CROSSING BORDERS IN NEURODEVELOPMENTAL DISORDERS

TOWARDS RATIONAL TREATMENTS AND STRATIFIED TRIAL DESIGNS

UMC Utrecht Brain Center

Dorinde Marije van Andel

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Towards rational treatments and stratified trial designs

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CROSSING BORDERS IN NEURODEVELOPMENTAL DISORDERS

Towards rational treatments and stratified trial designs

ONTWIKKELINGSSTOORNISSEN ONBEGRENSD BENADEREN

Naar gerichte behandeling en gestratificeerde klinische trials

(met een samenvatting in het Nederlands)

Proefschrift

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door

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geboren op 27 januari 1991
te Zeewolde

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CONTENTS

"A perfection of means, and confusion of aims, seems to be our main problem."
— Albert Einstein

CHAPTER 1

Introduction

INTRODUCTION

As a psychologist by training, I had never imagined spending my cherished weekends contemplating how the mammalian brain regulates intraneuronal chloride. Let alone write a dissertation about it. And yet here we are.

Over the last five years, I was privileged to study children who generously offered me a glimpse in their world and their everyday challenges. Their generosity allowed me to investigate the multifarious facets of dealing with the consequences of neurodevelopmental disorders (NDDs). And they are not alone. To date, approximately 1 in 6 children (~17%) are diagnosed with a developmental disability and most require interventions to address developmental and behavioral challenges[1]. For a clinical diagnosis (i.e., a diagnostic classification based on a predefined set of observable behaviors), most clinicians rely on the psychiatric handbook Diagnostic and Statistical Manual (DSM)[2] or the International Classification of Diseases (ICD)[3]. However, there is a growing appreciation that NDD classification share 'trans-diagnostic' mechanistic factors and manifest with considerable phenotypic overlap. Ongoing genetic and other neuroscientific studies have increased this awareness and the trend is now to move away from discrete entities towards considering NDDs as spectrum disorders, e.g., autism spectrum disorder (ASD). We are therefore entering exciting times for child psychiatry, as these advances will reshape our current approaches to diagnostic evaluation and intervention to help children with NDDs. However, significant challenges lie ahead in implementing science into clinical practice.

Neurodevelopmental disorders

The term neurodevelopmental disorders (NDDs) has been applied to a broad group of neurological and psychiatric conditions with onset in the developmental period[2]. NDDs typically manifest in childhood (before puberty) and can be lifelong conditions affecting personal, academic, social, or occupational functioning. The clinical course is different compared to many other psychiatric disorders since they show a steady course rather than relapsing or remitting patterns such as with psychosis or depression. NDDs are associated with a strong genetic component[4] but often have a multi-factorial etiology. The DSM-5 groups autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), intellectual disabilities, communication disorders, specific learning disorder, and motor disorder as NDDs[2]. Although the DSM-5 classifies these conditions as distinct entities, they show a considerable amount of overlap in clinical characteristics and different NDDs frequently co-occur. At the same time, they are highly heterogeneous in terms of treatment response and outcomes. NDD diagnoses can be accompanied by the specifier "associated with a known medical or genetic condition or environmental factor"[5, p. 3], expressing factors associated with etiology or clinical course. These may include genetic disorders (e.g., tuberous sclerosis complex [TSC]), medical conditions (e.g., epilepsy), and environmental factors (e.g., a very low birth weight). Epilepsy is also often regarded as NDD, since several complex developmental processes contribute to epileptogenesis, epilepsy is a common comorbidity in the other DSM-5 classified NDDs and they often share a genetic basis[6-8]. In this thesis, we also view epilepsy as NDD.

The currently held view on treatment: one-size-fits all and symptom-based

Historically, pharmacological interventions in NDDs, are applied on the basis of diagnostic ascertainment to mitigate the most problematic symptoms such as temper tantrums in ASD or inattention and impulsivity in attention-deficit/hyperactivity disorder (ADHD). However, this approach is increasingly criticized to ignore the established heterogeneity in clinical manifestations and underlying etiologies. Indeed astonishingly, despite this recognition of phenotypic overlap between conditions and heterogeneity within conditions, the field is *still* often searching for the 'silver bullet' effective treatment for all children classified with a specific disorder: the **'one-size-fits-all' approach**. In contrast, the genetic architecture of ASD indicates that the pathophysiology is complex, diverse and associated with more than hundreds of causal genetic alterations[9, 10]. Together with the heterogeneity in clinical manifestations of NDDs, it is imperative that we need to seek more individually tailored and mechanism-based treatments.

Indeed, advances in neuroscience have provided ideas about common mechanistic pathways in NDDs, yet most treatment developments still focus on **symptomatic instead of rational, mechanism-based treatments**, e.g., treatments targeting identified mechanistic pathways. At present, registered pharmacological treatment options are empirically derived and are used to suppress co-occurring symptoms (e.g., selective serotonin reuptake inhibitors for anxiety) while approved mechanism-based treatments are still lacking. The large number of monogenetic disorders associated with NDDs like tuberous sclerosis complex (TSC)[11] (see text box below) have proven a strong entry point to gain understanding of underlying biological mechanisms of both disorders. Clinical trials on cohorts with these single-gene disorders instead of diagnostically ascertained cohorts have shown promise to validate the discovery of novel pharmacological targets and may also benefit other patients with shared (dysfunctional) mechanistic pathways.

An example of a rational treatment target that has progressed from experimental research, is the development of pharmacological therapies involving mammalian-target-of-rapamycin (mTOR) inhibitors for TSC. Preclincial research had revealed the critical role of mTOR overactivation in the molecular pathophysiology of TSC. This finding opened the way for mTOR inhibition, for instance by administering compounds such as everolimus or sirolimus, as targeted treatment[12]. mTOR inhibitors may especially be recommended when specific features observed on MRI evaluations or the presence of intractable seizures are present, as an example of tailored

treatment with a genetic disorder[12, 13]. The use of an mTOR inhibitor in TSC is a good example of a treatment targeting etiological pathophysiology, contrasting traditional symptomatic treatment in TSC, for instance by administration of antiepileptic drugs (AEDs) for manifestations of seizures.

Fortunately, experimental studies and clinical trials have also suggested rational treatment approaches for other NDDs. This promise followed upon the prominent hypothesis that NDDs are based on aberrant neuronal network organization and activity, which has provided a framework for rational treatments development[14]. Along these lines, innovative rational treatments have emerged in the last decade some of which are based upon repurposing of existing pharmacological agents. These drugs may facilitate implementation as its mechanistic targets and side effects are often already established and they do not have to go through the drug discovery regulatory pipeline.

The excitation/inhibition (E/I) imbalance hypothesis and rationale for NDD treatments

Almost 20 years ago, Rubenstein and Merzenich where the first to postulate the E/I imbalance hypothesis[15]. Motivated in part by clinical observations of comorbid epilepsy and epileptiform abnormalities in ASD, they proposed that ASD and related disorders were characterized by an increased ratio of E/I in key neural circuits, driven by a combination of genetic and environmental factors. The authors hypothesized that a change in E/I balance was caused by a reduction in signal-to-noise ratio in neural circuitry, in turn leading to hyper-excitability of these circuits. This may be a reductionist theory of the concept as excitation and inhibition are highly multidimensional and complex entities, but it has been a worthwhile concept to further our understanding of NDDs. Since Rubenstein and Merzenich first postulated their theory, a growing body of preclinical and clinical data has supported and refined the E/I imbalance hypothesis.

E/I imbalance in NDDs

E/I imbalances have frequently been observed in animal models of ASD and the pharmacological correction of E/I imbalances resulted in normalization of autistic-like phenotypes (for a review see[16]). Indeed, over the last 15 years, other studies have shown that E/I is an important pathway implicated in the pathogenesis of ASD. Results from the recent and largest exome sequencing study of ASD to date identified 102 ASD risk genes[17]. The majority of these genes is expressed in cortical tissue and associated with either excitatory or inhibitory neuronal lineages[17]. These findings have been regarded as compelling support for the E/I imbalance hypothesis in ASD.

Evidence for E/I imbalance in NDDs other than ASD has also been found. For instance, seizures and epilepsy have traditionally been considered the result of elevated E/I[18-20], with increased excitation and/or decreased inhibition leading to uncontrolled excitation (for a more recent view on E/I imbalance in epilepsy see[21]). Studies have further shown alterations in E/I balance in monogenetic NDDs such as TSC[22-24], Fragile-X syndrome[25, 26], and Rett syndrome[27-29] – all characterized by high incidences of ASD diagnoses. In most of these, prominent deficits in inhibitory GABAergic signaling have been identified, suggesting that GABAergic signaling may serve as a common pathway to E/I imbalances and atypical brain function across NDDs.

Targeting E/I via chloride

A specific GABAergic mechanism leading to E/I imbalance in NDDs has been suggested in the form of altered chloride homeostasis. In normal development, a developmental sequence occurs around birth, which is characterized by a dramatic decrease in chloride concentration in neuronal cells[30]. This maturational downregulation of chloride levels causes a shift in the so-called polarity of GABAergic transmission from excitatory (depolarizing) to inhibitory (hyperpolarizing): also referred to as the GABA-shift[31]. Thus, although GABA is characterized as the main inhibitory transmitter in the brain, during development it initially depolarizes and excites immature neurons. Since GABAergic inhibition has an important role in maintaining E/I balance for proper neuronal growth, and synapse and circuit development, alterations in polarity may have wide-ranging consequences. Indeed, in ASD[32], epilepsy[33, 34], Rett syndrome[35] and Down syndrome[36], the GABA shift was found to be abolished and excitatory GABAergic signaling was established in neuronal circuits. The GABA shift is mediated predominantly by a change in the expression of two chloride co-transporters: the Na^+-K^+-$2Cl^-$ (NKCC1) importer and K^+-Cl^- (KCC2) exporter[37, 38]: developing and immature neurons have higher intracellular chloride concentrations compared to mature neurons (see Figure 1). This system provides a possible avenue to ameliorate symptoms by influencing GABAergic signaling via treatment aiming to restore chloride regulation. Indeed, this is already possible using existing, well-studied pharmacological agents.

Figure 1. The GABA developmental shift

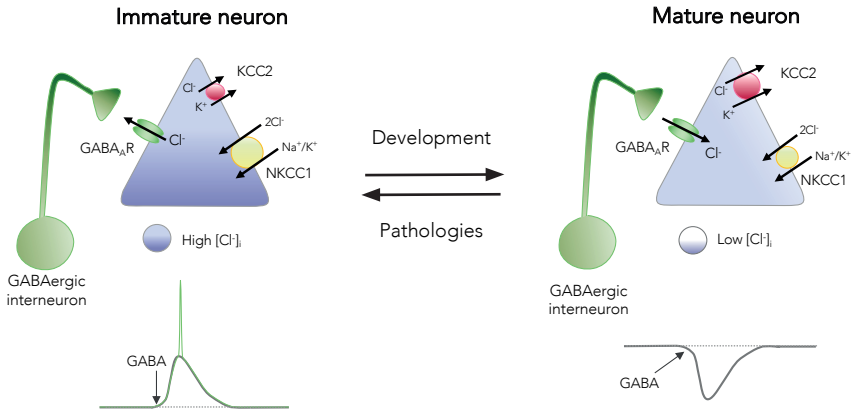

Figure adapted from Ben-Ari & Lemonnier (2021)[43] with permission from the authors

Bumetanide as a potential E/I restoring treatment

The selective NKCC1 antagonist bumetanide is the most studied chloride influencing pharmacological agent. Blocking NKCC1 chloride import can lower chloride concentrations and potentially reinstate GABAergic hyperpolarization (favoring inhibition) instead of depolarization (favoring excitation)[39, 40]. Bumetanide is a registered loop diuretic that has been used for almost 50 years in adults and children with a variety of edematous conditions[41]. Bumetanide has a mild side effect profile with diuretic effects such as electrolyte imbalance and hypokalemia that can be safely monitored when kidney function is normal[42].

Ben-Ari and Lemonnier pioneered the potential of bumetanide to ameliorate behavioral manifestations of NDDs. In a recent paper[43] they summarized the history of using bumetanide for ASD symptomatology, while emphasizing its unique potential. Their journey and hypotheses can be summarized in the following simplified steps:

1) Ben-Ari and Lemonnier suggest that ASD develops in utero and impacts brain development which leads to poorly formed or misconnected neurons and networks which therefore remain immature resulting in disturbance of the oscillations needed for behavior[44]; 2) Experimental data had shown immature neurons to have depolarizing and excitatory features due to the inhibitory neurotransmitter GABA, as a result of elevated intracellular chloride levels[38, 45]; 3) Novel experimental data showed that benzodiazepines had a paradoxical effect when GABA had excitatory

effects[46]; 4) Along these lines, Ben-Ari and Lemonnier hypothesized that when ASD is characterized by immature and/or misplaced neurons, a pharmacological agent with properties to restore low intracellular chloride levels could lead to symptomatic improvements.

In earlier studies we and others have shown that a (history of) paradoxical response to GABA–enforcing drugs, such as benzodiazepines, may be a prognostic marker of depolarizing GABA activity and may predict the efficacy of bumetanide treatment[47, 48]. Bumetanide has indeed been shown to reinstate GABAergic inhibition in several mouse models of ASD[30, 32]. More importantly, case reports, followed by clinical trials with bumetanide in children and adolescents showed reduced severity of ASD symptoms[49-52], more spontaneous eye contact and a normalization of amygdala activation in response to eye contact[53]. A recent bumetanide trial added an exploratory neuroimaging marker of E/I balance by measuring GABA and glutamate concentrations in the insular and visual cortex with magnetic resonance spectroscopy[52]. Along a reduction of ASD symptoms, they found decreased GABA/glutamate ratios in the insular cortex that were associated with clinical improvement.

Finally, in addition to behavioral effects, several lines of evidence suggest that in several disorders bumetanide also has an effect on cognitive information processing. For instance, after bumetanide treatment, individuals with ASD showed improvements on an emotional face processing task[54] and patients with drug-resistant epilepsy showed improved memory functioning[55]. Similarly, animal studies demonstrated improvements in (predominantly) memory functioning in a valproate induced rat model[56], and in Down syndrome[57] and Huntington's disease mouse models[58] after bumetanide treatment. Together, the results of these preclinical and clinical studies strongly imply that bumetanide has clinical potential for several NDDs.

Stratified approaches and appropriate outcome measures to improve new clinical trials

Unlike the rest of traditional medicine, treatment selection in current child psychiatry does not rely on (biological) tests to select treatment nor does consensus exist on how to establish efficacy. Although there is an ongoing debate on the use and validity of biological tests as screening and/or diagnostic tools in psychiatry, no one can argue against the use of more selective tests that predict potentially beneficial or adverse responses to specific pharmacological therapies. Such a **stratified approach** in psychiatry has been attracting more and more interest. There are various approaches and levels of analysis when it comes to stratification strategies in NDDs such as those

relying on genetics, neurophysiology, cognition and behavior. Defining subtypes within heterogenous NDDs can provide a basis for stratification which may ultimately improve clinical outcomes across conventional diagnostic boundaries. Moreover, targeted interventions for stratified subgroups with identified mechanistic alterations (which can be biological, cognitive, or otherwise) may be more likely to produce positive treatment outcomes.

