he regularly spits out or regurgitates the IP.

8.6 Follow-up of subjects withdrawn from treatment

In case a subject discontinues before the end of the study, no replacement is needed due to the methods that were selected for our statistical plan.

8.7 Premature termination of the study

If the study is terminated prematurely, for whatever reason, the accredited METC will be notified immediately.

9. SAFETY REPORTING

9.1 Section 10 WMO event

In accordance to section 10, subsection 1, of the WMO, the investigator will inform the subjects and the reviewing accredited METC if anything occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal. The study will be suspended pending further review by the accredited METC, except insofar as suspension would jeopardise the subjects' health. The investigator will take care that all subjects are kept informed.

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the investigational product. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded. Severity of the adverse event is rated according to the NCI Common Terminology for Adverse Events (CTCAE) rating scale. The scale consists of 5 grades:

Adverse Events:

Grade 1 Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; no intervention indicated

Grade 2 Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental ADL

Serious Adverse Events:

Grade 3 Severe or medically significant but not immediately life-threatening; hospitalization or prolongation of hospitalization indicated; disabling; limiting self care ADL

Grade 4 Life-threatening consequences; urgent intervention indicated.

Grade 5 Death related to AE.

9.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that at any dose:

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect;
- Any other important medical event that may not result in death, be life threatening, or require hospitalization, may be considered a serious adverse experience when, based upon appropriate medical judgement, the event may jeopardize the subject or may require an intervention to prevent one of the outcomes listed above.

The sponsor will report the SAEs through the web portal *ToetsingOnline* to the accredited METC that approved the protocol, within 15 days after the sponsor has first knowledge of the serious adverse events.

SAEs that result in death or are life threatening should be reported expedited. The expedited reporting will occur not later than 7 days after the responsible investigator has first knowledge of the adverse event. This is for a preliminary report with another 8 days for completion of the report.

9.2.3 Suspected unexpected serious adverse reactions (SUSARs)

Adverse reactions are all untoward and unintended responses to an investigational product related to any dose administered.

Unexpected adverse reactions are SUSARs if the following three conditions are met:

1. the event must be serious (see chapter 9.2.2);
2. there must be a certain degree of probability that the event is a harmful and an undesirable reaction to the medicinal product under investigation, regardless of the administered dose;
3. the adverse reaction must be unexpected, that is to say, the nature and severity of the adverse reaction are not in agreement with the product information as recorded in:
   - Summary of Product Characteristics (SPC) for an authorised medicinal product;
   - Investigator's Brochure for an unauthorised medicinal product.

The sponsor will report expedited the following SUSARs through the web portal *ToetsingOnline* to the METC:

- SUSARs that have arisen in the clinical trial that was assessed by the METC;
- SUSARs that have arisen in other clinical trials of the same sponsor and with the same medicinal product, and that could have consequences for the safety of the subjects involved in the clinical trial that was assessed by the METC.

The remaining SUSARs are recorded in an overview list (line-listing) that will be submitted once every half year to the METC. This line-listing provides an overview of all SUSARs from the study medicine, accompanied by a brief report highlighting the main points of concern.
The expedited reporting of SUSARs through the web portal ToetsingOnline is sufficient as notification to the competent authority.

The sponsor will report expedited all SUSARs to the competent authorities in other Member States, according to the requirements of the Member States.

The expedited reporting will occur not later than 15 days after the sponsor has first knowledge of the adverse reactions. For fatal or life threatening cases the term will be maximal 7 days for a preliminary report with another 8 days for completion of the report.

Please refer to section 8.2 for the unblinding procedure.

9.3 Annual safety report

In addition to the expedited reporting of SUSARs, the sponsor will submit, once a year throughout the clinical trial, a safety report to the accredited METC, competent authority, and competent authorities of the concerned Member States.
This safety report consists of:

- a list of all suspected (unexpected or expected) serious adverse reactions, along with an aggregated summary table of all reported serious adverse reactions, ordered by organ system, per study;
- a report concerning the safety of the subjects, consisting of a complete safety analysis and an evaluation of the balance between the efficacy and the harmfulness of the medicine under investigation.

9.4 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. SAEs need to be reported till end of study within the Netherlands, as defined in the protocol

9.5 Data Safety Monitoring Board (DSMB)

An independent DSMB will be established to perform ongoing safety surveillance and to perform interim analyses on the safety data, this committee should be an independent committee. The composition, roles and responsibilities of this committee are described in the appendix of the DSM charter (Appendix 2).
The advice(s) of the DSMB will only be sent to the sponsor of the study. Should the sponsor decide not to fully implement the advice of the DSMB, the sponsor will send the advice to the reviewing METC, including a note to substantiate why (part of) the advice of the DSMB will not be followed.

10. STATISTICAL ANALYSIs

A significance level of [alpha] = 0.05 will be used for all analyses. P values will be adjusted for multiple comparisons using the Bonferroni correction. SPSS version 22 will be used for most analyses except the mixed effects modelling, which will be done with R version 3.1.2.

10.1 Primary study parameter(s)

Summary statistics including mean and standard deviation for the continuous SRS variables and frequencies for categorical variables will be compared. Effect analysis will be done on an intention-to-treat basis. Linear mixed effects models will be used to assess differences in changes in clinical outcomes over time. T-tests will be used to test for differences in baseline demographics between the treatment and placebo groups.

10.2 Secondary study parameter(s)

To assess the relation between treatment outcome and cognitive and neurophysiological (EEG) markers, we will do both linear and ordinal regression. For the ordinal regression, the

treatment outcome will be categorized into improved, unchanged and deteriorated, using an assumed clinically relevant difference of 16 points on the SRS as a cut-off point.

10.3 Other study parameters
n/a

10.4 Interim analysis
n/a

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement
The study will be conducted according to the principles of the Declaration of Helsinki, version of Fortaleza, 2013, and in accordance with the Medical Research Involving Human Subjects Act (WMO).

11.2 Recruitment and consent

Recruitment
Patients will be recruited from the patient population referred the department of developmental disorders (DDD) of the UMC Utrecht. In addition, we will advertise the study via the website of the Dutch Autism Association / Balans (parent organization), the website of the Division Brain of the UMCU, the website of Rhino (a training institute for psychologists and other professionals involved in the care for children with ASD), stichting oval (parent organization) and via the LinkedIn group of Dutch Child and Adolescent Psychiatry.

The principal investigator, a child psychiatrist (HB), will identify patients with ASD who could be considered for participation in this study. For each patient considered, eligibility criteria will be checked for compliance, and any possible deviations will be identified prior to that patient's inclusion in the study.

The Investigator will enter the details (date the patient was considered for the study, whether the patient was included in the study and their respective randomization number or reason why the patient was not included in the study) of each potentially suitable patient onto a Prestudy Screening log and will explain the details of the study to the patient and the patient's parent(s). The patient (if able and as per local laws and regulations) and the patient's parent(s) will be given the information letters to read for further information about the study.

Consent
Each potentially eligible patient (and their parents) will be informed by the Investigator (JS) of the study's objectives and overall requirements. Prior to conducting any of the pre-entry tests not performed routinely in the treatment of the patient, the Investigator will explain the study fully to the patient and his/her parent(s) using the PICD. Following this information, the patients and his/her parents are given at least 2 weeks to read the information and decide

whether to participate or not. If a parent is willing for the patient to participate in the study, they will be requested give written informed consent for children under 12 years of age. In case of patients 12 years or older, patients themselves need to also sign consent. The informed consent will be signed and personally dated by a single or both parents according to local laws and regulations and by the Investigator.
A copy of the signed form(s) will be provided to the parent(s) and the original(s) retained with the source documents.

11.3 Objection by minors or incapacitated subjects

We will work according to the 'Verzet' act of the 'Nederlandse Vereniging van Kindergeneeskunde'. For each individual participant, a record will be kept including information about the investigation sessions and any details of interest.

11.4 Benefits and risks assessment, group relatedness

The proposed study could establish a novel safe and groundbreaking treatment for children with autism spectrum disorders, for which there is no existing pharmacological treatment. As we emphasized in this protocol, there are so far no clear targets for autism spectrum disorder treatments, but this should not imply that novel treatment developments will be delayed and that these devastating disorders remain outcasts. Consistent with this notion, the intended research could lead to elucidation of a new therapeutic concept as well as an opportunity for further, advanced investigation of the neurophysiological mechanism of ASD.

This study will be performed among children with ASD as this disorder manifests from young age and if untreated will devastate the lives of these children. In addition, it is well recognized that intervention at the intended ages (and far less at later or adult ages) may enhance plasticity of the brain and restore important developmental capabilities for learning or behavioral adaptation.