The successful implementation of novel treatments also depends on the availability of relevant and valid outcome measures that are sensitive to detect change due to treatment. In this context, a patient or caregiver-oriented assessment in clinical trials ideally focusses on the complaints and symptoms of most concern to the patient or caregiver. However, in DSM-based clinical practice this is not always the case as concerns voiced by caregivers or patients are often not comprised by the defining diagnostic criteria. This also affects clinical trials: regulatory bodies mandate the use of standardized diagnostic measures as primary endpoints. Many outcomes that are currently used in clinical trials were initially developed as a screening tool or characterization measure and are not always psychometrically appropriate: they are often lengthy, not well suited to detect a change, or do not have good test-retest reliability. Another problem is that these scales generally focus on symptoms that do often not echo the complaints individual patients and caregivers struggle with in their daily lives, also referred to as limited ecological validity (i.e., the extent to which a trial endpoint measure extrapolates to (dys)functioning in everyday life).

The alternative would be to have methodology to rate individualized, participant-chosen target symptoms, which is sometimes performed as secondary, exploratory analysis[59]. In recent years, this has culminated in the increasing interest to use Patient-Reported Outcome (PRO) measures to assess health-related outcomes in intervention studies. However, a variety of different PROs are currently being used, leading to incomparable findings. Also, many PRO measures lack measurement precision and have a relatively high respondent burden[60]. Thus, more satisfactory alternatives are needed.

To sum up these present-day challenges: a diagnosis is often being equated to a disorder and treatment development uses a one-size-fits-all approach in which factors that determine an individual's outcome are being largely unknown or ignored. The following paragraphs and text boxes will further introduce concepts that are relevant to the research comprising this dissertation.

Concepts and definitions

Autism spectrum disorder (ASD)

- ASD is characterized by the presence of persistent problems in two domains: social communication and restricted, repetitive, or unusual (sensory–motor) behaviors[2]
- The estimated prevalence of ASD in developed countries is ~1.5%, with males being up to 4 times more affected than females[61]
- People with ASD have varying phenotypes and ASD is seen as a spectrum ranging from very mild to severe
- To date, there are no reliable biomarkers to diagnose ASD, hence a classification is merely based on behavior
- Although the neural mechanisms underlying manifestations in ASD remain largely unknown, twin and family studies have shown a strong genetic component with heritability estimates ranging from 50-95%[62, 63]
- Up to 70% of individuals with ASD meet criteria for at least one other psychiatric condition, with anxiety disorders and ADHD being the most prevalent co-occurring disorders[64]
- Tantrums, mood lability, self-injury and aggressive behaviors towards others are also frequently occurring symptoms and more common in ASD compared to other developmental disorders[65-67]. Together, these behaviors are often referred to as irritability. Irritability negatively impacts quality of life, family and community interactions and hampers receptivity to therapy[66, 68]

Current pharmacological treatment options for ASD

- Despite the multitude of intervention trials that have been conducted in ASD, pharmacological interventions for core symptoms are currently lacking and treatment options are limited to co-occurring symptoms and diagnoses
- Risperidone and aripiprazole show moderate to large effect sizes in trials targeting ASD-related irritability[66]. Risperidone and aripiprazole (atypical antipsychotics) are the only approved drugs to treat ASD-related irritability
- Although effective for irritability and agitation in children and adolescents with ASD[69, 70], atypical antipsychotics have serious side effects, including sedation, weight gain, extrapyramidal effects and elevated risk of diabetes[71, 72]
- Other pharmacological treatments that are frequently used to manage co-occurring symptoms are: sleep medication for sleep disturbances, psychostimulants for ADHD symptoms and antidepressants for anxiety[73-75]

Tuberous sclerosis complex (TSC)

- TSC is a rare genetic disorder caused by loss-of-function mutations in either of the *TSC1* (encoding hamartin) or *TSC2* (encoding tuberin) genes[76, 77]
- Patients with the TSC phenotype and no identifiable mutation in these genes also exist (10–15% of TSC patients), although the majority of these may be identified with mosaic and intronic mutations in *TSC1* or *TSC2* genes after full gene coverage[78]
- The TSC protein complex (TSC1 and TSC2) regulates the mammalian-target-of-rapamycin (mTOR) pathway, involved in protein synthesis, and therefore functions as a tumor suppressor[79, 80]
- TSC affects multiple organ systems with hallmark features of benign tumors in the brain, heart, lungs, skin, eyes and kidneys. The development of cardiac rhabdomyomas and cortical tubers are main characteristics of TSC and occur during embryogenesis[81, 82]
- The brain is generally the most severely affected system resulting in epilepsy (in 80-90%[83, 84]), cognitive disability and broad neuropsychiatric manifestations which have been denoted as TSC-associated neuropsychiatric disorders (TAND)[85]
- TAND comprise a range of developmental, behavioral and affective symptoms manifesting in ~90% of TSC patients[86, 87]. The most commonly associated NDD is ASD, with an estimated prevalence of 36% in TSC[11]
- The clinical impact of TAND is increasingly recognized by clinicians and researchers, but still less than ~20% receives treatment for TAND symptoms[86, 87], emphasizing the need for better treatment development[88]

Current treatment options for TSC

- Although the mTOR inhibitor everolimus seems promising for treating some manifestations of TSC[89] and may reduce seizure frequency in young children[90], no effect on autistic symptoms and cognitive functioning in TSC patients have been found[91]
- Trials showed that while everolimus treatment can be considered safe, it also has systemic side effects[89, 90]
- There have been studies showing rapamycin-therapy-sensitive periods for ASD-like behavior in a $Tsc1^{-/-}$ mouse model of TSC and ASD[92, 93]. This supports the hypothesis that the timing of therapeutic interventions is crucial in ameliorating TSC-associated sequelae and TAND[93]

Epilepsy

- Epilepsy is a chronic neurological disorder characterized by a persisting predisposition to generate epileptic seizures[94]
- Epileptic seizures are the result of abnormal excessive or synchronous neuronal activity in the brain
- The estimated world-wide prevalence of epilepsy is 0.64% (i.e., 6.38 per 1000 persons)[95] and is slightly higher in males than in females (although this ratio may vary for individual seizure types)[96, 97]
- Approximately 70-80% of epilepsy cases are thought to result from one or more genetic factors and ~20-30% is caused by acquired conditions (e.g., stroke, head injury or tumor)[98]
- Epilepsy and ASD commonly co-occur and several biological pathways have been identified likely perturbated in both disease processes. For instance, genetic and chromosomal abnormalities[99] and metabolic conditions[100, 101] have been indicated as factors that predispose to both epilepsy and ASD
- There are numerous antiepileptic drugs available (e.g., valproate, carbamazepine, oxcarbazepine, vigabatrin) which achieve seizure freedom in around two-thirds of patients, with treatment of choice depending on the type of seizures or syndrome[102, 103]

Attention-deficit hyperactivity disorder (ADHD)

- ADHD is defined in the DSM-5 by developmentally inappropriate inattention, impulsivity and hyperactivity[5]
- The estimated world-wide prevalence of ADHD is 7.2%[104] with males being 3 times more affected than females in community samples and 5 to 9 times more affected in clinical samples[105, 106]
- Pharmacological treatments with stimulants have proven efficacious in the short-term[107] and are widely used[108]
- There are limitations of treatment with stimulants as the long term-effectiveness remains to be proven[109] and adverse events on growth, sleep and appetite are common[110, 111]
- Genetic factors play a substantial role in the etiology of ADHD as family, twin and adoption studies have shown heritability estimates of 60 to 90%[112, 113]
- Environmental risk factors include extreme early adversity, pre and postnatal exposure to lead and low birth weight[114]
- Neuropsychological deficits in the domains of executive functioning and (spatial) working memory are often present at group level[115, 116]

Scope of this thesis

The research comprising this thesis is focused on a rational treatment candidate studied in children with different NDDs, to acknowledge the extensive heterogeneity of etiologies and clinical manifestations of NDDs and to accommodate different aspects of stratification strategies. The overarching hypothesis of the thesis is that increased phenotypic and/or etiological homogeneity translates to reduced variability in treatment response. To address the before mentioned present-day challenges in child psychiatry (i.e., the one-size-fits-all approach and focus on symptom-based treatments) and to come to effective and tailored treatments for NDDs, we carried out three clinical trials and a qualitative study by (i) adopting different stratification strategies and (ii) evaluating bumetanide, a mechanism-based treatment option and finally (iii) identifying patient/parent-relevant treatment outcomes. Text box 1 provides a brief background and introduction to these studies.

Text box 1. Brief background to the studies comprising this thesis

Chapters

2

TSC open-label trial (BATSCH)

TSC patients aged 8-21 years were included in this open-label pilot study (n=15). Use of concomitant antiepileptic and psychoactive drugs was allowed. Event-related potential (ERP) and resting state measures were collected with electroencephalography (EEG), cognitive tasks (if obtainable) and questionnaire data on behavior. The primary outcome was difference in irritable behavior after 91 days bumetanide treatment.

3 & 4

ASD randomized controlled trial (BAMBI)

Children aged 7-15 years with ASD and IQ≥55 were included in this randomized controlled trial (RCT) (n=92). ERP and resting state measures were collected with EEG, cognitive tasks and questionnaire data on behavior. The primary outcome was difference in social behavior after 91 days bumetanide treatment.

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Cross-NDD randomized controlled trial (BASCET)

Children with a neurodevelopmental disorder (ASD, ADHD and/or epilepsy) stratified on sensory reactivity problems aged 5-15 years and IQ≥55 were included in this RCT (n=38). Use of concomitant antiepileptic and psychoactive drugs was allowed, except stimulants. The primary outcome was difference in irritable behavior after 91 days of bumetanide treatment.

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Sensory reactivity PROM-study

In this qualitative study we identified relevant parent reported outcomes for sensory reactivity problems in children with ASD. Interviews with clinicians and focus groups and interviews with caregivers of children with ASD were used in this initial phase for concept elicitation. We subsequently developed a Sensory-Reactivity PROM set by comparing these outcomes to PROMIS item-banks and other validated PROMs. The aim was to develop a relevant, less time-consuming and user-friendly set that can be readily implemented in clinical trials targeting sensory reactivity.

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"An approximate answer to the right question is worth a great deal more than a precise answer to the wrong question."
— John Tukey

CHAPTER 2

Effects of Bumetanide on Neurodevelopmental Impairments in Patients with Tuberous Sclerosis Complex: an Open-Label Pilot Study

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ABSTRACT

Background Tuberous sclerosis complex (TSC) is an autosomal dominant disease that affects multiple organs including the brain. TSC is strongly associated with broad neurodevelopmental disorders, including autism spectrum disorder symptomatology. Preclinical TSC studies have indicated altered neuronal chloride homeostasis affecting the polarity of γ-aminobutyric acid (GABA)ergic transmission as a potential treatment target. Bumetanide, a selective NKCC1 chloride importer antagonist, may attenuate depolarizing GABA action, and in that way reduce disease burden. In this open-label pilot study we tested the effect of bumetanide on a variety of neurophysiological, cognitive and behavioral measures in children with TSC.

Methods Participants were treated with bumetanide (2dd0.5-1.0mg) for 13 weeks in an open-label trial. The Aberrant Behavior Checklist-Irritability (ABC-I) subscale was chosen as the primary endpoint. Secondary endpoints included other behavioral questionnaires in addition to event related potentials (ERP) and neuropsychological tests if tolerated. Additionally, treatment effect on seizure frequency and quality of life was assessed. Endpoint data were collected at baseline, after 91 days treatment and after a 28-day wash-out period.

Results Fifteen patients (8-21 years old) with TSC were included of which 13 patients completed the study. Treatment was well-tolerated with only expected adverse events due to the diuretic effects of bumetanide. Irritable behavior (ABC-I) showed significant improvement after treatment in 11 out of 13 patients ($t(12)=4.41$, $p=.001$, $d=.773$). A favorable effect was also found for social behavior (Social Responsiveness Scale) ($t(11)=4.01$, $p=.002$, $d=.549$) and hyperactive behavior (ABC-hyperactivity subscale) ($t(12)=3.65$, $p=.003$, $d=.686$). Moreover, patients rated their own health related quality of life higher after treatment. At baseline, TSC patients showed several atypical ERPs versus typically developing peers of which prepulse inhibition was significantly decreased in the TSC group. Neuropsychological measurements showed no change and bumetanide had no effect on seizure frequency.

Limitations The sample size and open-label design of this pilot study warrants caution when interpreting outcome measures.

Conclusions Bumetanide treatment is a potential treatment to alleviate the behavioral burden and quality of life associated with TSC. More elaborate trials are needed to determine the application and effect size of bumetanide for the TSC population.

Trial registration EU Clinical Trial Register, EudraCT 2016-002408-13. Registered 25 July 2016.

Keywords Tuberous Sclerosis Complex, bumetanide, open-label, NKCC1 antagonist, TAND, irritability, ERP, neurocognitive task

BACKGROUND

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder generally caused by inactivating mutations in *TSC1* (encoding hamartin) or *TSC2* (encoding tuberin) genes[1]. The *TSC1-TSC2* protein complex is required for suppression of mammalian-target-of-rapamycin (mTOR) activity and therefore referred to as a tumor suppressor. Almost every organ can be affected in patients with TSC with hallmark features of benign tumors in vital organs including the brain[1]. As a consequence of brain involvement, TSC is strongly associated with a broad range of neurodevelopmental and psychiatric symptoms. Epilepsy is estimated to occur in 72-85% of patients[2], of which the majority responds insufficiently to antiepileptic drugs (AEDs)[3]. The broad neuropsychiatric manifestations have been denoted as *TSC associated neuropsychiatric disorders* (TAND)[4] and comprise a range of developmental, behavioral and affective symptoms affecting approximately 90% of TSC patients[2, 5]. The most commonly associated neurodevelopmental disorder is autism spectrum disorder (ASD), diagnosed in 40-50% of TSC patients[5]. The clinical impact of TAND is increasingly addressed by clinicians and researchers, but still less than 20% is estimated to receive treatment for these specific symptoms[2, 5]. A large extent of TAND seems to remain unrecognized further emphasizing the need for better triage and treatment[6].

In search for treatments correcting or decreasing the neurological consequences of TSC, animal models and human remnant tissue samples from surgeries have progressed our understanding of its pathophysiology. Correction of the mTOR pathway has been a dominant theme in TSC research. Indeed, over-activation of the mTOR signaling pathway is a direct result of loss of *TSC1-TSC2* function in TSC. mTOR inhibitors like rapamycin analogues may therefore modify the TSC phenotype and several studies are currently investigating the benefit of mTOR inhibitors to treat TAND symptoms[7-9]. Yet, a recently published randomized controlled trial with mTOR inhibitor everolimus showed no effect on autistic symptoms and cognitive functioning in 4-17 year old TSC patients[10].

Another more recently proposed treatment target in TSC is chloride homeostasis. Several studies have implicated altered regulation of neuronal chloride levels in and around tubers through analysis of chloride transporter activity. More specifically, altered activity ratios between the chloride importer Na(+)-K(+)-2Cl(-) cotransporter (NKCC1) and chloride exporter Na-Cl cotransporter (KCC2) have been found[11] that may affect g-aminobutyric acid (GABA) polarity and cause unwanted depolarizing effects of GABAergic transmission[12]. For instance, Talos and colleagues[11] showed

that cortical tubers in human TSC specimens (*n*=14), collected after surgery or post-mortem, demonstrated a decreased expression of $GABA_A$α1 receptor, increased NKCC1, and decreased KCC2 levels compared to nontuberal TSC tissue and tissue from controls (*n*=10). They additionally recorded $GABA_A$R responses in cortical tissue from a single TSC patient and an epilepsy case control. The neurons from the cortical tuber slices appeared to be characterized by depolarizing $GABA_A$R-mediated responses, in contrast to hyperpolarizing $GABA_A$R-mediated currents in neurons from the non-TSC epilepsy case control. Ruffolo et al[13] investigated GABAergic transmission in TSC by injecting *Xenopus* oocytes with membranes from TSC cortical tubers (*n*=7) and control tissues (*n*=9) at different pre- and postnatal ages. They reported that hyperpolarized $GABA_A$ reversal potential was abolished in TSC tuber tissue and this was accompanied by an elevated NKCC1/KCC2 ratio in RNA expression.