Bumetanide has been used for over 20 years in the treatment of treatment of fluid retention (edema) in congestive heart failure, liver disease (cirrhosis) and kidney disease including nephrotic syndrome. Its safety and efficacy is well-established. The current study could demonstrate an added value of bumetanide addition regarding symptom improvement. This could prevent or shorten hospitalizations, with the associated high costs for society as well as the negative impact on the patients' lives.

In the face of the limited additional burden for the patient when participating in the current trial as compared to routine treatment, and the possible positive outcome for future treatment, offering participation to selected patients appears to be justified

Routine care consists of less extensive monitoring of symptomatology and (neuro)cognitive functioning compared to the tests proposed in this study. Therefore, enrolled patients may benefit from extended clinical examinations during study participation.

The data obtained from genetic analyses will serve primarily the research purposes of this study. All data will be saved under anonymous codes to prevent potential linking of psychiatric or biologic information to individuals. At the informed consent form parents can indicate to wish to be informed on incidental findings with clinical relevance.

11.5 Compensation for injury

The sponsor/investigator has a liability insurance which is in accordance with article 7 of the WMO.
The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study.
The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

11.6 Incentives

All participants will get a small reward for participating in the EEG assessment. We have a present-box available where they can choose a small gift (approximately €1 per gift).

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

Privacy laws and regulations will be adhered to during the complete study. The collection and processing of participants' personal information will be limited to what is necessary to ensure the study's scientific practicability, the evaluation of efficacy, adherence, side effects and the investigational product's safety. Information collected about participants during this clinical investigation will be treated confidentially. The investigator or her co-workers will collect data and transfer it without recording the patient's name or date of birth. Instead data will be coded with a participant identification number.

The file with the key to the code will be managed by one person. The source documents will be kept in a locked file cabinet in the office of the study coordinator with limited access of the research personnel. In accordance with national laws and guidelines and the specifications of the ICH-GCP guidelines, the investigators are obligated to archive all documents pertaining to the study for the legally required time period.
The acquired data of team 1 will be entered into an electronic case record form (eCRF) that is accessible via the internet, called research Online. The eCRF will be build and supported by the department of data management of the Julius Center (supervisor Diane van der Doest). Their specialized data management tool will be used called Research Online 2.0 (RO2). RO2 is an advanced web based electronic data capture system for collecting, managing and reporting high quality clinical research data according to ICH-GCP standards. It allows for creating databases for a variety of study designs including clinical trials. Investigators of team I will receive personal user names and passwords for this purpose, and data will be encrypted for transfer.

Team 2 will also enter data and examination results into a similar Research online based eCRF that is accessible via the internet. Here, members of team II will also receive personal usernames and passwords and data will be encrypted for transfer and will not be visible for team I. For more detail on the handling and storage of collected data can be found in the separate SOP on Data Management. For more information about the eCRF software Research Online and the department of data management of the Julius Center, see http://www.juliussupport.nl/Fields-of-Expertise/Data-management.
In addition, we will use SLIM (Study Logistics and Information Manager) as a software system that enables the GCP-compliant registration and access to personal data of study subjects for digital and paper communication purposes. It also stores informed consent details as well as in- and exclusion data. Finally, it records the dropout or opt out status of a subject. SLIM for this study will be developed and supported by the same datamanagement team of the Julius Center (supervisor Diane van der Doest), who will support Research Online (see above). Parents will be given the choice to be informed about their appointments for this study by email or by regular paper mail.

12. 2 Monitoring and Quality Assurance

Associated investigators will be carefully selected and comprehensively informed and trained regarding Good Clinical Practice (GCP), all study procedures and the required examinations and documentation.

All monitoring activities will be in line with national laws and guidelines and the specifications of the ICH-GCP guidelines.
Study monitors will visit the study site at regular intervals to monitor the execution of the study. Monitors will have access to all documents that are needed to perform their task according to the above mentioned guidelines. Monitors will check whether requirements to conduct the study are met and study procedures are followed correctly, and will check the study site's documentation, the participants' source data, eCRF entries, and the correct maintenance of the Investigator Site File. Investigators will permit trial-related monitoring, audits, ERB reviews and regulatory inspections, providing direct access to source data and study documents.

Monitoring will be conducted by Julius Clinical, an academic research organization (ARO) that combines strong scientific leadership and operational excellence to conduct innovative national and global clinical trials. Julius Clinical originates from the Clinical Trial Services Unit based at the University Medical Center Utrecht (UMC Utrecht), and was transferred into a separate legal entity, with head office in Zeist, The Netherlands. Julius Clinical remains closely linked to her parent organization, the UMCU Utrecht.

Monitoring activities that will be performed for this study are:

- Presence and completeness of Trial Master File (TMF) and/or Investigator File (IF); Investigators are responsible for completing the TMF/IF and for assembling the essential documents
- Monitoring of the rate of inclusion and the percentage of drop-outs
- 25% check on Informed Consent Forms (ICFs)(completeness & availability) for monitored participants
- 100% verification of existence and identity of the participant for monitored participants
- 100% check on Serious Adverse Event (SAE) procedure for all reported SAEs
- 100% check on in- and exclusion criteria for the first 10 enrolled subjects per site and 25% of the remaining enrolled subjects per site; a random selection will be made upfront
- 25% verification of endpoints, see monitorplan
- 25% SDV of the first 3 patients per site; thereafter complete SDV of 20% of the remaining patients, including check on missed SAEs, see monitorplan
- Check query process and assist investigators in solving unanswered queries, if applicable
- Check the presence of instructions for the execution of study procedures and if study personnel has been properly trained on these procedures; check equipment and facilities
- Check on GLP/GMP certification of laboratory/ Pharmacy

The monitor will write a monitoring visit report, using Julius Clinical SOPs and report templates, and send a copy by e-mail to the coordinating investigator and the quality manager of the appropriate division after every visit (within 15 working days). A detailed monitoring plan and contract with Julius Clinical BV is attached can be found in the monitoring plan attached to this protocol.

12.3 Amendments

A 'substantial amendment' is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect to a significant degree:

- the safety or physical or mental integrity of the subjects of the trial;
- the scientific value of the trial;
- the conduct or management of the trial; or
- the quality or safety of any intervention used in the trial.

All substantial amendments will be notified to the METC and to the competent authority.

Non-substantial amendments will not be notified to the accredited METC and the competent authority, but will be recorded and filed by the sponsor.

12.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

12.5 End of study report

The sponsor will notify the accredited METC and the competent authority of the end of the study within a period of 90 days. The end of the study is defined as the last patient's last visit.

In case the study is ended prematurely, the sponsor will notify the accredited METC and the competent authority within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC and the Competent Authority.

12.6 Public disclosure and publication policy

The results of the study will be submitted for publication in an international peer-reviewed journal adhering to applicable privacy laws and regulations. Publication strategy will be determined by the principal investigator together with Neurochlore.

13. STRUCTURED RISK ANALYSIS

13.1Potential issues of concern

a. Level of knowledge about mechanism of action

Bumetanide is an inhibitor of the Na-K-Cl co-transporters called NKCC1 and NKCC2. NKCC1 is widely distributed throughout the body, especially in exocrine glands and is also expressed in many regions of the brain. NKCC2 is specifically found in cells of the thick ascending limb of the loop of Henle in nephrons, the basic functional units of the kidney. Bumetanide has been used since several decades as a loop diuretic used in the treatment of edema, including that associated with congestive heart failure or hepatic or renal disease, and hypertension. It belongs to the same family as Furosemide. Furosemide also antagonizes the chloride exporter KCC2 and would therefore exert a chloride lowering effect [15] [16] and has therefore no purpose in this context. Pharmacological and clinical studies have shown that 1 mg Bumetanide has a diuretic potency equivalent to approximately 40 mg furosemide. The site of Bumetanide action is the ascending limb of the loop of Henle. Recent studies have shown Bumetanide also blocks the NKCC1 co-transporter in the brain, and decreases internal chloride concentration in neurons. This concentration change makes the action of GABA more hyperpolarizing, which may be useful for treatment of ASD.

Due to overexpression of neuronal NKCC1 and low expression of KCC2 (K+ Cl− co-transporter isoform 2), *immature* neurons have a high intracellular Cl− concentration, rendering GABAa receptor-mediated Cl− currents depolarising (excitatory state) instead of hyperpolarising (inhibitory state) as in *mature* neurons [11,54,55]. This may at least partially explain the high incidence of seizures and poor response to conventional AEDs in the newborn infants. The switch from excitatory to inhibitory function is assumed to happen around birth but varies between species and between different brain regions. Indeed, the levels of intracellular chloride have been shown to be elevated in immature neurons and are progressively reduced in a brain structure and neuronal age dependent developmental sequence [56,57].