These findings of altered GABAergic transmission and chloride transporter activity may constitute a treatment target to decrease disease burden in TSC[11]. Bumetanide is a selective NKCC1 antagonist and has been used as a diuretic drug for decades. In addition, bumetanide regulates neuronal chloride ion concentration by inhibiting the Na(+)-K(+)-2Cl(-) cotransporter (NKCC) and may therefore have therapeutic potential by reinstating hyperpolarizing GABA-activated currents[14]. Favorable clinical effects of correction of chloride homeostasis and GABAergic transmission through bumetanide has also been indicated in other experimental models of epilepsy and neurodevelopmental disorders, most notably ASD[15, 16]. In childhood epilepsy, including some specific genetic forms of epilepsy, bumetanide has been suggested to reduce seizure frequency[17, 18].

Following the available preclinical and clinical evidence for efficacy of bumetanide in TSC and other neurodevelopmental disorders, we hypothesized that bumetanide may alleviate TAND manifestations in TSC. We conducted an open-label study to explore the effects of bumetanide on behavior, cognition and event related potentials (ERPs) in a sample of children and adolescents with TSC.

METHODS

The medical ethical committee (METC) of the UMC Utrecht approved the trial protocol and the study is conducted according to the principles of the Declaration of Helsinki, version of Fortaleza, 2013, the International Conference on Harmonisation – Good Clinical Practice (ICH-GCP) and in accordance with the Medical Research Involving Human Subjects Act (WMO). Written informed consent was obtained from all parents and participants.

Participants

Male and female TSC patients were recruited via the database of the TSC center of excellence of the department of Pediatric Neurology of the UMC Utrecht and via online advertisement on the website of the Dutch TSC patient organization (https://stsn.nl/). Potential participants were screened for eligibility by a child psychiatrist (HB) and child neurologist (FJ). Children with a definite TSC diagnosis based on genetic or clinical diagnostic criteria (as established by the 2012 International Tuberous Sclerosis Complex Consensus Conference[19]), between 8 and 21 years old, and >30 kg were eligible as participants. Patients with intellectual disability (ID [TIQ≤70]) and without ID (IQ>70) were both included, to study a representative sample. Use of concomitant antiepileptic and psychoactive drugs was allowed, when being taken on a stable regime at least 8 weeks prior to baseline. Exclusion criteria were renal and liver insufficiencies, serious unstable illnesses (including gastroenterological, respiratory, cardiovascular, endocrinologic, immunologic, hematologic disease, dehydration or hypotension, electrolyte disturbances), treatment with NSAIDS, aminoglycosides, digitals, antihypertensive agents, indomethacin, probenecid, acetazolamide, lithium, other diuretics, stimulants (like methylphenidate and dexamphetamine) and drugs known to have a nephrotoxic potential.

To compare ERP measures of our sample at baseline with typically developing (TD) children, data from a TD control group collected at the department of psychiatry at the UMC Utrecht between 2015 and 2018 consisting of 39 children (49% male) aged 7-15 years old (M=12.9; SD=3.8) with no history of medical or developmental or learning problems (TIQ M=118.2; SD=14.6) was used. This control group was tested in the same EEG lab using identical ERP paradigms and conditions.

Design

All participants and their parent(s) visited the outpatient Psychiatry department of the UMC Utrecht for a screening visit and baseline measurements between March 2017 and April 2018. This visit included a detailed interview of medical and family history (to complete the data already collected in the database and medical records), a physical examination, blood and urine analysis and if obtainable an IQ-estimation assessment. The total study assessment period consisted of 8 to 11 study visits: visit 1 (week-4, screening and baseline assessment), visit 2-3 (neuropsychological testing and EEG on separate days), visit 4 (day 4), visit 5 (day 7), visit 6 (day 14), visit 7 (day 28), visit 8 (day 56), visit 9-10 (day 91, end of treatment and neuropsychological measurements and EEG) and visit 11 (day 119, end of wash-out and EEG).

Treatment

Patients were treated with bumetanide CF 1.0 mg tablets (RVG 23140) for 91 days, as add-on treatment. They received 0.5 mg (i.e., half a tablet) bumetanide twice daily (breakfast and afternoon) as starting dosage, which was increased to 1.0 mg twice daily if blood electrolytes were normal at visit day 7. This dose was selected as this presented the most favorable benefit/risk ratio in the phase IIB bumetanide RCT (n=88) for children with neurodevelopmental disorders (i.e., ASD)[20]. Due to expected hypokalemia, all participants received oral potassium-chloride supplements. To evaluate the tolerability and safety of bumetanide in TSC, blood analysis, physical examination of vital signs, epilepsy diary assessment and report of adverse events were carried out at day 4, 7, 14, 28 and 56. Blood analysis included sodium, potassium, chloride, uric acid, urea, creatinine, glucose, estimated glomerular filtration rate, hematocrit, hemoglobin, erythrocytes, leukocytes, thrombocytes and total protein. At day 91 and 119 only physical examination was carried out.

Endpoint measurements

Behavioral and quality of life (QOL) questionnaires

Clinical endpoint questionnaires included the Aberrant Behavior Checklist (ABC)[21], Social Responsiveness Scale-2 (SRS)[22], Sensory Profile-2 (SP-NL)[23], Sensory Profile School Companion (SP-SC)[24], Repetitive Behavior Scale-Revised (RBS-R)[25] and Behavior Rating Inventory of Executive Function (BRIEF; versions parent and teacher reported)[26]. In addition to these validated questionnaires, the TAND-checklist[5] was administered during an interview for additional evaluation of the broad TSC related psychiatric manifestations. Although the checklist was originally developed as screening tool to describe and evaluate the multidimensional TAND symptoms, we quantified the prevalence of TAND symptoms on the basis of parental reported incidence. Parent's QOL was assessed by the World Health Organization QOL (WHOQOL – BREF)[27] and EQ-5D-5L[28] whereas the EQ-5D-Youth[29] and Pediatric Quality of Life Inventory (PedsQL)[30] focused on health-related QOL of the patients. In order to provide meaningful interpretation of the data, we compared raw scores instead of normative data (i.e., not corrected for calendar age).

ERP measurements

Neurophysiological effects of treatment were measured using electroencephalography (EEG) to assess automatic responses to auditory stimuli (paradigms have previously been described as part of the Copenhagen Psychophysiological Test Battery[31-33]. A prepulse inhibition of the startle reflex paradigm (PPI) to measure sensorimotor

gating, a P50 suppression task to measure sensory gating, and a mismatch negativity (MMN) oddball paradigm was used to evaluate automatic auditory discrimination. All measurements took place in the morning and participants were asked to refrain from consuming caffeinated beverages and foods, albeit being allowed to take their regular medication. Participants were seated and requested to sit still while auditory stimuli were presented through tubal insert earphones (EARtone®, Etymotic Research) by a computer running Presentation® software (Neurobehavioral System Inc.). For details about the ERP paradigms, acquisition and (pre)processing see Additional File 1. Not all participants were capable of understanding the instructions and cooperating in the ERP assessment; a total of 9/13 patients underwent ERP measurements.

Neuropsychological measurements

Attentional, memory and flexibility skills were tested, as these areas were considered of particular concern according to the consensus clinical guidelines for the assessment of cognitive and behavioral problems in TSC[34]. The battery included a baseline reaction time task, a Go No-Go task, different memory tasks and an auditory and visual set shifting task (Amsterdam Neuropsychological Test Battery[35]). A total of 7/13 patients completed all neuropsychological measurements.

Statistical analyses

Statistical analyses were performed using IBM SPSS Version 25.0. Descriptive analyses, Kolmogorov-Smirnov tests and histograms were used to identify the distribution of outcome responses on behavioral questionnaire data. When data were normally distributed, paired-samples Student's *t*-tests were used to compare results before and after treatment (i.e., D0 versus D91); otherwise Wilcoxon signed-rank tests were used. Comparisons between D0 and D119 and D91 and D119 were made to explore possible alterations after wash-out. Wilcoxon singed-rank tests were used to compare the patients' EEG-data between these time points and Mann Whitney tests (exact statistic) to compare TSC patients with the TD control group at baseline. Tests were two-sized and *p*-values <.05 were considered significant for main analyses. Bonferroni corrections $(1-(1-\alpha)^{1/n})$ were applied when multiple comparisons across secondary behavioral subscales or ERP parameters were performed. Effect sizes for significant effects were calculated using Cohen's *d*[36] with the following formulas respectively for within-subject (treatment effect) and between-subject effects (group difference ERP measures): $d = \frac{|m1 - m2|}{\sqrt{s_1^2 + s_2^2 - (2rs_1s_2)}}$ and $d = \frac{|m1 - m2|}{\sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_{1+}n_{2-}2}}}$.

Due to the limited sample size, exploratory analyses were used for neuropsychological data.

RESULTS

Cohort characteristics

Fifty-two potential participants were screened for eligibility by telephone and 17 patients who met inclusion criteria consented and attended the baseline visit (Figure 1). After this visit, 15 patients enrolled in the study and started treatment. One patient was lost to follow-up (after completing 91 days treatment) and one patient dropped out at day 50 due to aggressive behavior. Thus, a total of 13 patients completed the study, see Table 1 for characteristics. Nine were able to comply with EEG-assessment and in 7 cognitive functioning could reliably be assessed.

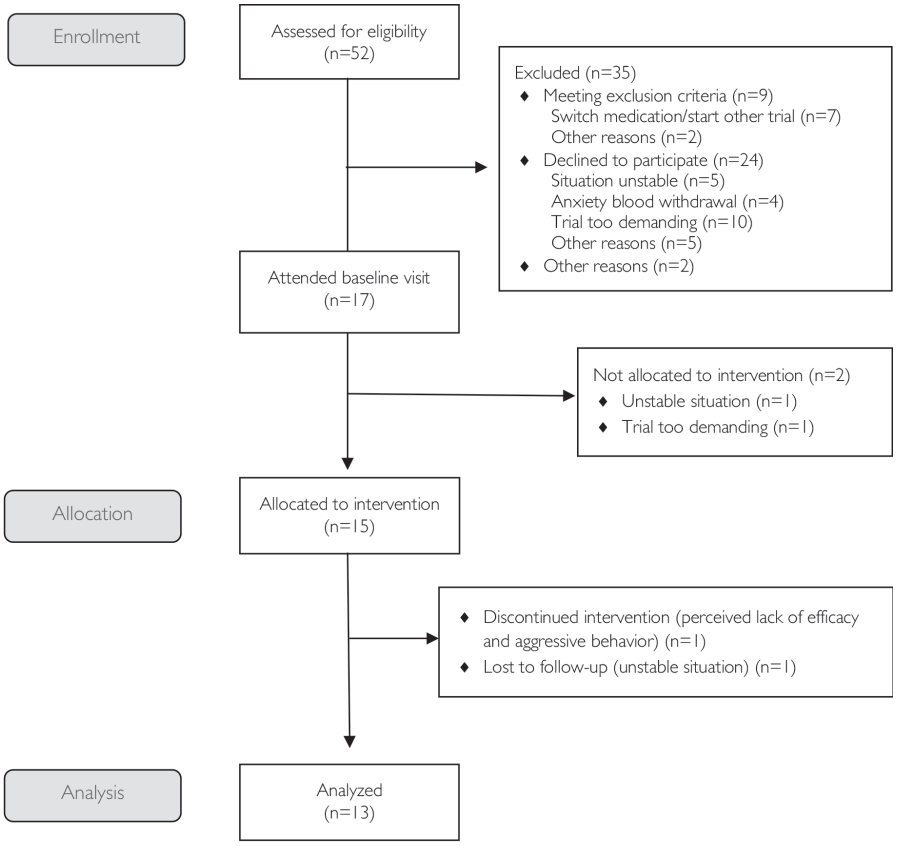


Figure 1. Flow diagram of BATSCH-study

Table 1. Cohort characteristics

Patient	Age	Sex	TIQ	Gene	Mutation	Epilepsy	Seizure control[1]	Medication	Psychiatric diagnoses[2]
1	8.3	M	82	TSC-2	De novo	Focal (M)	Yes	VPA, OXC	-
2	9.4	F	<40	TSC-2	De novo	Focal (N-M)	No	VPA, LTG, VGB	ASD
3	9.5	M	107	TSC-1	Familial	No	NA	NA	ASD
4	10.1	M	<40	TSC-2	De novo	Focal (M)	No	VPA, FBM	ASD
5	10.7	M	107	NMI	NA	Focal (M)	Yes	OXC	-
6	11.2	M	47	TSC-2	Familial	Focal (M)	Yes	VPA	ASD
7	11.8	F	80	TSC-1	Familial	No	NA	NA	ASD
	12.6	M	49	NMI	NA	Focal (M)	Yes	LEV, RAM	-
8	13.7	F	88	TSC-1	Familial	CR	NA	NA	-
9	13.8	F	51	NT	NA	Focal (M)	No	OXC	ADD, MD
	14.0	F	63	TSC-2	De novo	Focal (M)	Yes	OXC	Selective mutism
10	14.4	M	<40	TSC-2	De novo	Focal (N-M)	No	VPA	ASD
11	16.3	F	70	TSC-2	De novo	No	NA	ESC	ASD, MD
12	17.6	F	88	TSC-2	Familial	No	NA	ESC	Anxiety, MD
13	21.3	M	49	TSC-2	De novo	Focal (M)	No	LEV	-

Abbreviations: ASD: autism spectrum disorder; F: female; M: male; TIQ: total intelligence quotient; NA: not applicable; NT: not tested; NMI: no mutation identified; CR: complete remission; ESC: escitalopram; OXC: oxcarbazepine; VPA: valproic acid; FBM: felbamate; LEV: levetiracetam; LTG: lamotrigine; VGB: vigabatrin; RAM: Ramipril; N-M: non-motor; MD: mood disorder. Note: The shaded rows include 2 patients that did not complete the study and were not included in analyses.[1]Seizure-free for >1 year; [2]Expert clinical diagnoses

Behavioral questionnaires

Following the 3-month bumetanide treatment, we found a significant reduction of clinical and behavioral symptoms as measured with several endpoint questionnaires. A significant effect was obtained for the primary endpoint, the Aberrant Behavior Checklist Irritability subscale (ABC-I) score, indicating a reduction of irritable behavior after treatment (D0: M=14.2, SD=7.6 versus D91: M=8.3, SD=7.5; $t(12)=4.41$, $p=.001$, $d=.773$). This effect persisted after wash-out (D0: M=14.2, SD=7.6 versus D119: M=8.8, SD=6.2; $t(12)=3.81$, $p=.002$, $d=.776$) with no change observed between D91 and D119 (D91 versus D119; $t(12)=-3.93$, $p=.701$). Improvement in hyperactive behavior was observed through reduction of the Hyperactivity subscale of the ABC (ABC-H) (D0: M=11.6 SD=7.1 versus D91: M=7.2, SD=5.6; $t(12)=3.65$, $p=.003$ [adjusted significance level $p<.0125$], $d=.686$). No improvement was noted on the other ABC-subscales (i.e., Lethargy, Stereotyped Behavior and Inappropriate Speech: $p>.137$; $d<.333$).