Recurrent seizures and other traumatic insults can lead to down-regulation of KCC2 and to a re-establishment of NKCC1-dependent depolarizing GABAergic signaling [58]. Several in vitro and in vivo studies suggest that bumetanide can switch the GABA equilibrium potential of immature neurons or abnormal mature neurons from depolarizing to hyperpolarizing, resulting in a reduced neuronal firing [11,54,55,58] (see Fig. 3). In addition, chloride levels are increased in epilepsies and a variety of brain disorders and lesions including cerebrovascular infarcts, spinal cord lesions, etc.[54,55,58-61]. Furthermore, bumetanide can augment phenobarbital anticonvulsive action in different rodent models including a hypoxic rat model [62,63].
These observations suggest that drugs that reduce intracellular chloride levels may be helpful in reinstating normal/low levels of chloride and thereby a powerful GABAergic inhibition.

b. Previous exposure of human beings with the test product(s) and/or products with a similar biological mechanism

Bumetanide, was first introduced in the seventees for diuretic use and was widely used for the treatment of fluid retention. In the early 1980s, several articles or reviews summarized the safety and the efficacy of Bumetanide in patients with renal or hepatic dysfunction. Diuresis begins within 30–60 minutes following oral administration and within a few minutes following IV administration. Peak diuretic activity generally occurs within 1–2 hours following oral and within 15–30 minutes after IV administration. Diuresis is dose-dependent and generally complete within 4–6 hours following oral. Bumetanide in newborns and children

was shown to be efficacious and generally well tolerated. The published studies indicate that acute or repeated administrations (several weeks) of Bumetanide treatments at dosages between 0.06 and 7.2 mg in newborns, toddlers and children result in a significant diuretic activity associated with loss of electrolytes. Despite the lack of direct comparison between the age groups, Bumetanide effect is anticipated to be similar in children above 2 years of age and adults.For details on the clinical experience with bumetanide in different age groups we refer to sections 5.1 and 5.2 of the IB.

In addition to its use for conditions associated with fluid overload, there is clinical experience with bumetanide as an agent for ASD and seizure management.

Bumetanide application in patients with epilepsy
In adults, bumetanide at total daily dose of 2 mg was associated with a reduction of seizure frequency and EEG discharges in three patients with temporal lobe epilepsy over a 4-month period [64]. However, it is not specified how the dose was divided over the day and how the drug was administered. Considering the half-life in adults, that dose appears to be rather small. In infants, published a single case study of a 6-week old infant with seizures due to meningitis, showing that a single dose of 0.1 mg/kg i.v. bumetanide was associated with a reduction of electrographic seizures by less than 50% (36 ± 7 seizures per hour in a 2-h window before treatment compared to 21 ± 7 seizures in the 2-h window following the administration of bumetanide) [65].

Bumetanide application in patients with ASD
A pilot study[36] and a confirmatory randomized study[1] have assessed the safety and efficacy of Bumetanide in ASD children.

The pilot study investigated the efficacy and safety of Bumetanide in 5 children (3.5 to 11.5 years of age) with ASD who received 0.5 mg Burinex® (half a 1 mg tablet per dosing) twice a day (bid) (morning and evening) for 90 days [36]. An improvement in total scores of the efficacy scales measured SRS, (Childhood Autism rating Scale [CARS], Aberrant Behaviour Checklist [ABC], Clinical Global Impressions [CGI], Regulation Disorder Evaluation Grid [RDEG] and the Repetitive and Restricted Behaviour [RRB] scale) was observed for all children at the end of the 90 day treatment. Bumetanide provided better cognitive regulation in keeping with the improved presence reported by the parents. The results on the SRS and subscales of ABC suggested an amelioration of states of vigilance and social interactions, stereotypic movements and hyperactivity.
Commencing 1 week after the treatment and once monthly, a clinical surveillance was made including research of dehydration, orthostatic hypotension, hyper-sensitivity, cramps, asthenia, diarrhoea, myalgia, arthralgia, nausea and dizziness. The levels of sodium and potassium remained stable (tests made 1 week and 2 months after the start of the treatment). No serious adverse effect related to the treatment were observed.
A confirmatory randomized double-blind placebo-controlled study was performed in 60 ASD children receiving Bumetanide capsules (2010; clinical registration number NCT01078714) [66].Patients were diagnosed with ASD by a clinical child psychiatrist using International Classification Disease (ICD 10, 92). Diagnosis was confirmed by ADI-R (Autism Diagnostic Interview - revised). Patients eligible for enrolment were 3 to 10 years old, with a diagnosis of autism (F84.0) or Asperger syndrome (F84.5) and a CARS score ≥ 30. Exclusion criteria

included history of seizures or other neurological disorders and treatment with other treatments (notably psychotropic agents, including risperidone) with the exception of melatonin to improve sleep disorders.
The primary endpoint was the childhood autism rating scale (CARS) measured before and after 90 days of treatment. The secondary endpoints were CGI and ABC measured before and after 90 days of treatment. Patients received a capsule containing either 0.5 mg Bumetanide or placebo (lactose) twice daily (morning and evening).
Bumetanide treatment resulted in a statistically significant decrease in CARS total score, with an average improvement of 1.8 units for the placebo group and 5.6 units for the Bumetanide group (p=0.004). Of the 27 patients for whom assessments of CARS were available on Day 90, 23 patients (85%) were considered to have improved in the Bumetanide group. The most improved items were relationships with people, imitation, and visual responsiveness.
The average CGI disease severity index score, calculated on the basis of all available data, was greater in the Bumetanide group compared with the placebo group (2.04 versus 1.56, p=0.017) suggesting a clinical amelioration.
Parents attested to the positive impacts of Bumetanide on the behaviour of their children, described as a 'greater presence' of the child, facilitated visual communication and social exchanges. This positive opinion resulted in 92% of parents requesting to continue treatment at the end of the 90 day treatment.
Bumetanide treatment was well tolerated. Body weights and sodium levels were not altered during the study. The most frequent AE was a decrease in plasma potassium level. A total of 22 of the 59 children (37%) and 6 out of 19 adolescents (32%) experienced decreased kalemia. Rapid improvements were observed after giving oral potassium supplements. Increase in uric acid occurred in 4 patients (1 child, 3 adolescents). During the treatment, dosage adjustments (0.5 to 2 mg daily) were made by the physicians based on ASD improvement and potassium plasma level.

In addition, we have published A case of a 10-year-old girl with ASD, epilepsy, cortical dysplasia, and a 15q11.2 duplication who had exhibited marked behavioral arousal after previous treatment with clobazam, a benzodiazepine was described (Bruining et al, 2015). We hypothesized that this response indicated the presence of depolarizing excitatory GABA and started Bumetanide treatment with monitoring of behavior, cognition, and EEG. The Bumetanide treatment (90 day, 0.5 mg twice daily) resulted in a marked clinical improvement in sensory behaviors, rigidity, and memory performance, which was substantiated by questionnaires and cognitive assessments. At baseline, the girl's EEG showed a depression in absolute α power, an electrographic sign previously related to ASD, which was normalized with Bumetanide. The treatment did not cause adverse effects, hypokalemia or discomfort through diuretic effects.

c. Can the primary or secondary mechanism be induced in animals and/or in *ex-vivo* human cell material?

Diuretics like Bumetanide or furosemide are organic anions that reversibly inhibit NKCC1 transporters in a variety of preparations by a concentration dependant mechanism, with a half inhibitory constant (IC_{50}) ranging from 0.1 µM to 10 µM in mammalian cells (fibroblasts, neurones, tumor cells, transfected HEK293 cells) [61,67-71]. The conclusion is that sensitivity of NKCC1 to Bumetanide varies over two orders of magnitude depending on level of activation the co-transporter, the experimental conditions and on the cell type [72].

In the brain, NKCC1 expression is responsible for altering responses to the GABA neurotransmitter from excitatory to inhibitory, which was suggested to be important for early neuronal development [69]. Bumetanide blocks the NKCC1 co-transporter, and thus decreases internal chloride concentration in neurons. This concentration change makes the action of GABA more hyperpolarizing; this is the initial triggering event for explaining the efficacy of Bumetanide in ASD [56].
The researchers and founders of Neurochlore have conducted in vitro pharmacological studies to assess the activity of Bumetanide on NKCC1 and the GABAergic system. A tabulated summary of the studies is provided (Table 3).