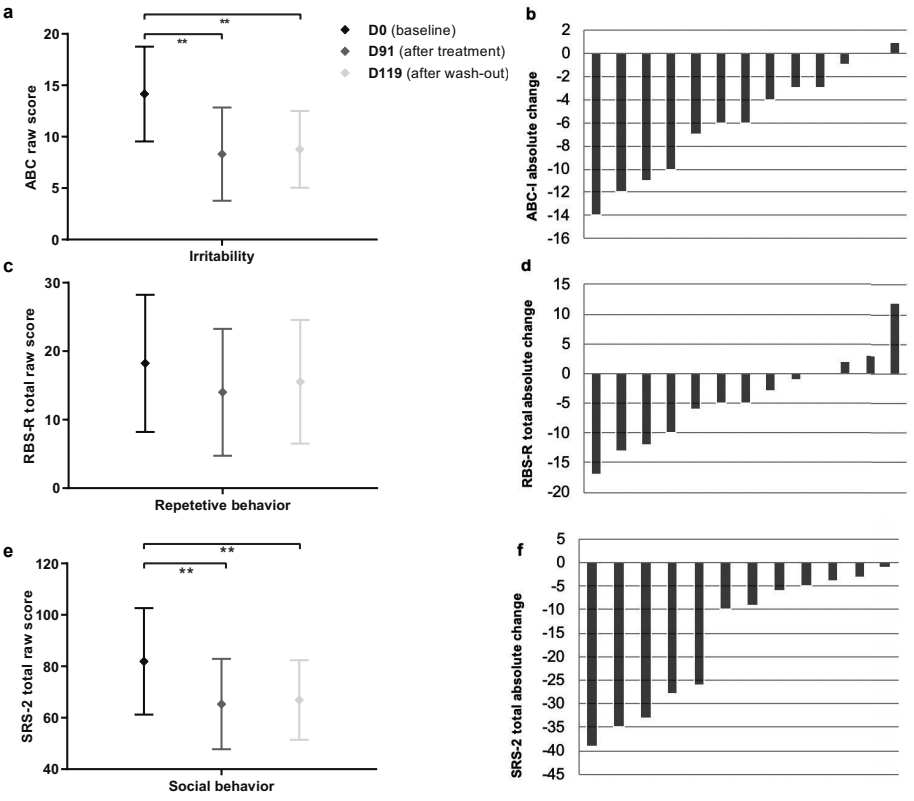


Figure 2. Treatment effect measured by behavioral questionnaires. Left panes show change after treatment and wash-out for a) ABC Irritability subscale; c) RBS-R total score; and e) SRS-2 total score. Right panes show absolute change per patient after treatment for b) ABC Irritability subscale; d) RBS-R total score; and f) SRS-2 total score. **: significance level p<.01

The RBS-R total score indicated no change in repetitive behavior (Z=-1.885; p=.059). Although an improvement in compulsive behavior was indicated on the RBS-R subscale "Compulsive Behavior", this did not survive multiple correction (Z=-2.448; p=.014 [adjusted significance level p<.0083]). Social behavioral improvements through treatment were observed on total scores of the SRS-2 (D0: M=81.9, SD=32.6 versus D91: M=65.3, SD=27.6; t(11)=4.01, p=.002, d=.549). This improvement persisted after wash-out (D0: M=81.9, SD=32.6 versus D119: M=66.9, SD=24.3; t(11)=3.27, p=.007, d=.522); and showed no difference between D91 and D119 (t(11)=-.54, p=.598). Furthermore, improvement was observed on the SRS-2 subscale "Social Communication" (D0: M=24.8, SD=12.9 versus D91: M=18.7, SD=12.0;

t(11)=4.65, *p*<.001 [adjusted significance level *p*<.01], *d*=.481), but did not survive multiple correction on the following subscales: "Social Cognition" (D0: M=17.7, SD=6.3 versus D91: M=15.2, SD=5.3; *t*(11)=2.28, *p*=.044 [adjusted significance level *p*<.01]), "Social Awareness" (Z=2.044; *p*=.041 [adjusted significance level *p*<.01]) and "Autistic Preoccupations" (Z=2.587; *p*=.01 [adjusted significance level *p*<.01]). Figure 2 provides an overview of treatment effect measured by the ABC, SRS-2 and RBS-R questionnaires.

To assess effects on sensory processing difficulties, quadrant and section (A-D) scores of the Sensory Profile-2 (SP-NL) were analyzed, showing no change after treatment (*p>.013* [adjusted significance level *p*<.0125]). Finally, the parent reported Behavior Rating Inventory of Executive Function (BRIEF) showed improvement after treatment (D0: M=142.8, SD=20.2 versus D91: M=130.1, SD=18.9; *t*(9)=3.125, *p*=.012, *d*=.649), although the questionnaire could be analyzed in only 10 patients as many items were rated as "not applicable". The SP School Companion and teacher reported BRIEF could not be analyzed due to many missing observations.

In addition to the validated questionnaires, we also applied the TAND-checklist before and after treatment to expand the exploration of possible effects on other psychiatric manifestations associated with TSC. Figure 3 shows the percentages of reported symptoms at baseline, after treatment and wash-out. At baseline, the most reported symptoms in the behavioral dimension of TAND were difficulty paying attention or concentrating (76,9%), temper tantrums (69,2%), poor eye contact (69,2%), difficulties getting on with other people of similar age (69,2%), anxiety, very rigid or inflexible about how to do things or not liking change in routines (69,2%), impulsivity (69,2%) and sleep difficulties (69,2%).

After treatment substantially less temper tantrums (-38,4) and aggressive outbursts (-38,4) were reported. Repetitive behavior did not improve. On the cognitive dimension (section 7), parents reported most problems to be apparent in attention (84,6%) executive skills (76,9%) and multi-tasking (76,9%) (see Figure 4) but no improvements were found after treatment.

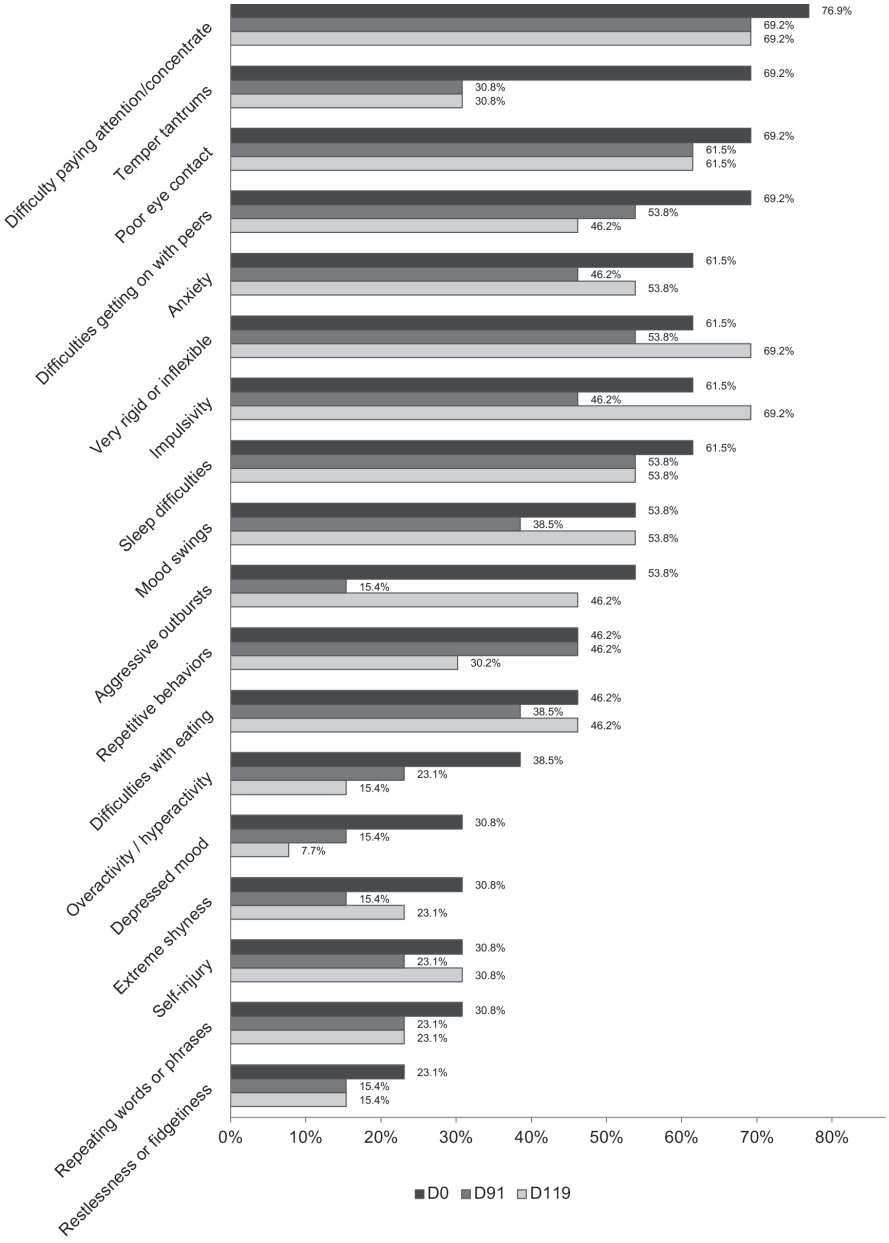


Figure 3. Percentage of parents reporting concerns/difficulties from section 3 of the TAND-checklist at baseline (D0), after treatment (D91) and after wash-out (D119)

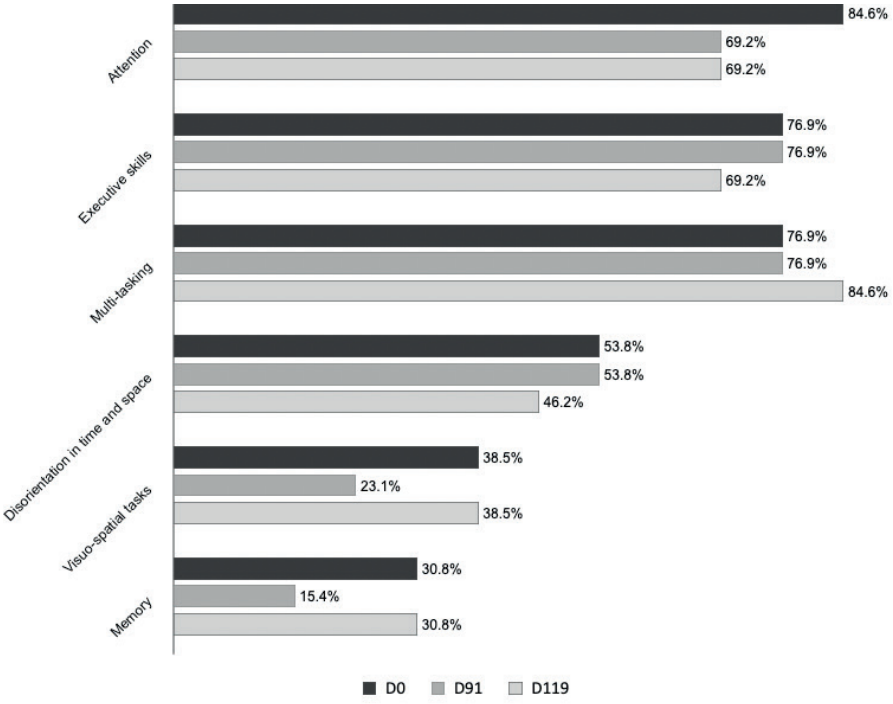


Figure 4. Percentage of parents reporting difficulties in cognitive skills from section 7 of the TAND-checklist at baseline (D0), after treatment (D91) and after wash-out (D119)

Quality of life questionnaires

Patient reported scales showed that a significantly higher quality of life score on the EQ-5D-Youth was given after treatment (D0: M=69.5, SD=12.3 versus D91: M=77.0, SD=11.1; $t(9)=-2.42$, $p=.038$). The self and proxy-reported subscales of the PedsQL (i.e., Physical Functioning, Emotional Functioning, Social Functioning and School Functioning) showed no change after treatment. Before treatment, parents rated their own general health on the 0-100 scale of the EQ-5D-5L with an average of 81.3 (fathers) and 81.1 (mothers), which remained stable after their child participated in the trial (81.1 and 80.4, respectively; $n=12$). Reports in the 4 domains of the WHOQOL-BREF (i.e., Physical Health, Psychological, Social Relationships and Environment) also remained stable during treatment.

ERP measurements

PPI

Table 2 and Figure 5a show the main results from the PPI paradigm - assessing sensorimotor gating - with four prepulse-pulse trials. At baseline, the TSC group showed only prepulse *facilitation* instead of *inhibition*: all trial types therefore showed decreased PPI in TSC patients compared to TD controls (76dB/120ms U=35, p=.004, d=-1.643; 76dB/60ms: U=39, p=.007, d=-1.188; 85dB/120ms: U=46, p=.017 and 85dB/60ms: U=51, p=.03), although this only survived Bonferroni correction in the two 76dB trial types, not in the two 85dB trial types (adjusted significance level p<.0125). The habituation coefficient showed no difference between groups at baseline (U=47, p=.650; see Table 2). However, where the TD group showed habituation starting from the third trial in block 1, the TSC group only showed *sensitization* (meaning an increase in startle amplitude compared to the first pulse alone trial) within the first habituation block, albeit not surviving multiple correction when comparing individual trials (trial 4: U=37, p=.026 and trial 7: U=36, p=.022; adjusted significance level p<.0125).

Treatment with bumetanide showed a tendency to reduce prepulse facilitation for both 120ms trials, which only reached significance for the 85dB/120ms trial type, but did not survive Bonferroni correction (Z=2.45, p=.014, adjusted significance level p<.0125). No change was observed in the other PPI trials (p>.140). Figure 5b shows the average startle amplitudes in the habituation paradigm. Visual inspection suggested normalization of habituation after treatment although this did not reach statistical significance (Z=1.214, p=.225). There was a significant difference between habituation after treatment and wash-out (Z=2.366, p=.016, d=.773).

Table 2. Habituation and percentage prepulse-pulse inhibition (PPI)

	TD (n=31)	**TSC (n=7)**		
Time point	**D0**	**D0**	**D91**	**D119**
Trial	Mean % (SD)	Mean % (SD)		
85dB/120ms	34 (33)	-9 (52)	19 (25)[2]	1 (55)
85dB/60ms	30 (35)	-13 (45)	-15 (16)	-12 (45)
76dB/120ms	32 (27)*[1]	-24 (58)	13 (30)	3 (21)
76dB/60ms	13 (49)*[1]	-44 (55)	-26 (34)	-14 (20)
Habituation coefficient	-4.52	-1.91	-6.63*[2]	-.78*[2]

Abbreviations. TD: typically developing controls; TSC: tuberous sclerosis complex. Notes. *: significant after Bonferroni correction ; 1: group effect;2: treatment effect

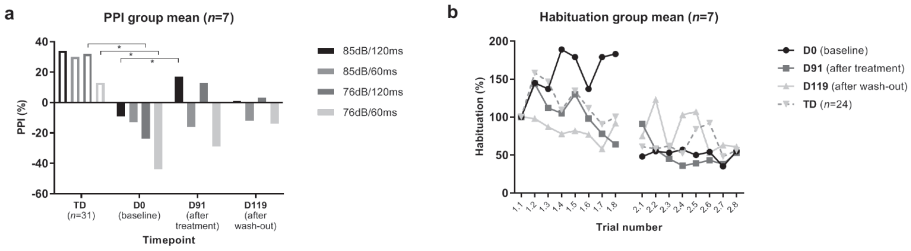


Figure 5. a) Mean percentage PPI in the TD group and TSC patients for all four prepulse–pulse trials. Significantly less PPI was found in TSC compared to TD in the two 76dB types at baseline. Improvement was found after treatment in trial type 85dB/120ms. b) Startle amplitude measured with electromyography for the 8 trials of blocks 1 and 2 in a habituation paradigm. Increased sensitization is apparent in the TSC group at baseline and habituation changes after treatment (D91) and wash-out (D119).

P50

Table 3 and Figure 6 show the grand averages for the P50 suppression paradigm – assessing sensory gating. No baseline differences were found between the TSC and TD group for the S1 amplitude (U=122.5, p=.593), S2 amplitude (U=137.5, p=.956) and the S1/S2 ratio (U=110.5, p=.357). After treatment or wash-out, no changes were found on the S1 amplitude, S2 amplitude and S1/S2 ratio (p>.123).

Table 3. P50 suppression amplitudes of S1, S2 and S1/S2 and latencies at different time points

	TD (*n*=31)	**TSC (*n*=7)**		
Time point	**D0**	**D0**	**D91**	**D119**
Trial	Mean amplitude (SD)	Mean amplitude (SD)		
S1 amplitude	1.65 (1.01)	1.42 (.68)	1.98 (1.26)	1.67 (1.38)
S1 latency	61.35 (10.39)	62.67 (11.40)	63.33 (9.06)	62.67 (9.95)
S2 amplitude	1.00 (1.04)	.88 (.70)	1.02 (.89)	.74 (.52)
S2 latency	59.27 (15.45)	65 (9.97)	67.75 (14.44)	66.22 (14.12)
S1/S2 ratio	.58 (.55)	.73 (.50)	.81 (.85)	.65 (.52)

Abbreviations. TD: typically developing controls; TSC: tuberous sclerosis complex.

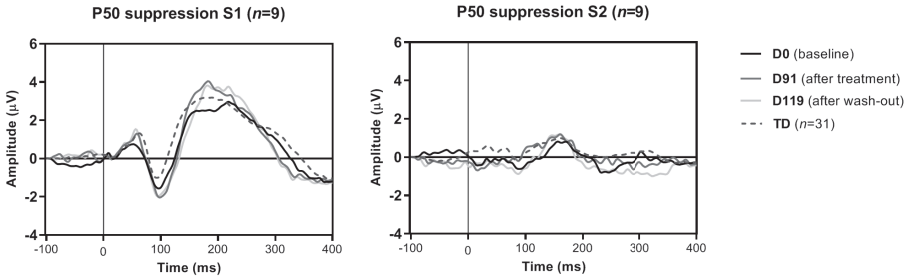


Figure 6. Grand average of lead Cz of the P50 suppression paradigm, for the a) conditioning ("S1") and b) testing stimuli ("S2") showing no difference between group and time points.

MMN

Table 4 and Figure 7 present the grand averages of the mismatch negativity paradigm, evaluating automatic auditory discrimination, for the four different stimuli (i.e., standard, frequency deviant, duration deviant and frequency/duration deviant). Compared to TD, the TSC group at baseline showed enhanced automatic discrimination of frequency deviant tones (U=53, p=.005 [adjusted significance level p<.0125], d=.893). For the other deviant tones, no significant difference was found at baseline (U>127, p>.702). The only effect after treatment on MMN was found for duration MMN; which was enhanced compared to baseline (D0 vs D91: Z=1.96, p=.05, d=1.061) and returned to baseline values after wash-out (D0 vs D119: Z=.56, p=.575). The MMN due to the other deviants showed no difference between treatment time points (p>.161) meaning the increase in frequency MMN persisted after treatment and wash-out.

Table 4. Mismatch negativity (MMN) mean amplitudes and latencies

	TD (*n*=35)		**TSC (*n*=8)**					
Time point	**D0**		**D0**		**D91**		**D119**	
Deviant type	Amp (SD)	Lat (SD)	Amp (SD)	Lat (SD)	Amp (SD)	Lat (SD)	Amp (SD)	Lat (SD)
Standard	-1.82 (.92)	255 (21)	-1.45 (.46)	253 (34)	-1.51 (.52)	261 (12)	-1.65 (.74)	258 (34)
Frequency	-1.68 (1.22)*[1]	138 (40)	-3.03 (1.75)*	137 (23)	-2.75 (1.71)	136 (28)	-2.23 (.70)	125 (28)
Duration	-1.91 (1.31)	193 (49)	-1.71 (1.5)[2]	206 (28)	-3.11 (1.10)[2]	206 (31)	-1.92 (1.15)	203 (20)
Freq/Dur	-2.35 (1.15)	130 (29)	-2.28 (1.32)	125 (34)	-2.64 (1.52)	116 (16)	-2.46 (1.19)	122 (25)

Abbreviations. MMN: mismatch negativity; Amp: amplitude; Lat: latency; SD: standard deviation; Freq/Dur: frequency duration deviant; TD: typically developing control group. Note. * Significant with Bonferroni correction; 1: group effect;2: treatment effect

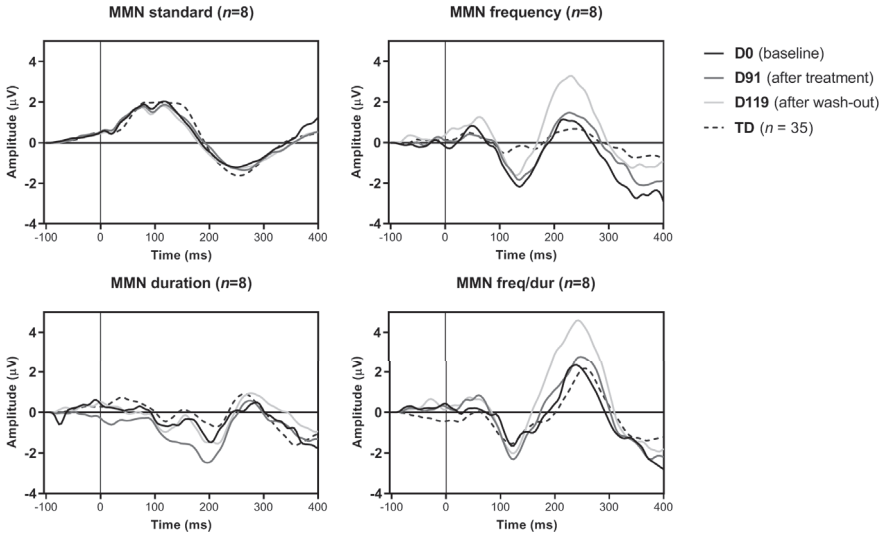


Figure 7. Grand averages of lead FCz of the mismatch negativity paradigm, for the a) standard stimulus; b) frequency deviant; c) duration deviant and d) frequency/duration deviant. Dashed lines represent data from the typically developing control group (n=35).

Neuropsychological measurements

No changes were observed with neuropsychological measurements after treatment. Additional file 2 shows all results obtained with the different neuropsychological tests.

Safety and tolerability

No serious adverse events (SAEs) were reported during the trial. Bumetanide was well tolerated by the majority of patients. As expected from the diuretic effect, hypokalemia was the most commonly observed adverse event, in spite of oral potassium-chloride supplements. Table 5 provides an overview of all reported adverse events in all patients enrolled and the (expected) relationship with the investigational product. Details on the blood safety analyses (i.e., potassium and sodium) are provided in Additional file 3.

Table 5. Adverse events

Event	Severity	Intervention relationship[a]	Expected	# Participants
Blood analyses				
Leukocytopenia	Mild	2-3	Yes	1
Hypokalemia	Mild	1-2	Yes	7
Hyponatremia	Mild	2	Yes	1
Hypovolemia	Mild	1	Yes	1
Gastrointestinal symptoms				
Nausea/vomiting	Mild	3	Yes	1
Obstipation	Mild	2	Yes	2
Decreased appetite	Mild	2	No	1
Weight loss	Mild	2	Yes	1
Genitourinary				
Diuresis	Mild	1	Yes	1
Infections				
Viral infection	Mild	3	No	1
Urinary tract infection	Mild	2	No	1
Behavioral symptoms				
Aggression	Moderate	2	No	2
Irritability	Mild	2	No	2
Anxiety	Mild	2	No	1
Musculoskeletal				1
Medial malleolus fracture	Moderate	3	No	1
Humerus fracture	Moderate	3	No	1
Other				
Hypotension	Mild	1	Yes	1
Hypothermia	Mild	3	No	1
Dehydration	Mild	1	Yes	1
Palpitations	Mild	2	No	1

Note: [a]1: Definitely related; 2: Possibly related; 3: Not related

Seizure frequency

Seizure frequency did not change after 3 months bumetanide treatment. Changes in seizure frequency from D-28 to D91 are depicted in Additional file 4 for all patients with uncontrolled epilepsy.

DISCUSSION

This open-label pilot study tested the effect of 91 days of bidaily 0.5-1.0mg bumetanide treatment on behavior, cognition and ERP parameters in a sample of children and adolescents with TSC. In general, bumetanide was well tolerated by the majority

of participants and no serious adverse events occurred. In this sample, we found significant improvement on the primary endpoint, the aberrant behavior checklist (ABC) – irritability subscale. Another main finding included significant improvement on social behavior (SRS-2). Beneficial effects of bumetanide were also indicated through quality of life assessments: patients rated their own health-related quality of life significantly higher after bumetanide treatment, together with parents reporting fewer problems with self-esteem in their children.

Although these are promising results, we should acknowledge that this is an open-label study with limited sample size. However, many parents noticed strong amelioration of behavioral manifestations as expressed in the preliminary evaluations made with the TAND-checklist and substantiated by the ABC-I, most notably improvements in the number and duration of temper tantrums and aggressive outbursts. Another main parental concern according to the TAND-checklist were social symptoms, such as difficulties getting on with peers and poor eye contact – which indeed improved according to SRS-2 results. In addition, according to the TAND-checklist and QoL questionnaires the familial situation also seemed to improve, showing reduction of familial stress and improved relationships between parents through treatment. These results may be consistent with previously reported effects of bumetanide on autism spectrum disorder (ASD) symptomatology[20, 37, 38], a neurodevelopmental disorder strongly associated with TSC.

Using the ERP analyses, we found changes between TD and TSC samples for PPI and MMN both at baseline and through treatment. This may support the hypothesis that the effect of bumetanide on TAND may be mediated through alterations in neurophysiology.

The origins of the ERPs changes in TSC are complex to interpret since MMN may also be sensitive to (localized) epileptic activity and antiepileptic treatment[39, 40]. However, we do expect these effects to be limited because dosage of antiepileptic treatment was stable prior and during the study. To our knowledge, no other trials have studied the effect of bumetanide on ERPs. It should be noted that ERP parameters are variable even in a genetic disorder as TSC perhaps due to differences in phenotypic expression including the brain malformations. Both the neuropsychological test battery as well as the parent-reported cognitive skills (TAND-checklist) indicated no changes in cognitive functioning through treatment.

Limitations

As mentioned, an important limitation of this study is the single-arm open-label design and absence of a placebo group. TSC is a rare genetic disorder and patients often experience extensive physical burden and unstable disease courses, therefore recruitment in placebo-controlled trials is highly challenging, with subsequently small sample sizes, insufficient to draw firm conclusions. Although a TD control group with a similar mean age (i.e., 12.9 years) was included to compare ERP measurements, this group had a more narrow age range (7-15 years) and no repeated EEG measurements were performed. We could only administer our testing battery in patients with an IQ above 70; therefore we cannot extrapolate the cognitive and ERP findings to TSC patients with intellectual disability. Another caveat may be that 91 days of bumetanide is not long enough to mediate significant effects on cognitive function tests or effects are too subtle to detect with our test battery in the exceptional context of a hospital environment. Researchers testing everolimus in TSC have also suggested that age of administration is an important factor in the treatment of TAND symptoms[9], as TAND symptoms initially present early (i.e., within 2 years of age) in life. Another limitation is that assessment of TSC and epilepsy-specific symptoms could have been carried out by using more sensitive outcome measures, such as using the quality of life in childhood epilepsy (QOLCE) and Quality of Life in Epilepsy Inventory for Adolescents-48 (QOLIE-AD-48), which has shown good psychometric properties[41]. An additional uncontrolled factor is the variety of co-medication used in 11 out of 14 patients, complicating the interpretation of direct effects of bumetanide. It should be noted that dosage of co-medication was kept stable during the study period. It is not possible yet to measure neuronal chloride concentrations in the clinical situation, thus it remains unclear whether treatment effects are exerted via correction of chloride homeostasis. Moreover, studies have shown that bumetanide may have limited penetrance in the brain so that we cannot rule out that peripheral effects also contribute to the observed changes[42].

Despite these limitations, this study indicates a favorable effect of bumetanide in TSC on clinically important behavioral symptoms. These findings may be followed-up in more elaborate studies either in a larger multicenter randomized controlled design or a multiple *n*=1 design (e.g., [43]) given the rarity of the genetic disorder.

Conclusion

This pilot study indicates potential efficacy of bumetanide on behavioral problems in young patients with TSC. Bumetanide improved irritable, explosive and social behavior in the majority of patients in this sample and treatment was well tolerated.

SUPPLEMENTAL MATERIAL

ADDITIONAL FILE 1

Supplementary methods

1. EEG recording

EEG was recorded from 64 electrodes placed in a cap (10-20 layout) with BioSemi® hardware (Amsterdam, Netherlands). Data were recorded with a sampling rate of 2048Hz. Electrooculography electrodes were placed above and below the right eye and beside both eyes to record eye movement and reference electrodes were located on the left and right mastoid. All auditory stimuli were presented using a computer running Presentation software (soundcard: Creative soundblaster 5.1) and were presented through stereo insert earphones (Eartone ABR).

2. ERP paradigms

PPI. The PPI paradigm started with 5 minutes of acclimation to a continuous background noise (70dB white noise) after which three blocks of stimuli were superimposed. Blocks 1 and 3 were identical and were used to assess habituation and sensitization of the acoustic startle reflex. These two blocks consisted of eight pulse-alone trials of white noise (115dB, 20ms), instant rise and fall. Block 2 consisted of 50 trials presented in a pseudorandomized order and were used to assess PPI: two intensities prepulse stimuli were used (6 and 15dB above the 70dB background, both with 20ms duration), while also two stimulus onset asynchronies (SOA) of 60 and 120ms were used in the trials. The session consisted of 10 pulse alone and 10 of each prepulse–pulse combination (76dB/60ms, 76dB/120ms, 85dB/60ms, 85dB/120ms) which were presented in a pseudo-randomized order (two of the same trial types were never directly following each other). Intertrial intervals in all blocks were randomized between 10 and 20s. The complete paradigm took approximately 25 min.

P50 suppression. The P50 paradigm is comprised of 40 paired clicks (1.5ms in duration and 80dB) presented binaurally and repeated in 3 blocks. Subjects were instructed to count the number of clicks. Interstimulus interval (ISI) was consistently 500ms and click pairs were separated by 10s. The total duration of the P50 suppression task is approximately 21 minutes.