Table 1: Main pharmacodynamics studies supporting the use of Bumetanide in ASD

Reference	**Experimental conditions**	**Dose/drug**	**Notable findings**
Tyzio, 2006[57]	Hippocampal slices *in vitro* from embryonic day (E) 18 to E21 (birth) rat.	Bumetanide 10µM	At E18, GABA is depolarized and Bumetanide hyperpolarizes it. During delivery the GABA action is transiently inhibitory and Bumetanide does not affect it.
Rheims, 2008a[55]	Neocortical slices from P1 to P15 Swiss mice.	Bumetanide 10µM	Bumetanide reduces intracellular chloride ([Cl-]i), GABA depolarization, and network oscillations.
Rheims, 2008b[55]	*N*eocortical slice from P6 to P17 Swiss mice.	Bumetanide 10µM	Bumetanide blocks epileptic seizures in the neocortex where GABA is excitatory.
Nardou, 2009[68]	Hippocampal slices from P7–P8 neonatal Wistar rats.	Bumetanide 10µM	Bumetanide reduces [Cl-]i and blocks spontaneous network activities.
Nardou, 2009[68]	Hippocampal intact formations and slices from P7–P8 Wistar rats.	Bumetanide 10µM	In epileptic tissue, Bumetanide reduces [Cl-]i and the excitatory action of GABA.
Valeeva, 2010[73]	Hippocampal slices from P2–P6 Wistar rats.	Bumetanide 0,2-10µM	At 0.2–1 µM, Bumetanide reducing $[Cl^-]_i$ modifies action potential properties

			that desynchronized neuronal network. At 5–10 µM, Bumetanide completely blocked network activities.
Mazzuca, 2011[74]	Experiment *in vivo* from P0-P2 rat and primary cultures of trigeminal neurons dissociated from newborn rats.	Bumetanide 5 µmol/kg (1,8 mg/kg)	Bumetanide has an analgesic action in newborn pups *in vivo*. It reduced $[Cl^-]_i$ reducing the depolarizing action of GABA *in vitro*.
Tyzio, 2011[75]	Wistar rats P5–P7 neocortical and hippocampal slices	Bumetanide 10µM	Bumetanide fully blocked the generation of spikes evoked by a depolarizing GABA.
Nardou, 2011[61]	Hippocampal intact formations and slices from P7–P8 mice wild type and knock-out for NKCC1.	Bumetanide 10µM	Bumetanide has no effect in knock-out mice of NKCC1.

The activity of Bumetanide on the GABAergic system was studied with brain slice preparations. Slices are prepared and neuronal recordings made notably using single GABA and NMDA channel recordings. Using this approach, Bumetanide was found to reduce significantly the driving force of GABAergic currents and to convert the depolarization to a hyperpolarization and a powerful inhibition [57]. Bumetanide also reduced the duration required for a neuron to recuperate the chloride levels after a strong stimulation by GABA and a large chloride influx. These effects were abolished in NKCC1 knockout animals indicating that they are mediated by this co-transporter (Nardou, 2011). These experiments were conducted at 10 µM.

The actions of Bumetanide was studied ex vivo on two models of autism[13]:

- The in utero valproic acid model (VPA): The offspring of women taking this medication for mental illness or epilepsy during early pregnancy are at elevated risk for autism. In pregnant rats, a single injection of VPA results in behavioral abnormalities such as increased stereotypic/repetitive behavior, decreased social interaction, altered sensitivity to sensory stimuli, impaired PPI, elevated anxiety, impaired reversal learning, altered eye blink conditioning, and enhanced fear memory processing, all of which are consistent with autism.

- Fragile X mice: Fragile X syndrome is caused by mutations in the FMR1 gene on the X chromosome. The disorder shares a number of symptoms in common with autism, mental retardation, attention deficit hyperactivity disorder, and epilepsy. Fmr1 KO mice display some core behavioral features of autism including impaired social interaction and repetitive behavior. Expression of other autism-related symptoms such as anxiety and hyperactivity depends on the genetic background.

In both models, immature hippocampal neurons recorded during the first weeks postnatally have indeed a higher concentration of chloride and depolarizing actions of GABA than match-aged control animals. Most intriguingly, it was observed that the dramatic oxytocin mediated loss of intracellular chloride that occurs in naive animals is abolished in Fragile X and VPA pups. These studies provide a substantial confirmation that the regulation of intracellular chloride is mal operative in autism in the hippocampus.

Bumetanide has also been shown to efficiently reduce intracellular chloride and shift GABA actions after spinal cord lesions [59]. Also, Neurochlore has recently found significant reduction of intracellular chloride levels in cortical neurons of two animal models in slices Fragile X and in utero valproate [13]
In keeping with this, Sipila et al, (2006) showed that Bumetanide, completely and reversibly blocks hippocampal sharp waves in the neonate (postnatal days 7-9) rat hippocampus in vivo, suggesting a central action of the diuretic.
Bumetanide appears to be an efficient drug to enhance GABAergic inhibition in a variety of pathological conditions. Taken together, these studies show that 30 years after the registration of Bumetanide as a diuretic, this molecule also belongs to the class of psychotropic agents.

d. Selectivity of the mechanism to target tissue in animals and/or human beings

Bumetanide toxicity has been extensively assessed in several animal species (see also section 4 of the IB). The toxicity of Bumetanide was investigated in rats, rabbits, dogs and monkeys. Doses of 10-140 mg/kg/day in rat, 0.1-1.2 mg/kg in dog and 0.1-5 mg/kg/day in monkey were tested.
Long term toxicity studies are available in 3 animal species, rat (13-26-78 weeks), dog (4-26-52 weeks) and (26 weeks) baboons. These studies indicate that Bumetanide is safe and that toxicity is for the most part the direct consequence of the potent diuretic activity of the compound. The toxicity profile of Bumetanide is similar to that reported for other loop diuretics such as furosemide. No severe toxicity or deterioration of clinical condition was apparent. In most repeated dose studies decreased body weight gain was recorded. Serum chemistry changes concerned decreases in electrolytes. They were noted in all tested species. Haematological examinations showed no changes. Hemoconcentration was the consequence of the increased urine excretion.
In long-term studies, Bumetanide produced effects similar in the rat and the dog but larger dosages of up to 140 mg/kg per day have been used in rat studies as compared to sub-mg/kg doses in dog studies. This discrepancy is explained by a rapid metabolism in rats. In contrast, Bumetanide is excreted largely unchanged in the dog and in man. The rat and dog are the two species in which most of the preclinical toxicologic evaluation of Bumetanide was performed.
The fact that the rat and dog metabolize Bumetanide differently is of value in that the dog, like the human, excretes most of the administered Bumetanide as unchanged drug, and the

rat, like the human, metabolizes Bumetanide by oxidation of the N-butyl side chain. Therefore, the rat and dog would appear to be suitable and complementary models for predicting Bumetanide toxicity in humans.
Kidney can be considered as the target tissue for toxicity. Distribution studies in dogs with 14C-Bumetanide have shown a high kidney exposure to Bumetanide. Furthermore, kidney changes were seen at the microscopic examination in all treatment groups in one 13-week study in male rats.
Despite the penetration of Bumetanide into brain, toxic effects on the CNS were not evident in short-term and long-term tests. Sub-chronic and chronic toxicology data with Bumetanide are listed in Table 5 in the IB and described in section 4.2 of the IB.
Main Reproduction toxicity studies with Bumetanide (oral administration) are listed in table 6 of the IB. No teratogenic potential was shown in mice, rats, rabbits, and hamster, but a slight embryolethal effect was reported in rats and rabbits. There was no Bumetanide related effect on fertility and peri-post-natal development in rats. The genotoxic potential of Bumetanide was re-evaluated according to current ICH standards. No evidence of genotoxicity was found. The predictability of the toxicity studies with Bumetanide was good since adverse effects experienced by patients with fluid retention are also related to its diuretic activity.

e. Analysis of potential effect

Bumetanide, was first introduced in the seventees for diuretic use and was widely used for the treatment of fluid retention. In the early 1980s, several articles or reviews summarized the safety and the efficacy of Bumetanide in patients with renal or hepatic dysfunction.
Diuresis begins within 30–60 minutes following oral administration and within a few minutes following IV administration. Peak diuretic activity generally occurs within 1–2 hours following oral and within 15–30 minutes after IV administration. Diuresis is dose-dependent and generally complete within 4–6 hours following oral.
Four studies have assessed the safety and efficacy of Bumetanide during long term treatments in adults with congestive heart failure or renal disease. Table 7 in the IB summarizes the safety data. These studies show that bumetanide is generally well-tolerated and that ASEs were related to the diuretic activity of the drug.
A review based on 58 clinical studies (493 patients) has studied the adverse reactions to Bumetanide. Results were compared with those in 220 patients treated with furosemide[7].
Both agents were administered to patients with edema secondary to congestive heart failure, and renal and hepatic disease.
The adverse reactions were listed according to the organ system involved. Those found to have occurred with the greatest frequency involved the special sense organs (impaired hearing, vertigo) and the skin (rash, pruritus, hives, sweating) and musculoskeletal (muscle weakness, cramps, arthritic pain) systems. Some of the adverse reactions reported weakness, hypotension, polyuria, dehydration, xerostomia are simply manifestations of the pharmacologic activity of the test drugs.