MMN. The MMN paradigm consisted of 1800 trials with an intensity of 75dB and an ISI randomly varying between 400-500ms. The task stimuli were subdivided into 1500 trials (83,3%) of standard tones with a frequency of 1000Hz and duration of 50ms;

100 trials (5,5%) of frequency deviant tones (1200Hz, 50ms); duration deviant tones (1000Hz, 100ms) and frequency/duration deviant tones (1200Hz, 100ms). Subjects were asked to ignore the stimuli and watched a muted documentary on a screen in front of them. The total duration is approximately 14 minutes.

3. EEG processing

The EEG signals were pre-processed, averaged and analyzed using Brain Electrical Source Analysis (BESA)-software (version 6, MEGIS Software GmbH, Gräfeelfing, Germany). Preprocessing of the data started with resampling from the original 2048Hz to 250Hz for MMN, 500Hz for P50 and 1000Hz for PPI to allow for easier file handling. PPI was epoched at -50 to 250ms, band-pass filtered at 25-250Hz and measured bipolarly from both eye electrodes below the right eye.

P50 and MMN were differently processed since they were analyzed over the scalp electrodes. Electrodes with aberrant signals were manually interpolated or removed when >6 channels were affected and eye-blinks were removed by BESAs internal scripts. Third, the data were epoched (from 100ms prestimulus to 900ms poststimulus for MMN and -100 to 400ms for P50) and corrected for movement (or other paradigm unrelated) artefacts, by removing those epochs from the database that contained amplitude differences between maximum and minimum exceeding 75μV, in the for P50, MMN relevant scoring windows (see below). Only data from the electrodes relevant for this study were analyzed (i.e., where the maximum activity for the ERPs was found): the midline electrodes Cz (for P50), FCz (for freqMMN, durMMN, freqdurMMN) and both eye-electrodes for PPI. P50 and MMN data were band-pass filtered (0.5Hz- 70Hz for P50 suppression data, 0.5Hz- 40Hz for MMN data), and grand average reference was used as a reference.

For PPI, startle magnitude was scored as the highest absolute amplitude within a window between 20 and 120ms following the startle eliciting pulse, whereas PPI was expressed as [(1 – (PP/PA)) × 100%]; with PP = the average startle amplitude to prepulse–pulse trials, and PA = the average startle amplitude to pulse alone trials in block 2. Habituation was defined as the β-coefficient of the linear trend line through the points of trials 4–8 in block 1 and trials 1–8 in block 3.

For P50, the amplitude was defined as the largest trough to peak amplitude within an interval of 40–90ms following the first (S1) stimulus in each paired click. The P50 amplitude following the second (S2) stimulus was identified as the largest trough to peak amplitude within an interval of 10ms of the latency of the maximum P50

amplitude to the C-stimulus. P50 suppression was expressed as the ratio "S2/S1". P50 waves were manually scored by BO and JS.

For MMN, amplitude and mean amplitude were scored for each of the three deviant types, expressed as the average ERP to the relevant deviant stimuli, subtracted with the average ERP to standard stimuli for each subject separately. MMN amplitudes were then scored as the minimum amplitude in a window between 75 and 200ms for frequency and the combined frequency-duration deviants, 180 and 340sec for standard tones and in a window between 100-270ms for duration deviants.

ADDITIONAL FILE 2

Neuropsychological test battery results

Table 1. Raw scores on auditory and visual memory tasks at baseline and after 3 months bumetanide treatment

	15-WT								**RVDLT**				**WISC-DS**						**WNV-SSp**					
	Total recall		Trial 1-5		Trial 1		DR		Trial 1-5		Recognition		Forwards		Backwards		Total		Forwards		Backwards		Total	
	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91
	27	25	40	44	2	4	10	15	30	46	35	35	10	7	7	3	17	10	6	7	2	3	8	10
	30	30	50	50	6	3	11	9	40	36	44	40	8	8	4	6	12	14	10	6	8	7	18	13
	30	30	58	49	3	5	8	11	29	36	39	45	9	11	3	7	12	18	8	6	8	6	16	12
	30	30	41	48	4	4	6	11	38	41	41	40	8	8	3	4	11	12	6	5	4	6	10	11
	22	28	38	35	2	2	5	3	19	13	34	30	6	5	2	2	8	7	6	4	2	2	8	6
	30	30	49	63	6	5	12	10	43	37	42	45	10	8	6	6	16	14	5	5	5	8	10	13
	29	29	43	41	6	3	11	11	39	40	39	43	7	6	4	3	11	9	6	6	6	6	12	12
M	28	29	46	47	4	4	9	10	34	36	39	40	8	8	4	4	12	12	7	6	5	5	12	11

Abbreviations: 15-WT: 15 Word Task; RVDLT: Rey Visual Design Learning Test; WISC-DS: Wechsler Intelligence Scale for Children-Digit Span; DR: Delayed Recall; M: Mean; WNV-SSp: Wechsler Non-Verbal Scale of Ability subtest Spatial Span

Table 2. Raw scores on the Amsterdam Neuropsychological Test (ANT) battery at baseline and after 3 months bumetanide treatment

	Baseline speed				**Go No-Go**											
	RT (ms)		Stability (ms)		RT (ms)		Missed		FA		RT Bias (ms)		Missed Bias		FA Bias	
	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91
	225	248	46	75	383	347	0	0	1	0	382	321	0	0	2	1
	253	264	45	55	305	302	0	0	0	0	358	390	0	0	0	2
	336	315	191	163	474	484	0	0	1	0	468	494	0	2	1	0
	324	346	172	111	381	394	0	0	4	0	376	413	0	0	5	0
	395	412	196	156	548	502	1	0	5	7	452	560	0	1	6	7
	394	312	443	91	412	353	0	0	0	0	427	384	0	0	0	1
	372	322	169	80	354	377	0	0	0	0	354	349	0	0	1	2
M	328	317	180	104	408	394	.14	0	1.57	1	402	416	0	0.43	2.14	1.86
(SD)	(67)	(54)	(133)	(41)	(80)	(73)					(46)	(84)				

	SSA (ms)								**SSV (ms)**							
	1		2		3 Compatible		3 Incompatible		1		2		3 Compatible		3 Incompatible	
	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91	D0	D91
	351	414	463	625	1241	962	1160	912	523	336	1175	550	1190	1474	951	1334
	358	503	620	674	774	765	833	986	473	523	969	751	1363	1500	1520	1864
	707	483	950	580	1048	1016	1201	1288	575	465	1125	1085	1263	1194	1758	1148
	528	501	752	688	868	851	884	825	667	553	729	890	945	947	1073	976
	-	704	-	1378	-	727	-	1147	1011	566	1218	601	1946	710	1200	1024
	383	392	665	595	1038	818	1171	1026	565	557	914	1022	1931	1613	2024	2045
	373	357	604	579	935	724	991	811	502	544	2092	1090	1523	1515	2000	1657
M	450	479	676	731	984	838	1040	999	617	506	1175	856	1452	1279	1504	1435
(SD)	(142)	(114)	(164)	(288)	(163)	(114)	(159)	(173)	(185)	(82)	(439)	(226)	(376)	(340)	(441)	(424)

ADDITIONAL FILE 3

Blood safety checks

Table 3. Blood safety checks: potassium

Patient	Baseline	D4	D7	D14	D28	D56
1	4,0	3,8	4,2	3,4	3,8	3,8
2	3,9	4,2	3,7	4,1	3,8	4,1
3	4,7[a]	4,0[a]	4,1[a]	4,0[a]	5,2[a]	4,1[a]
-	4,3	4,0[b]	4,2[b]		3,8[b]	
4	4,2	4,1	4,0	4,1	4,3	3,6
5	4,4	3,5[b]	3,9[b]	3,4[b]	3,4[b]	3,5[b]
6	3,7	3,4	3,6	3,4	3,5	3,6
7	3,9	3,8[b]	3,5[b]	3,2[b]	3,9[b]	3,2[b]
8	4,4	4,7	3,7[b]	4,0	3,8	4,4
9	3,9	4,0[b]	3,9[b]	4,2	3,9[b]	4,2[b]
10	4,1	3,4[b]	4,1[b]	3,8[b]	3,8[b]	4,3[b]
-	4,9	4,4[b]	4,1[b]	4,1[b]	3,6[b]	Drop-out
11	4,0	3,9	4,0	4,1	4,1	3,9
12	4,0[b]	4,2[b]	3,5[b]	3,7[b]	3,6[b]	3,3[b]
13	3,9	4,4[a]	4,1	3,8	3,9	4,4[a]

Note: [a]Capillary finger-prick blood draw; [b]blood samples at general practitioner.

Table 4. Blood safety checks: sodium

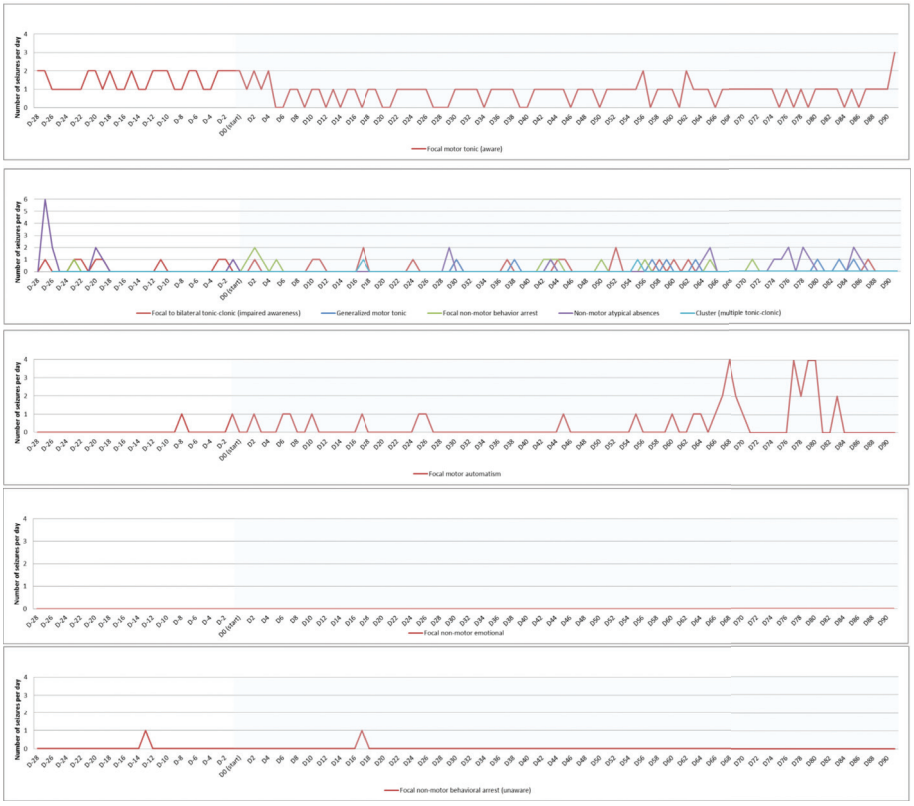
Patient	Baseline	D4	D7	D14	D28	D56
1	137	137	139	140	136	138
2	138	137	138	139	138	138
3	135[a]	136[a]	132[a]	138[a]	140[a]	137[a]
-	138	139[b]	140[b]		142[b]	
4	138	140	136	139	138	135
5	137	141[b]	141[b]	142[b]	141[b]	142[b]
6	139	140	139	139	142	143
7	136	142[b]	142[b]	141[b]	144[b]	137[b]
8	135	137	138	134	139	137
9	137	140[b]	143[b]	141[b]	141[b]	143[b]
10	139	143[b]	141[b]	148[b]	143[b]	141[b]
-	140	140[b]	142[b]	140[b]	142[b]	Drop-out
11	136	137	137	137	137	136
12	139[b]	142[b]	144[b]	144[b]	142[b]	141[b]
13	137	137[a]	138	139	140	138[a]

Note: [a]Capillary finger-prick blood draw; [b]blood samples at general practitioner.

ADDITIONAL FILE 4

Seizure frequency

Seizure frequency per seizure type as reported by parents 28 days prior to and during treatment phase (D-28 to D91)



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""*Medical science has made such tremendous progress that there is hardly a healthy human left."* — Aldous Huxley

CHAPTER 3

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

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ABSTRACT

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

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

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

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

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

Keywords ASD, bumetanide, RCT, children, SRS

INTRODUCTION

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

For instance, compelling evidence shows that deficits in GABAergic inhibition can contribute to ASD development[8]. The efficacy of GABAergic inhibition depends on the regulation of the intracellular neuronal chloride ([Cl−]i) concentrations[9, 10]. Pathologically high [Cl-]i can reverse the polarity of GABA binding its receptor from inhibition to excitation, a converging mechanism that has been linked to a variety of disorders including ASD[10]. Elevated levels of neuronal chloride and excitatory actions of GABA receptor signaling have been established in animal models of ASD and associated conditions. These observations have raised interest in the development of pharmacological treatments that restore chloride homeostasis and consequently GABAergic inhibition in pathological conditions[10]. The [Cl-]i is predominantly regulated by the chloride importer NKCC1 and chloride exporter KCC2 and the best studied agent is bumetanide, a selective NKCC1 antagonist[11]. Bumetanide is approved since many decades as a safe loop diuretic to treat conditions of hypervolemia with a mild adverse effect profile, which facilitates its application in neurological disorders[10, 11].

Following a pilot study[12], Lemonnier et al. conducted two consecutive placebo controlled randomized trials testing bumetanide (1-4 mg/day for three months) in 60 and 88 participants respectively[13, 14]. Both trials showed a significant reduction in their primary outcome of broad ASD symptomatology. Further anecdotal evidence supported this evidence through a case study in fragile X syndrome (FRX)[15] and from studies testing bumetanide on emotion recognition in functional neuroimaging and eye-tracking[16, 17]. These results are promising, although several methodological and mechanistic concerns have been raised. Both studies used the Childhood Autistic Rating Scale (CARS) as primary outcome, a diagnostic screening questionnaire. No

prior bumetanide trials included outcomes on the core domain of repetitive behaviors or atypical reactivity to sensory input or determined levels of cognitive functioning or comorbidities in their participants. Another problem is that most plasma bumetanide is protein bound and rather low concentrations were found to diffuse into the brain[11]. Therefore, brain penetrance of bumetanide across an intact blood-brain barrier may be limited and behavioral improvements through peripheral effects have been suggested. Furthermore, the clinical and etiological diversity in ASD may preclude that agents targeting specific elements of GABAergic signaling may only be effective in particular subgroups[18, 19].

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

METHOD

Study Design and Participants

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

Eligible participants were children aged 7-15 years with an expert confirmed ASD diagnosis according to DSM-IV-TR[21] (i.e. autism, Asperger syndrome or PDD-NOS) or DSM-5[22] criteria. Children were enrolled when the expert diagnosis was accompanied by a clinical threshold score on the Autism Diagnostic Observation Schedule (ADOS-2 module 3 or 4 ≥ 6)[23] or the Social Responsiveness Scale-2 (SRS-2; t-score ≥60)[24]. Given the 85% sensitivity of the ADOS-2[23], children with an expert diagnosis of ASD and either an ADOS-2-score <6, or a SRS-2 t-score <60, were evaluated for second opinion by an independent in-house child-psychiatrist. When ASD diagnosis was confirmed,

children could advance to treatment allocation. Exclusion criteria were an IQ<55; psychoactive medication use less than eight weeks prior to screening visit (except chronic melatonin treatment); start of any new therapy for developmental disorder problems (e.g. cognitive behavioral therapy); comorbid neurological disorders; chronic renal disease; unstable serious illness; NSAID treatment; and/or documented history of hypersensitivity reaction to sulphonamide derivatives. Furthermore, children were allowed to receive care as usual restricted to stable frequency of supportive care initiated minimally two months prior to randomization (e.g. physiotherapy, education support), but excluding behavioral, cognitive behavioral therapy, family therapy, or any other kind of psychological intervention. No amendments to eligibility criteria were made.