Precautions and safety alerts

The SmPC reports reactions including abdominal pain, vomiting, dyspepsia, diarrhoea, stomach and muscle cramps, arthralgia, dizziness, fatigue, hypotension, headache, nausea, encephalopathy (in patients with pre-existing hepatic disease), fluid and electrolyte depletion, dehydration, hyperuricaemia, raised blood urea and serum creatinine, hyperglycaemia, abnormalities of serum levels of hepatic enzymes, skin rashes, pruritus, urticaria, thrombocytopenia, gynaecomastia and painful breasts.

Hearing disturbance after administration of Burinex is rare and reversible.

The decreases in electrolytes and more specifically in potassium should be measured periodically and potassium supplements or potassium sparing diuretics added if necessary. Periodic determinations of other electrolytes are advised in patients treated with high doses or for prolonged periods, particularly in those on low-salt diets.
Hyperuricemia may occur; it has been asymptomatic in cases reported to date.
Bumetanide may increase urinary calcium excretion with resultant hypocalcemia.

A Safety Labeling change was approved by FDA Center for Drug Evaluation and Research (CDER) in January 2010 about serious skin reactions (Stevens-Johnson syndrome, toxic epidermal necrolysis) in association with Bumetanide use.

Tolerability and safety in newborns and children with edema or seizure
Bumetanide is used in preterm and full term newborns to treat fluid volume overload due to cardiac or pulmonary diseases. Many full-term newborns with refractory seizures have hypoxic-ischemic encephalopathy from perinatal asphyxia. Despite limited publications, there is significant evidence to show that the paediatric populations respond to diuretics in general and Bumetanide in particular in a manner comparable to adults. The main differences are related to pharmacokinetics parameters in newborns compared with older ages; the diuretic activity of Bumetanide in newborns is in accordance with the presence of the NKCC1 co-transporter in kidney.
Published studies with Bumetanide in children or adolescents are scarce. Bumetanide is not presently recommended for the treatment of edema in children below 12 years of age until further studies are conducted. Most of prescriptions are off label in this age group.
The table 8 in the IB summarizes the main published studies. Bumetanide in newborns and children was shown to be efficacious and generally well tolerated. The published studies indicate that acute or repeated administrations (several weeks) of Bumetanide treatments at dosages between 0.06 and 7.2 mg in newborns, toddlers and children result in a significant diuretic activity associated with loss of electrolytes. Despite the lack of direct comparison between the age groups, Bumetanide effect is anticipated to be similar in children above 2 years of age and adults.

Recently a trial tested the use of bumetanide for treatment of neonatal seizures (NEMO trial). In this study, the dose and feasibility of intravenous bumetanide was tested as an add-on to phenobarbital for treatment of neonatal seizures. In this study, the dose and feasibility of intravenous bumetanide was tested as an add-on to phenobarbital for treatment of neonatal seizures. In this open-label, dose finding, and feasibility phase 1/2 trial, full-term infants were recruited younger than 48 h who had hypoxic ischaemic encephalopathy and electrographic seizures not responding to a loading-dose of phenobarbital. Newborn babies were allocated to receive an additional dose of phenobarbital and one of four bumetanide dose levels by use of a bivariate Bayesian sequential dose-escalation design to assess safety and efficacy. No short-term dose-limiting toxic effects, but three of 11 surviving infants had hearing impairment confirmed on auditory testing between 17 and 108 days of age [76]. The most common non-serious adverse reactions were moderate dehydration in one, mild hypotension in seven, and mild to moderate electrolyte disturbances in 12 infants. The trial was stopped early because of possible increased risk for hearing loss. A causal link between bumetanide and hearing loss was refuted on the basis of the occurrence in 3 of 11 children and the

possibility of interaction with aminoglycosides, which had been given in 2 of 3 children in which hearing loss was established[76]. Following this study, researchers of the same consortium conducted a Pilot evaluation of the population pharmacokinetics of bumetanide in term newborn infants with seizures [77]. In this study ,fourteen infants were included, 13 of them being cooled (a common intervention in this disorder). No relationship was found between bumetanide exposure and its efficacy (reduction in seizure burden) or its toxicity (hearing loss).

Tolerability and Safety in ASD Children

Pilot studies and a confirmatory randomized study have assessed the safety and efficacy of Bumetanide in ASD children [1,22,36].

The most important data for safety and tolerability of bumetanide in ASD come from the RCT in 2010 by Lemonnier et al[1]. This confirmatory randomized double-blind placebo-controlled study was performed in 60 ASD children (clinical registration number NCT01078714). Patients eligible for enrolment were 3 to 10 years old, with a diagnosis of autism (F84,0) or Asperger syndrome (F84,5) and a Childhood Autism Rating Scale (CARS) superior or equal to 30 (see table 9 in the IB). Exclusion criteria included history of seizures or other neurological disorders and treatment with other treatments (notably psychotropic agents including risperidone) with the exception of melatonin to improve sleep disorders. The primary endpoint was CARS measured before and after 90 days of treatment. Bumetanide treatment resulted in a statistically significant decrease in CARS total score. Six patients (3 each from placebo and Bumetanide treatment arms) withdrew from the study because of adverse effects (4) or joined decision between the parents and the physicians (2): 4 patients (2 placebo, 2 Bumetanide) because of adverse effects (2 enuresis, 1 placebo and 1 Bumetanide; 1 eczema, placebo, 1 hypokalaemia (3.0 mEq/mL), Bumetanide; 2 patients (1 placebo, 1 Bumetanide) because of agitation-related behavior following risperidone discontinuation according to inclusion criteria). Thus, 54 patients completed 90 days of treatment. Body weights were not modified. Sodium was not altered. A slight decrease in mean potassium level was observed in Bumetanide treated patients compared with placebo (see figure 4 in the IB). Decrease in kalemia (3.5-3.1 mM) occurred in 6 patients receiving Bumetanide (22 %).

Bumetanide treated patients events including hypokalaemia, asthenia, abdominal pain and nausea or consent withdrawal. Presently, all patients have completed the 90 day treatment period. Given the 3:1 randomisation ratio, approximately 66 of these patients had received Bumetanide. As expected, Bumetanide exerts significant effects on urinary excretion and blood electrolytes. The most frequent adverse events measured are mild (K <3.5 mEq/mL and > 3 mEq/mL) or moderate (K ≥2.0 mEq/mL and ≤3.0 mEq/mL) hypokalaemia and dehydration mainly at the beginning of the treatment period. The decrease in blood potassium is not associated with cardiac dysrhythmia. A mild decrease in blood chloride for half of patients experiencing hypokalaemia is observed. Natremia is not affected. Other noticeable adverse effects are fatigue and asthenia (12 %), loss of appetite (11%), abdominal pain (10%). Approximately 9% of the patients enrolled have experienced enuresis, increased diuresis, or polyuria. No other clinically important issue has arisen from the ongoing clinical trial.

During the reporting period (January – December 2014), 3 related serious adverse reactions (SAR) were reported:Two patients (six- and 10- year old male patients) were hospitalised

due to hypokalaemia. The potassium level was 2.19 mEq/mL and 2.40 mEq/mL, respectively. The patients received intravenous or oral potassium and study drug was discontinued. A 9-year-old male experienced placebo or Bumetanide solution overdose due to parent's inattentiveness. He was hospitalised due to this accidental ingestion of 25 mL of Bumetanide/placebo, i.e. approximately 10-12 mg. He had 3 important micturitions. The blood electrolyte profile was normal. The day after, the event was considered resolved and the patient was discharged.

f. Pharmacokinetic considerations

Bumetanide is well absorbed after oral administration with a bioavailability reaching between 80 and 95%. The elimination half-life ranges from between 0.75 to 2.5 hours. No active metabolites are known. Bumetanide in animals and adults is poorly distributed due to high protein binding. Renal excretion accounts for approximately half the clearance with hepatic excretion responsible for the other half. Several studies have described the lower clearance of Bumetanide in newborns compared with adults due to incomplete kidney maturation. It has been shown to increase proportionally with postnatal age [15,78-83].

Absorption

Several pharmacokinetic studies have shown that following single oral dose (0.5 to 2 mg) to healthy volunteers, absorption of Bumetanide is rapid and almost complete. Bioavailability of oral doses has been recorded at more than 90%. The extent of bioavailability of Bumetanide from the tablet and oral solution dosage forms are equivalent. Peak plasma concentrations of 30 to 50 ng/mL usually occur 0.5 to 4 hours after administration (see Figure 5 and Table 10 in the IB). There is an increase in half-life and a reduced plasma clearance in the presence of renal or hepatic disease.

Metabolism:

Bumetanide is partially metabolized by oxidation in the liver to at least 5 metabolites [82] Major urinary metabolite is the 3′-alcohol derivative. The major metabolite excreted in bile and/or feces is the 2′-alcohol derivative. Minor metabolites include the 4′-alcohol, N-desbutyl, and 3′-acid derivatives.

Metabolites in urine and bile are present as conjugates, principally glucuronide conjugates. Conjugates of Bumetanide and its metabolites do not appear in feces.

Elimination

Bumetanide is rapidly eliminated from the body after intravenous, intramuscular, oral solution, and oral tablet administrations, with half-lives ranging from 0.4 to 1.7 h. Bumetanide and its metabolites are excreted principally in urine (about 70-80 % in urine and 50 % unchanged; about 10- 20 % in feces, almost completely as metabolites apparently via biliary elimination) [84-86]. Pharmacokinetics in children and newborns.

Loop diuretics are used intravenously in paediatric patients to treat fluid overload in various disease states (Table 10). Small pharmacokinetic studies of intravenous Bumetanide in preterm and full-term neonates with respiratory disorders have reported an apparent half-life of approximately 6 hours with a wide range and a serum clearance ranging from 0.2 to 2.7 mL/min/kg. Elimination half-life decreased considerably during the first month of life, from a mean of approximately 6 hours at birth to approximately 2.4 hours at 1 month of age. Pharmacokinetic studies with furosemide or Bumetanide have shown that newborns do not eliminate loop diuretics efficiently due to decreased renal and metabolic clearance.

As stated within the CHMP "Concept paper on the impact of renal immaturity when investigating medicinal products intended for paediatric use" (CPMP/PEG/35132/03), the kidney reaches maturity until between one and two years of age. The paper highlights that adult levels of glomerular filtration rate (GFR) (120 ml/min/1.73 m2) are reached between one and two years of age. This suggests that the pharmacokinetics of Bumetanide in children (2-11 years) is close to that observed in adult.

Due to the targeted age range (7-15), we do not expect major changes in the pharmacokinetic parameters of Bumetanide solution 0.5 mg/mL compared with adults receiving Burinex®, 1 mg. The reason for this assumption is manyfold:

- same dose range as Burinex® , 1-2 mg daily
- high oral bioavailability after oral administration of Burinex® (>90 %),
- dose proportionnality of pharmacokinetics of Burinex® in the dose range 0.5-2 mg, mature kidneys over 2 years of age.

g. Study population

Ninety children with ASD will be included in the study in the age range of 7 to 15 years of age. The diagnosis should be made no more than three years ago. Patients are physically healthy and stable on relevant aspects, as determined during the screening visit. A potential subject who meets any of the following criteria will be excluded from participation in this study in case of:

1. Total IQ < 55 (WISC) and/or inability to comply with the protocol-specified procedures for the duration of the study, including treatment and blood sampling to control diuretic effects.
2. Serious, unstable illnesses including, gastroenterologic, respiratory, cardiovascular (arythmias, QT interval lengthening), endocrinologic, immunologic, hematologic disease, dehydration or hypotension; electrolyte disturbances (Na <133 mmol/L, K <3.5 mmol/L or Ca < 2.17 mmol/L (<13y) or <2.2 mmol/L (>13y);
3. Renal insufficiency (CKD st2-5; estimated glomerular filtration rate < 90 ml/min/1.73m2), congential or acquired renal disease with decreased concentration capacity (tubulopathy, diabetes insipidus) and liverinsufficiency interfering with excretion or metabolism of Bumetanide;
4. Neurological disorders such as epilepsy, seizures and microcephaly;
5. Behavioral treatment
6. Treatment with psychoactive medications, (antipsychotics, antidepressants, anxiolytic drugs, psychostimulant drugs or other medication with effect on the central nervous system, including anti-epileptic drugs) in the last 8 weeks prior to start of the study, except melatonin; no use of other psychoactive substances is allowed from 8 weeks prior to the pre-study evaluation until the endpoint measurements at the end of the washout period. If clinically feasible and desired by the patients and/or parents, then it is allowed to stop psychoactive medication to allow enrollment in the study after a 8 week washout period of their psychoactive medication.
7. Treatment with NSAIDS, aminoglycosides, digitalis, antihypertensive agents, indomethacin, probenecid, Lithium, other diuretics (e.g. furosemide, hydrochlorothiazide), drugs known to have a nephrotoxic potential
8. Documented history of hypersensitivity reaction to sulfonamide derivatives.

The condition of ASD is expected to be stable. No patients at intensive care will participate in the study. Girls in the included age range (7-15 yrs old) may have child bearing potential but pregnancy risk will be very low.

h. Interaction with other products

Drugs with ototoxic potential: the use of Bumetanide in patients to whom aminoglycoside antibiotics like amikacin (Amikin), streptomycin, neomycin, gentamicin (Garamycin), erythromycin (E-Mycin, Eryc), kanamycin (Kantrex), tobramycin (Nebcin), netilmycin (Netromycin), vancomycin (Vancocin)) should be avoided. In life-threatening conditions, Bumetanide treatment will be stopped and the patients withdrawn from the study.

The use of non-steroidal anti-inflammatory drugs should also be avoided.

Other drugs can be avoided including digitals, antihypertensive agents, indomethacin, probenecid, Lithium, drugs known to have a nephrotoxic potential. These medicines cannot be prescribed during the study due to the inclusion and exclusion criteria (see the study protocol for more details).

Cytochromes P450 involved in Bumetanide metabolism have not been identified.

i. Predictability of effect

In experimental animals, it has been shown that GABA excites immature neurons instead of inhibiting them because of elevated intracellular chloride that reverse the polarity of GABA actions and an abrupt shift leads to an excitatory /inhibitory shift of GABA actions [57]. Following this discovery, it was found that this shifts fails in a variety of brain disorders, including 2 animal models of ASD [13]. Consequently, it was established that the diuretic Bumetanide as a NKCC1 chloride importer antagonist reduces intracellular chloride thereby reinstalling physiological actions of GABA. Bumetanide also reduced in these ASD models aberrant brain oscillations and “autistic” behavior. Pursuing this line of approach, it was shown that bumetanide produces an improvement of behavioral impairment measured by CARS and CGI in ASD children (3-11years old) in a double blind randomized study with little or no side effects [1]. A favorable effect on behavioral and cognitive functioning was further confirmed in our case study [22].

In terms of accuracy of the primary endpoint measurement, although clinicians have been studying therapies for autism for over 50 years, it is important to note that there are currently no Health authorities-approved indications for the treatment of autism. So far no definitive instrument has been identified for assessing core features of autism in clinical drug trials in Autism, but some tools worthy of consideration have been identified. The SRS was chosen as the primary endpoint, because this questionnaire is an extensively validated quantitative trait measure of social-communicative and restricted/repetitive behavior deficits referable to core ASD symptoms, in which informants base their ratings on cumulative observations of the subject in the subject's natural social settings [37,38].

The monitoring of EEG/ERP and cognitive parameters will be used to capture markers of elevated E/I balance due to excitatory GABA and to establish electrophysiological markers

that can predict efficacy of bumetanide treatment. This is pivotal element of this study as no ASD trials before have used EEG to find prognostic biomarkers.

j. Can effects be managed?

There is no specific antidote or antagonist for bumetanide.

13. 2 Synthesis

We proposes to conduct a randomized clinical trial (BAMBI **study**) to confirm clinical efficacy of Bumetanide on autism morbidity in a substantial sample of children and adolescents with ASD. In addition, the aim will be to develop cognitive and neurophysiological prognostic markers to enhance the rational application of Bumetanide in ASD. This study will be conducted in collaboration with the Health technology assessment centre of the Julius Center. Funding was obtained from the rational treatment program (Goed Gebruik Geneesmideelen (GGG)) of the Dutch ZonMW funding body. This new trail builds on pre-existing pre-clinical and clinical research as well as on our experience with Bumetanide. An internal DSMB will be established to oversee the trial.

The main back ground characteristics of the project are:

- One out of 100/120 children affected by autism or related disorders,
- A strong family demand for efficient treatments,
- No existing pharmacological treatment acting on the causes of the disease,
- A clinical proof of concept already established by one clinical study in ASD Children,
- A Bumetanide safety profile established in newborn and adult patients since 1973 for fluid retention treatment.
- long term efficacy and safety of Bumetanide for children and adolescents with ASD.