Randomization and Masking

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

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

Procedures

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

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

After an early amendment to the protocol, safety visits were scheduled at D4, D7, D14, D28, D56, D91 and D119 at the department of pediatric nephrology of the Wilhelmina Children's Hospital. The initial protocol involved extra visits at D35 and blood analysis at D91 and D119. At all visits, adverse events, weight, height (monthly) and vital signs were checked. At D4, D7, D14, D28 and D56 blood was obtained and analyzed for adverse events (Supplementary Figure S1). Adverse events were documented according to severity, duration, attribution and outcome with the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) rating scale

and classified in Medical Dictionary for Regulatory Activities (MedDRA) categories. Hypokalemia was the main adverse event to be expected; hence a treatment protocol was formulated beforehand (Supplementary Table S2).

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

Outcomes

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

Statistical Analysis

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

We analyzed outcomes by modified intention-to-treat allocated participants (see results section for details)[26, 27]. Screening differences between randomized and eligible non-randomized participants were analyzed with appropriate t-statistics or Fisher's exact tests for dichotomized variables.

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

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

RESULTS

Participant Characteristics

Participants were enrolled between June 21st 2016 and December 6th 2018, the end of planned recruitment. A total of 267 caregivers contacted the research team to obtain the study information folder (Figure 1). Of these potential participants, 133 gave informed consent and 125 were assessed for eligibility. 32 of these participants did not advance to randomization for reasons of non-eligibility (n=13), requirement of immediate psychiatric intervention (n=9), inability to adhere to study protocol (n=6), or withdrawal of consent (n=3). Finally, 92 participants were randomly allocated to treatment (Table 1). There was no difference in baseline characteristics in eligible participants (n=110) who did and who did not advance to randomization ($p \geq 0.163$). Of the 92 participants who were randomized, all started allocated treatment and were included in the modified intention-to-treat analysis, excluding 10 allocated participants (Figure 1). A total of 47 children were allocated to bumetanide (15 female

children) and 45 to placebo (14 female children). Four participants discontinued treatment prior to collecting outcomes, two in each treatment arm. One participant in the placebo arm stopped because of a-specific somatic complaints and another due to intractable resistance to venipunctures. The two discontinued treatments in the bumetanide arm were due to inability to adhere to potassium supplementation and one due to a school crisis requiring immediate psychiatric intervention. During the trial nobody had to be unmasked. No further participants were lost to follow-up and all completed the trial, although some participants did not complete all behavioral outcome measures at different time points (D91 n = 3 placebo and n = 4 bumetanide; D119 n = 6 placebo, n = 4 bumetanide).

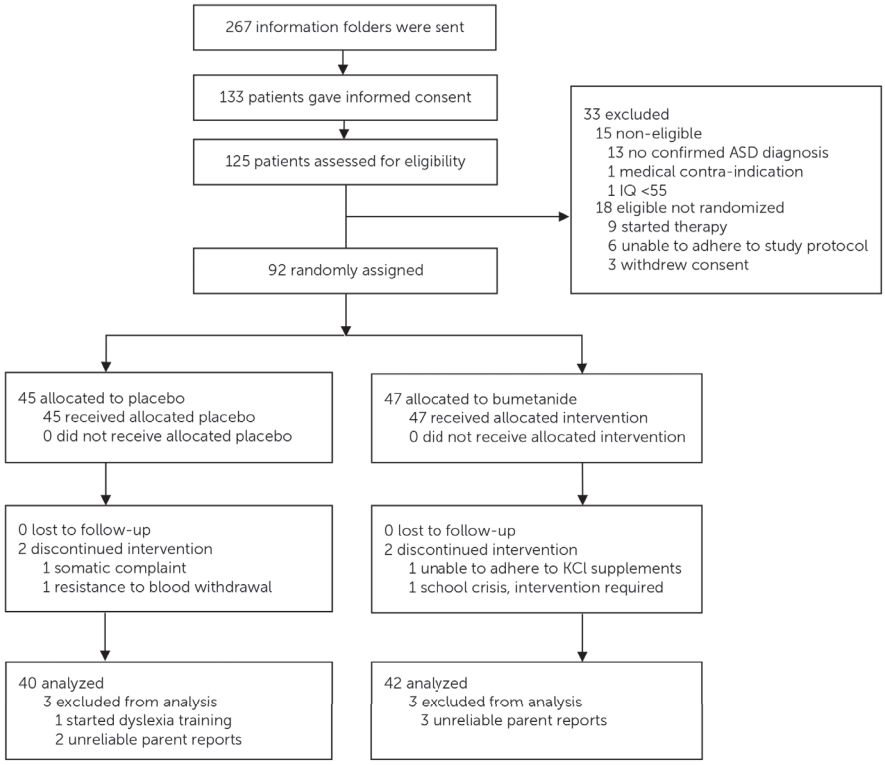


Figure 1. CONSORT Diagram of the Trial

Table 1. Baseline Characteristics of the Intention-to-Treat Population

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

Note: Data are mean (SD) or n (%). ADHD = attention-deficit/hyperactivity disorder; ADOS = Autism Diagnostic Observation Scale; AP = antipsychotics; IQ = intelligence Quotient; SRS = Social Responsiveness Scale (range 0-195; higher score is more affected); STM = stimulants.

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

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

After the last study visit of a participant, we inventoried the parent predictions of the treatment their child had received. In the bumetanide arm (n = 47), 30 parents thought to have been allocated to bumetanide, 11 parents expected to have been allocated to placebo, and 6 parents were uncertain. In the placebo arm (n = 45) 18 parents thought to have been allocated to bumetanide, 25 parents expected to have been allocated to placebo, and 2 parents were uncertain. There was fair agreement between expected and actual treatment allocation (κ = 0.312 [0 indicating effective masking, 1 indicating failure of masking], p = 0.004).

Outcomes

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

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

Table 2. Changes in Primary and Secondary Outcome Measures after Treatment and Washout

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

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

Individual changes in SRS-2 and RBS-R showed a conspicuous distribution (Figure 2). For both outcome measures, the nine participants with the largest improvement had been allocated to bumetanide treatment, which alludes to a responsive subset. Nevertheless, only two participants overlapped indicating limited phenotypic similarity and accordingly, a larger correlation between change in SRS-2 and RBS-R D91-D0 scores was found in the placebo treated group (r = 0.529, $p = .001$) than in the bumetanide treated group (r = .294, $p = .074$, Figure S2). No dose dependent relationship was found. Mean treatment dose showed no correlation with change in SRS-2 or RBS-R score in the bumetanide arm (respectively r =-0.191, $p = .251$; r = .016, $p = .926$).

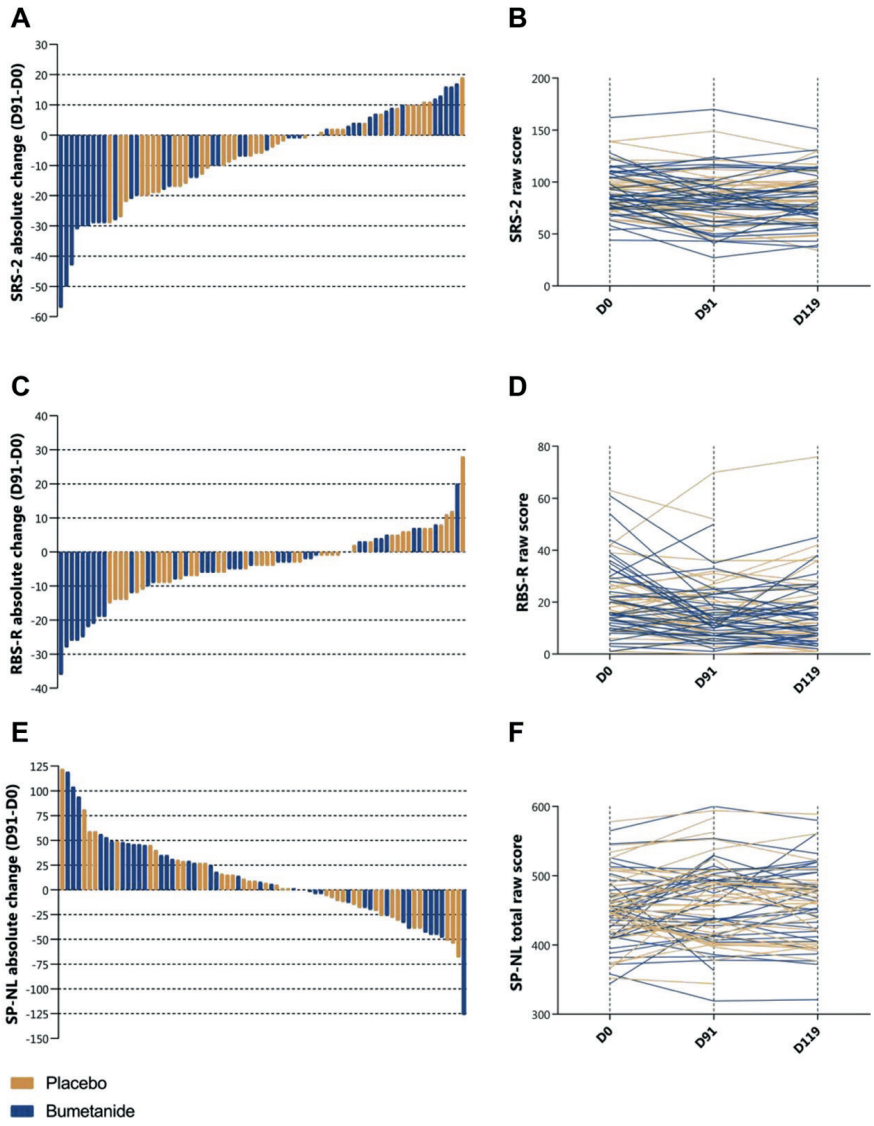


Figure 2. Individual Treatment Effects on the Autism Spectrum Disorder Core Domain Outcomes

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

Tolerability and Adverse Effects

Bumetanide was generally well tolerated. Adverse events occurring in more than 4% of the participants are listed in Table 3. All events were mild to moderate in intensity according to the CTCAE rating-scale and resolved. Three serious adverse events (SAEs) occurred: syncope after venipuncture requiring a short period of clinical observation (bumetanide arm), extended hospitalization after a Kieselbach coagulation (placebo arm), and the occurrence of an acute appendicitis requiring appendectomy (bumetanide arm). The SAEs were determined to be probably unrelated to treatment with the study medication, except for syncope, which was possibly related. Hypokalemia, orthostatic hypotension, dehydration and diuresis were the most frequent and expected adverse events despite being treated with the preventive measures in the protocol. Diuresis and hypokalemia also occurred independently. A total of 51% of the participants receiving bumetanide treatment developed hypokalemia against none in the placebo arm ($p < .0001$). Hypokalemia did not occur before D14 and potassium levels did not drop below 3.0mmol/L (Supplementary Table S4); 36% percent of the participants in the bumetanide arm developed orthostatic hypotension (bumetanide 17 [36%], placebo 5 [11%], $p = .007$). No paradoxical response nor deterioration of irritability was observed.

Table 3. Adverse Events Occurring in More Than 4% of Participants Classified in MedDRA Categories

	Bumetanide arm				**Placebo arm**			
Symptom	**No. of AEs**	**No. of part.**	**Severity**	**IR[a]**	**No. of AEs**	**No. of part.**	**Severity**	***p***
Total AE	276	46			161	43		
Metabolism and nutrition disorders								
Hypokalemia	31	24	Moderate	1	0	0	Moderate	<.0001
Dehydration	8	8	Moderate	1	1	1	Moderate	.031
Hypoglycemia	1	1	Mild	3	3	3	Mild	.617
Gastrointestinal disorders								
Vomiting	14	11	Mild	2	5	4	Mild	.089
Nausea	13	10	Mild	2	8	7	Mild	.594
Abdominal pain	17	13	Mild	3	14	11	Mild	.814
Diarrhea	3	3	Mild	3	5	5	Mild	.481
Obstipation	6	5	Moderate	2	1	1	Moderate	.204
Dyspepsia	4	4	Mild	2	1	1	Mild	.362
Gastroenteritis	3	3	Mild	3	2	1	Mild	.617

	Bumetanide arm				Placebo arm			
Symptom	**No. of AEs**	**No. of part.**	**Severity**	**IR[a]**	**No. of AEs**	**No. of part.**	**Severity**	***p***
Vascular disorder								
Orthostatic hypotension	22	17	Mild	1	7	5	Mild	.007
Epistaxis	3	3	Moderate	3	2	2	Moderate	1.000
Syncope	3	3	Mild	2	3	1	Mild	.617
Infections and infestations								
Common cold	21	19	Mild	3	16	13	Mild	.279
Otitis media	4	4	Moderate	3	2	2	Moderate	.677
Musculoskeletal and connective tissue disorders								
Myalgia	12	12	Mild	2	10	8	Mild	.452
Muscle cramp	5	4	Mild	2	5	5	Mild	.737
Renal and urinary disorders								
Dysuria	5	4	Mild	2	4	4	Mild	1.000
Enuresis[b]	2	2	Mild	1	1	1	Mild	1.000
Diuresis	14	14	Mild	1	4	4	Mild	.017
Nervous system disorders								
Headache	12	10	Mild	3	19	15	Moderate	.244
Dizziness	8	6	Mild	3	5	5	Mild	1.000
Blurred vision	2	2	Mild	3	3	3	Mild	.674
Psychiatric disorders								
Insomnia	10	9	Mild	3	6	6	Mild	.575
General disorders and administration site conditions								
Fatigue	6	6	Mild	2	5	5	Mild	1.000
Skin and subcutaneous tissue disorders								
Dermal abnormalities	14	12	Moderate	3	8	7	Moderate	.306
Injury, poisoning and procedural complications								
Injury	7	6	Moderate	3	3	3	Mild	.486

Note: Data are numbers (n). Differences were tested with Fisher Exact tests. Significance level is $p < .05$. AE = adverse event; IR = intervention relationship; Part = participants.
[a] 1: definitely related; 2: possibly related; 3: not related .
[b] Occurring in <4% of participants but listed as important expected AE.

In an attempt to account for potential unmasking through diuretic effects, we performed linear mixed model analysis comparing treatment effects in three groups: placebo, bumetanide with hypokalemia, and bumetanide without hypokalemia. A larger treatment effect in the bumetanide with hypokalemia group may be expected when masking would be compromised. No treatment effect difference was suggested

between participants with and without hypokalemia for all outcome measures (SRS-2 mean difference -0.65, 95% CI = -9.52 to 8.22, p = .884 and RBS-R mean difference 2.04, 95% CI = -2.64 to 6.72, p=0.387 and ABC-I mean difference 1.54, 95% CI = -1.49 to 4.57, p =.342 and SP-NL mean difference -4.92, 95% CI = -28.54 to 18.70, p = .679). The presence of diuresis neither showed a difference in treatment effect (SRS-2 mean difference -5.77, 95% CI = -15.43 to 3.90, p = .238 and RBS-R mean difference 1.80, 95% CI = -4.06 to 7.67, p = .541 and ABC-I mean difference 2.65, 95% CI = -0.98 to 6.29, p = .149 and SP-NL mean difference 0.55, 95% CI = -25.54 to 26.63, p = .967).