Hypotheses:
1. Thirteen weeks of treatment with Bumetanide will effectively reduce ASD-related behavioral symptoms and improve day-to-day functioning in comparison with usual care.
2. Efficacy of Bumetanide is associated with certain cognitive and psychophysiological parameters related to hyperexcitable networks in patients with ASD.

We consider that the available toxicology data are sufficient to allow conclusion on the relationship of the toxic effects and treatment with Bumetanide in the new target population. Whatever the age group, Bumetanide is well tolerated. The main adverse events are related to the diuretic activity of the molecule leading to a decrease in electrolytes. Hypokalaemia is frequently reported. Treatments could also result in an increase in blood uric acid. No serious adverse events were described after short or prolonged treatment. It is reported that oral or intravenous Bumetanide (0.5-2 mg) produced a dose related increase in diuresis, sodium, potassium and chloride excretion (Ramsay, 1978).
On a mg/kg basis, the daily dosages of Bumetanide in the different groups are the following: 0.02 to 0.2 mg/kg in adults with fluid retention{Dixon, 1981 #337;Handler, 1981 #335;Konecke, 1981 #336;Whelton, 1981 #338}, 0.005 to 0.2 mg in newborns (iv), toddlers and children with fluid retention{Marshall, 1998 #345;Ward, 1977 #341;Wells, 1992 #364}, 0.1 to 0.3 mg/kg (iv) in newborns with seizures{Pressler, 2015 #683} and 0.06 to 0.04 mg/kg

in ASD children{Bruining, 2015 #703;Lemonnier, 2010 #258;Lemonnier, 2012 #149}. The comparison shows that the proposed dosage for ASD children and adolescents is in the low end of the standard range of Bumetanide.
In summary, the rationale for the application and dosage of bumetanide is based upon the following elements:

- The dose range proposed for the treatment of ASD children and adolescent is the same as for the treatment of fluid retention,
- The toxicology findings are clinically relevant,
- Pharmacokinetics of Bumetanide in children and adolescents with mature glomerular filtration should be not different from the adults. The published data are in agreement with this assumption.
- The mechanism of action of Bumetanide is well-defined,
- Toxicology studies and the 40 years clinical experience with Bumetanide did not reveal any toxicity on the nervous, reproductive, pulmonary, cardiovascular and immune systems,
- Bumetanide is mainly prescribed in adult patients with fluid retention and altered renal or hepatic function. In the vast majority, the paediatric population display healthy hepatic and renal functions suggesting a better tolerance profile.
- Drugs with related chemical structure as Furosemide are prescribed for children with chronic diseases.
- Adverse effects of the diuretics are related to the prolonged increase in diuresis. They are predictable and manageable.

Due to the targeted age range (7-15 yrs of age), we do not expect major changes in the pharmacokinetic parameters of Bumetanide solution 0.5 mg/mL compared with adults receiving Burinex®, 1 mg. The reason for this assumption is manyfold:

- same dose range as Burinex® , 1-2 mg daily
- high oral bioavailability after oral administration of Burinex® (>90 %),
- dose proportionnality of pharmacokinetics of Burinex® in the dose range 0.5-2 mg,
- mature kidneys over 2 years of age.
- the Dutch pediatric formulary of medications recommends a starting dose between 0.01-0.1/kg/dose and 0.2 mg/kg/dose with a maximum dosage of 10 mg/day. Our proposed dosage is well within this range.

In conclusion, based on the preclinical and clinical data, we consider that the risk-benefit relationship of Bumetanide allows the initiation of phase II studies in ASD children and adolescents within the dose range. The results show that Bumetanide 1 to 2 mg twice daily was effective and generally well-tolerated in children with ASD

14. REFERENCES

<Include all key references published in peer reviews journals that are relevant for the study and are discussed in the protocol. Do make sure that the references are up to date.>

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APPENDIX 1: Instructions for Use of the Bumetanide Solution

Removed because of privacy

Appendix 2: internal DSMB charter

1. Roles and responsibilities DSMB

The aim of this DSMB is to safeguard the interests of trial participants, assess the safety of the interventions during the trial, and monitor the overall conduct of the clinical trial

This DSMB will:

a. Meet half-yearly (see DSMB Meetings) to review aggregate and individual subject data related to safety**,** data integrity and overall conduct of the trial.
b. Advise and monitor on evidence for treatment harm (eg toxicity data, SAEs, deaths)
c. Provide recommendations to continue or terminate the trial depending upon these analyses.
d. Communicate other recommendations or concerns as appropriate.
e. Operate according to the procedures described in this charter and all procedures of the DSMB.
f. Follow conflict of interest guidelines as detailed below (see DSMB Membership).
g. Comply with confidentiality procedures as described below (see Confidentiality).
h. Maintain documentation and records of all activities as described below (see DSMB Meetings, DSMB Reports).
i. Keep all of the information provided to the DSMB strictly confidential.

The sponsor will:

a. Assure the proper conduct of the study.
b. Assure collection of accurate and timely data (monitoring and data management).
c. Compile and report SAEs and SUSARs to the DSMB.
d. Promptly report potential safety concern(s) to the DSMB.
e. Prepare summary reports of relevant data for the DSMB. (This may include analyses not otherwise outlined in this charter based upon findings.)
f. Provide an independent facilitator for presentation of results during DSMB meetings if requested by the DSMB.

2. Practicalities prior to the start of the trial

The trial protocol will be reviewed by all members of the DSMB prior to agreeing to serve as a part of the DSMB for this study. The DSMB will meet prior to the start of the trial.

3. Composition of the DSMB

Prof. Dr. Ir. M.J.C. Eijkemans (chairman, biostatician, head department biostatistics and research Support, Julius Center, UMC Utrecht)
Dr. G.J. de Borst (associate professor vascular surgery, UMC Utrecht)
Prof. Dr.P. Blankestijn (head department nephrology, DIGD, UMC Utrecht)
Prof. Dr. L. Bont (Paediatric Infectious Diseases Specialist, head RSV Research Group, WKZ)
Dr. M.H.G. Langenberg (specialist internal medicine, oncologist, UMC Utrecht)

The DSMB for this Trial consists of 5 members. A secretary (Dr. G.C.M. van Baal, associate professor and statistician) will support the DSMB. The core members were appointed by the Executive Board of the University Medical Center Utrecht. See the attached CVs for previous relevant experience of each of the members.

The Internal DSMB of the University Medical Center was established in 2014 by the Executive Board of the University to facilitate efficient safety monitoring of low to moderate risk studies in the University Medical Center Utrecht. The standard operating procedure (SOP) of the Internal DSMB of the University Medical Center is attached to this charter; details are outlined in the present document. Most importantly, the DSMB has all powers referred to in the EMA-guidelines and the concept charter compiled by the Damocles study group.

The chairman has extensive experience serving on and chairing DSMBs and was elected by the other members of the 'core' DSMB.

As characteristic qualifications, members will:

- Work professionally and meet qualifications for their respective professions
- Comply with accepted practices of their respective professions.
- Comply with the conflict of interest policies specified by the Damocles Study Group to ensure that members do not have serious scientific, financial, personal, or other conflicts of interest related to the conduct, outcome, or impact of the study according to the guidelines specified below (e.g., engaged in any simultaneously occurring competitive trials in any role that could pose a conflict of interest for this study).
- Compy with confidentiality statements as laid down in the CAO 2013-2015, paragraph 9.8.
- Be independent from the trial.

DSMB members will sign a non-conflict of interest statement in regard to this study which will be on file with the secretary of the DSMB. Conflicts of interest and/or potential conflicts of interest will be reduced to the greatest extent that is consistent with assembling a highly competent DSMB. Any questions or concerns that arise regarding conflicts of interest will be addressed by the DSMB chairperson with input from other DSMB members as necessary.

4. Organisation of DSMB meetings

DSMB meetings will generally be conducted in face to face meetings and take place every three months. Meetings will be closed, unless attendance of the researchers in an open session is requested by the DSMB. The data for this study will be reviewed half-yearly, due

to mild-risk, the participation time of 5 months and the slow inclusion rate of approximately 1.5 per week.

The closed session will be restricted to the DSMB members, and the secretary of the internal DSMB, who will facilitate and record the meeting. Data which may compromise the integrity of the study (e.g., comparative data) will be analyzed and discussed only in the closed session.
The open session may be attended by representatives of the principal investigator. Data presented in the open session may include enrollment data, individual adverse event data, baseline characteristics, overall data accuracy and compliance data or issues, and other administrative data.

Following each meeting, a report separate from the minutes of the open and closed sessions will be sent to the principal investigator describing the DSMB recommendations and rationale for such.

5. Trial documentation and procedures to ensure confidentiality and proper communication

All data provided to the DSMB and all deliberations of the DSMB will be privileged and confidential. The DSMB will agree to use this information to accomplish the responsibilities of the DSMB and will not use it for other purposes without written consent from the principal investigator. No communication of the deliberations or recommendations of the DSMB, either written or oral, will occur except as required for the DSMB to fulfil its responsibilities. Individual DSMB members must not have direct communication regarding the study outside the DSMB except as needed to fulfil their responsibilities to the DSMB.

During the trial, none of the investigators will have knowledge of treatment allocation and event rates or complication rates according to treatment allocation. The DSMB will not be blinded to treatment allocation. Only the DSMB will have access to the deblinded data. The Trial team is responsible for identifying and circulating external evidence (e.g. from other trials, systematic reviews).

The DSMB will report recommendations in writing to the principal investigator. Reports to the DSMB will be sent at least 2 weeks before any meetings

Every person may write to the chair/ secretary of the DSMB to bring points of concern to the attention of the committee. Such points could refer to side effects, supplementary data from other studies, etc.

The secretary of the DSMB will store the papers safely after each meeting so they may check the next report against them, and retain these until discarded in accordance with applicable statutory regulation.

6. Decision making

The primary charge of the DSMB is to monitor the study for patient safety and provide recommendations to the trial team accordingly. Data from external evidence may also be used to provide a recommendation The DSMB will *not* monitor effectiveness outcomes to determine relative risk/benefit, futility, nor for early termination due to overwhelming effectiveness.

Critical decisions of the DSMB should be made by unanimous vote. However, if this is not possible, the chair's vote will decide.

Effort should be made for all members to attend. The secretary of the DSMB will try to ensure that a date is chosen to enable this. If, at short notice, any DSMB members cannot attend at all then the DSMB may still meet if at least one statistician and one clinician, including the chair will be present. If the DSMB is considering recommending major action after such a meeting the DSMB Chair should talk with the absent members as soon after the meeting as possible to check they agree. If they do not, a further teleconference should be arranged with the full DSMB.
If a member does not attend a meeting, it should be ensured that the member is available for the next meeting. If a member does not attend a second meeting, they will be asked if they wish to remain part of the DSMB. If a member does not attend a third meeting, they will be replaced.

Possible recommendations could include:

- No action needed, trial continues as planned
- The DSMB may give uncalled-for recommendations based for example on data from recently presented studies.
- The DSMB may give uncalled-for recommendations with regard to the (quality of the) execution of the trial.
- Early stopping due, to clear harm of a treatment

A stopping rule will not be provided as simulations have shown that there are no realistic scenarios in which inclusion in this study could be stopped prior to including all 90 subjects.

7. Reporting

Reports from the Trial team to the DSMB will include:

- Information on progress in data collection
- All safety data, including adverse events, SAEs and SUSARs
- The outcome of every randomization performed Data Management of the Julius Center (see protocol) will be sent to the secretary of the DSMB.

The DSMB will review the blinded data and discuss the analyses during the closed portion of the scheduled meeting. Should the committee find it necessary to unblind the study, this will be noted in the DSMB minutes and the available randomization data will be used to unblind the data where appropriate.

Minutes of the open session will be recorded by the secretary of the DSMB. Minutes will be finalized upon signature of the chairperson and maintained by the secretary of the DSMB in accordance with applicable statutory regulation.
Minutes from the closed session will be recorded separately from the minutes of the open session and stored securely by the secretary of the DSMB. Closed session minutes, finalized by signature of the chairperson, will be maintained in confidence and retained until discarded in accordance with applicable statutory regulation.

The DSMB will report its findings and recommendations by letter to the principal investigator within 2 weeks after the meeting. If these findings include serious and potentially consequential recommendations that require immediate action, the chairperson will also promptly contact the principal investigator by phone.
Requests for additional data by the DSMB members will be made to the DSMB chairperson or his or her designee, who will be responsible for communicating the request with the coordinating investigator.

The Trial team will review and respond to the DSMB recommendations. The recommendations of the DSMB will not be legally binding but require professional consideration by the recipients. If the DSMB recommends continuation of the study without modification, no formal response will be required. However, if the recommendations request action, such as a recommendation for termination of the study or modification of the protocol, the DSMB will request that the principal investigator provide a formal written response stating whether the recommendations will be followed and the plan for addressing the issues.

It is recognized that the principal investigator may need to consult with regulatory agencies or other consultants before finalizing the response to the DSMB. Upon receipt, the DSMB will consider the sponsor response and will attempt to resolve relevant issues, resulting in a final decision. Appropriate caution will be necessary during this process to avoid compromising study integrity or the ability of the principal investigator to manage the study, should the study continue. The sponsor will agree to disseminate the final decision to the appropriate regulatory agencies, IRB/EC, and investigators within an appropriate time.

If the DMSB has serious problems or concerns with the decision of the Trial team a meeting of these groups will be held. The information to be shown would depend upon the action proposed and the DSMBs concerns. Depending on the reason for the disagreement, confidential data may have to be revealed to all those attending such a meeting. The meeting will be chaired by the principal investigator an external expert who is not directly involved with the trial.

Public disclosure of the sponsor’s final decision or DSMB recommendations will be at the discretion of the sponsor or their designee. The DSMB will not make any public announcements either as a group or individually.

Appendix 3: Drug accountability calendar for participants (example)

Bumetanide kalender Deze kalender is van.....

Week 1

Dag	Datum	Dosis 1	Dosis 2	Bijzonderheden
1				
2				
3				
4				
5				
6				
7				

Appendix 4: minutes of meeting on the value of renal echography

Attendents: M. Lilien (pediatric nephrologist), M.Keijzer (pediatric nephrologist), H.Bruining (child psychiatrist), J.Sprengers (PhD student)

Ultrasound investigation of the kidneys in participant is conducted to evaluate kidney pathology induced by Bumetanide. The complication of concern is nephrocalcinosis, which results from calcinuria induced by diuretics. The incidence of nephrocalcinosis in children treated with diuretics is very low and results from chronic treatment. Considering the short treatment duration, the low incidence of nephrocalcinosis and the laboratory evaluation of calcinuria, ultrasound monitoring of renal pathology has no clinical relevance and increases burden for the participants. In addition, the phase IIb study has shown that none of the participants showed abnormalities on renal ultrasound. Anamnestic and laboratory evaluation of the participants suffices to screen for renal diseases as stated in the exclusion criteria. Echography has no added value in this regard. Taken together, the aforementioned arguments warrant the omission of renal ultrasound evaluation from the protocol.

Appendix 5: Minutes on ECG evaluation in the BAMBI study

Attending: Dr Strengers (pediatric cardiologist), Dr Krings (pediatric cardiologist), M.Korpershoek (resident pediatric cardiology) D. van Andel (Phd student psychiatry), J.Sprengers (PhD student psychiatry)

The meeting was scheduled on initiative of the department pediatric cardiology to discuss the value of ECG measurement in the BAMBI protocol, Two questions were discussed:

1. What is de value of ECG screening to evaluate the primary effects of bumetanide on the heart?
   The most common cardiac pathologies in children are congenital abnormalities. When clinically relevant these abnormalities are usually picked up before the age of seven. The most common abnormalities expected in the BAMBI participants are arrhythmias. These are not adequately detected by ECG. More relevant is careful cardiac history taking. When cardiac history is suspect for arrhythmias children should be referred to the pediatric cardiologist, who will perform ultrasound cardiac evaluation. ECG is not important to screen for cardiac disorders which exclude children from participation.

   To the knowledge of the cardiologists there is no cardiac interaction of bumetanide. Therefore ECG recording is not required.

   In addition, consultation with one of the residents hospital pharmacy (I.Favie 21-11-2016) revealed that screening on Pubmed or CredibleMeds revealed no record of Bumetanide increasing QTc risk.

   Taken together there is no evidence that bumetanide interacts directly with cardiac function. This warrants omission of ECG measurements to assess primary effects of bumetanide on the heart.

2. What is the value of ECG screening to evaluate the secondary effects of bumetanide on the heart?
   The main effect of bumetanide on the heart is mediated via electrolyte disturbances (most importantly hypokaliemia). Electrolyte disturbances are detected via laboratory screening as is secured via the study protocol. Following serious electrolyte disturbances an ECG recording is acquired. Nevertheless, treatment exists of correcting the electrolyte disturbances. Base level ECG recordings are not required to guide clinical evaluation following cardiac arrhythmias following electrolyte disturbances. Therefore, base level and post treatment ECG recording has no additional value in the current protocol and can be omitted.