DISCUSSION

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

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

It is important to note that the previous bumetanide RCT[14] included, on average, younger and more severely affected children (112.3 SRS-2 score versus 89.7 in our study) without characterization of IQ or comorbidity. We used a different statistical analysis to test superiority and to include baseline measurement of outcomes to correct for potential confounding and to optimize the statistical analysis for power[27-29]. We noted that in the BAMBI trial, younger children showed marginal significance toward more improvement on SRS-2, which may be consistent with better efficacy in younger

children with ASD. Indeed, there is a suggestion that efficacy of treatments targeting GABAergic inhibition is related to so-called windows of plasticity in which excitation-inhibition balance is expected to be crucial for functional brain development[37]. Our observed potential age effect seems consistent with the increasing notion that ASD trial drugs should not be abandoned purely on the basis of effects in adults, but always should also be tested in younger children. The previous RCT tested different dosages including a higher dosage regimen (2mg twice-daily) that contributed to a higher drop-out rate[14]. We therefore aimed for the suggested optimal dosage of 1.0mg twice-daily, and observed no dose-dependent effect in the currently applied range. The previous bumetanide studies have been criticized for potential unmasking through diuretic effects, which we tried to reduce through increased fluid intake instruction and supplementation of potassium in both treatment arms as well as organizing treatment surveillance through an independent team. We could therefore better analyze potential influences of diuretic effects on treatment outcome, and found no statistical indication that bumetanide treatment results were substantially influenced by their occurrence.

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

behavior profiles, which limits its usefulness in RCTs[39]. The second measure, the ABC-I, has been used to validate the use of antipsychotic drugs for irritability in ASD[1]. These studies used as an inclusion criterion a high threshold for baseline ABC-I scores that would have led to the exclusion of the majority of individuals in the current study population.

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

chloride in neuronal cells has been established only in animal models. Furthermore, limited penetrance of bumetanide across the blood-brain barrier has been indicated, implying that systemic non-neuronal effects of treatment should also be considered[11].

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

SUPPLEMENTAL MATERIAL

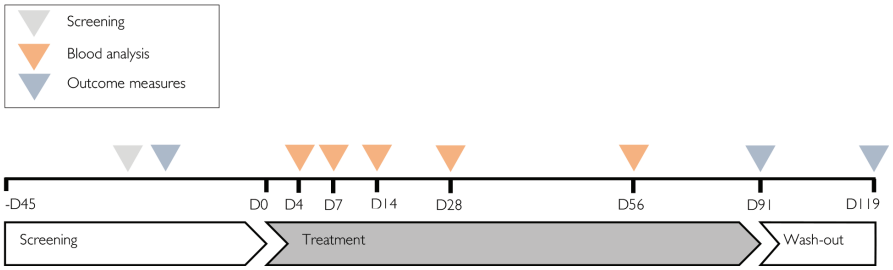


Figure S1. Overview of the Study Visits of the BAMBI trial

Table S1. Specification of Clinical Laboratory Tests Conducted During the BAMBI trial

Screening visit	
Blood analysis	Sodium, potassium, chloride, calcium, urea, creatinine, uric acid, alkaline phosphatase, gamma-glutamyltransferase aspartate aminotransferase, alanine aminotransferase, total protein, glucose, haemoglobin, haematocrit, erythrocytes, thrombocytes, leukocytes, aldosterone, renin activity, eGFR[a]
Urinary analysis	Creatinine, sodium, potassium, chloride, calcium, uric acid, total protein, micro-albumin
Control visit	
Blood analysis	Sodium, potassium, chloride, urea, creatinine, uric acid, total protein, glucose, haemoglobin, haematocrit, erythrocytes, thrombocytes, leukocytes, eGFR[a]

[a] eGFR = estimated Glomerular Filtration Rate and calculated by the modified Schwartz formula (36.5*height [cm] / serum creatinine [µmol/L])

Table S2. Schematic Overview of Pre-specified Hypokalaemia Treatment Protocol

[K^+]	**Medication dose: 2dd1ml**		**Medication dose: 2dd2ml**	
≥3.5	No changes, continue study med		No changes, continue study	
3.0-3.5	Increase KCl with 0.5mmol/kg/day Check [K^+] in 3-4 days		Increase KCl with 0.5mmol/kg/day ↓ med 2dd1ml Check [K^+] in 3-4 days [K^+] >3.5 med 2dd2ml	
2.5-3.0	Increase KCl with 0.5mmol/kg/day Stop med Check [K^+] in 3-4 days		Increase KCl with 0.5mmol/kg/day Stop med Check [K^+] in 3-4 days	
	[K+]↑ Med 2dd1ml Check 3-4da	[K+] =,↓ Stop study Check 2d	[K+]↑ Med 2dd1ml Check 3-4da	[K+] =,↓ Stop study Check 2d
<2.5	Stop study [$Mg2^+$], ECG, symptoms		Stop med [$Mg2^+$], ECG, symptoms	
	>70mmol + normal KCl +2mmol/kg/day Check 2d	<70mmol, abnormal Direct KCl 1mmol/kg Hospital admission 24h monitoring KCl i.v. via nephrologist	>70mmol + normal KCl +2mmol/kg/day Check 2d >3.5 med2dd1ml	<70mmol, abnormal Stop study Direct KCl 1mmol/kg Hospital admission 24h monitoring KCl i.v. via nephrologist

Note: [K^+] = potassium concentration in mmol/L; Med = medication; KCl = potassium chloride; ↑ = increased; ↓ = decreased; d=days; ECG = electrocardiogram; [$Mg2^+$]=magnesium concentration in mmol/L.
[a] After 2 subsequent [K^+] >3.5mmol increase med 2dd2ml.

Table S3. Mean and Standard Deviations of Subscales at Different Time Points

	Placebo arm			**Bumetanide arm**		
	Baseline	**D91**	**D119**	**Baseline**	**D91**	**D119**
SRS-2 subscales	n=37	n=37	n=32	n=38	n=38	n=36
Social Awareness	11.8 (3.1)	11.6 (3.5)	11.9 (3.4)	11.8 (2.5)	11.4 (3.5)	11.4 (3.1)
Social Cognition	17.2 (4.5)	16.8 (5.4)	16.4 (5.2)	18.0 (5.0)	16.2 (6.3)	15.9 (5.7)
Social Communication	30.4 (7.5)	28.2 (8.4)	28.7 (9.2)	30.3 (9.3)	26.9 (10.5)	27.4 (10.2)
Social Motivation	15.7 (5.0)	14.5 (5.5)	14.8 (5.0)	15.3 (5.7)	13.7 (6.2)	14.0 (6.7)
Autistic Preoccupations	15.6 (5.6)	14.5 (5.5)	14.5 (6.5)	16.1 (6.3)	13.3 (6.5)	13.5 (6.0)
RBS-R subscales	n=37	n=37	n=31	n=38	n=38	n=36
Stereotypic Behavior	2.8 (2.0)	2.6 (2.4)	2.6 (2.0)	3.0 (3.0)	1.8 (1.8)	2.2 (2.4)
Self-injurious Behavior	1.7 (2.4)	0.8 (1.4)	0.8 (1.3)	1.5 (2.5)	0.9 (1.9)	0.8 (1.8)
Compulsive Behavior	2.3 (2.3)	2.5 (3.1)	2.7 (3.9)	2.9 (2.5)	1.9 (1.8)	2.2 (2.3)
Ritualistic Behavior	4.4 (3.4)	3.8 (3.4)	4.2 (3.7)	4.7 (3.8)	3.2 (2.8)	3.0 (2.5)
Sameness Behavior	6.4 (5.0)	6.1 (5.4)	6.5 (6.2)	7.4 (5.0)	5.3 (4.4)	5.1 (4.2)
Restricted Interests	2.1 (2.0)	2.0 (1.8)	2.0 (1.9)	1.8 (1.6)	1.6 (1.6)	1.5 (1.3)
SP-NL quadrants	n=37	n=36	n=29	n=38	n=37	n=33
Low Registration	56.7 (9.5)	57.2 (10.0)	56.0 (8.0)	54.9 (10.7)	55.2 (11.7)	54.7 (12.8)
Sensation Seeking	101.2 (14.6)	104.9 (15.2)	104.3 (16.1)	101.3 (15.5)	105.3 (15.2)	104.7 (15.4)
Sensory Sensitivity	71.5 (10.3)	74.4 (11.5)	72.9 (10.3)	71.8 (10.8)	73.1 (11.2)	72.8 (11.2)
Sensation Avoiding	100.9 (14.5)	101.4 (16.1)	99.3 (13.6)	96.9 (17.1)	100.1 (16.5)	100.6 (16.3)
ABC subscales	n=37	n=37	n=32	n=37	n=38	n=36
Irritability	14.5 (7.9)	11.2 (7.2)	12.6 (7.0)	14.3 (8.2)	10.4 (8.5)	10.2 (7.1)
Lethargy	11.5 (6.0)	8.3 (6.1)	9.9 (6.5)	11.0 (9.3)	7.8 (7.8)	7.6 (7.0)
Stereotypy	3.5 (3.6)	2.8 (3.5)	2.3 (2.4)	3.6 (4.2)	2.6 (3.2)	2.5 (3.2)
Hyperactivity	19.8 (10.1)	16.2 (9.2)	17.0 (9.3)	19.8 (12.2)	15.0 (11.3)	14.5 (10.0)
Inappropriate Speech	3.7 (2.8)	3.7 (3.1)	3.2 (2.6)	3.7 (2.8)	3.3 (2.8)	2.8 (2.8)
ABC Total	53.1 (21.3)	42.2 (22.1)	45.3 (19.3)	52.5 (24.4)	39.1 (25.7)	38.0 (21.8)

Note: Data are mean (SD). SRS-2 = Social Responsiveness Scale-2; RBS-R = Repetitive Behaviors Scale-Revised; SP-NL = Sensory Profile-2; ABC = Aberrant Behavior Checklist.

Table S4. Individual Potassium Levels Split out per Treatment Arm

	BL	D4	D7	D14	Extra[1]	Extra[2]	D28	Extra[3]	D35	Extra[4]	D56	Extra[5]	Extra[6]	D91	D119
Bumetanide arm															
1	4.4	4.1	4.0	3.9			3.4	3.9	3.9		4.1			4.0	4.1
2	4.0	3.8	4.1	4.0			3.5		3.3	3.9	3.4	3.8		3.7	4.2
3	3.7	3.7	3.8	3.4	3.7		3.4		3.4	3.5	3.2	3.8	3.4	3.5	3.9
4	3.7	4.2	4.0	3.3	4.1		4.0		3.6						
5	4.2	4.4	4.1	4.5			3.7		3.6		4.0			3.9	4.1
6	4.0	3.9	3.7	3.4	4.1		3.5		3.5		3.7			3.7	3.9
7	3.8	4.5	4.1	3.5			4.1		3.9		3.8			3.9	4.0
8	3.9	4.8	4.4	4.6			5.7		4.0		4.1				
9	3.8	3.9	3.7	3.2	3.4	4.0	3.6		3.8		4.1				
10	3.5	3.8	4.2[a]	3.7			3.7				3.5				
11	3.9	3.8	3.7	3.7			3.6				3.9				
12	4.0	3.7	4.4	3.9			3.4	3.6							
13	4.2	3.8	3.9	3.8			3.7								
14	3.7[a]	4.1[a]	4.1[a]	4.1[a]			3.7	3.8			3.9				
15	4.1	4.2	4.0	3.9			3.9				4.1				
16	3.8	3.7	3.9	3.4	3.7		3.4				3.8				
17	4.1	3.9	3.7	3.4	3.5		3.4	4.1		3.7	3.7				
18	4.3	4.1	4.0	3.9			3.8				4.1[a]				
19	4.0	4.1	3.8	4.1			3.7				3.8				
20	3.6	4.4	4.0	3.8			3.7				3.9				
21	4.0	4.0	3.9	3.8			3.7				4.2				
22	4.0	4.0	3.9	4.1			3.9[a]	4.8			3.8				
23	4.0	3.7	3.8	3.6			3.7[a]	3.8			3.5				
24	3.8	3.6	3.9	3.3	3.7		3.5				3.7				
25	4.2		4.3	3.3	4.2[a]	3.8	3.8				3.6	4.1			
26	4.4	4.3	4.3	4.0			4.0				4.1				
27	3.7	3.6	4.1[a]	4.2			3.4	3.5			4.0				

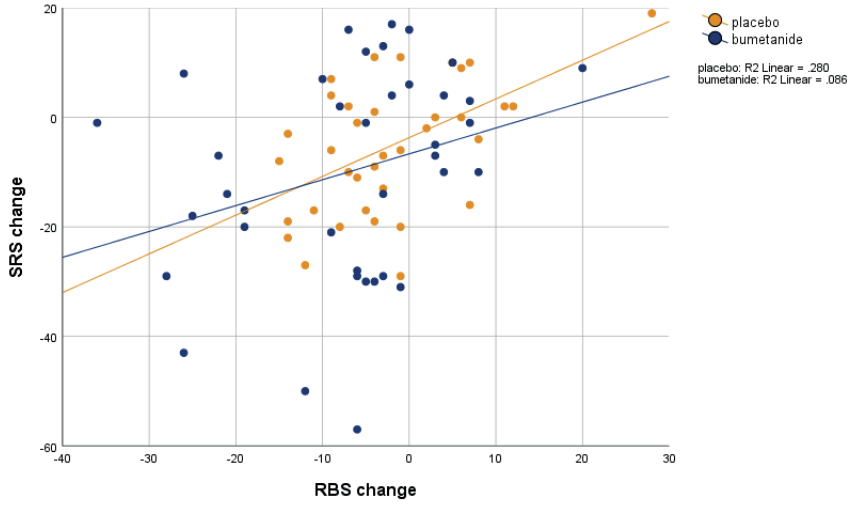
	BL	D4	D7	D14	Extra[1]	Extra[2]	D28	Extra[3]	D35	Extra[4]	D56	Extra[5]	Extra[6]	D91	D119
28	4.1		3.6	3.5			3.9				3.9				
29	4.0	3.9	3.8	4.1[a]	4.0		3.8				4.0				
30	3.8	3.5	3.5	3.7			3.5				3.3	3.2	3.3	3.7	
31	3.7	4.1	4.0	3.8			4.1				3.8				
32	3.9		3.9	3.5			3.9				3.7				
33	3.9	4.5	4.2	3.9			4.1				4.0				
34	3.9		3.8	3.8			3.6				3.3	4.3			
35	3.6	3.9	3.7	3.1	3.4		3.2	3.9[a]			3.9[a]				
36	3.6		3.7	3.4	3.6		3.3	3.5			3.4	3.9			
37	4.2	4.0	3.6	3.1	3.8		3.7				3.6				
38	4.3		3.8	3.6			3.6				3.9				
39	3.9		3.8	3.4			3.5				3.6				
40	4.5	4.7	4.7	4.0			3.3	3.6			3.8				
41	4.5		3.9	3.9			3.8	3.7			3.4	3.5			
42	4.0	3.6	3.8	3.3	3.3	3.6	4.1				4.3				
43	4.1	4.0	4.1	3.9			3.7				3.5				
44	4.2		3.6	3.5			3.4	4.3			4.2				
45	4.1		4.5[a]	4.5[a]	3.5		3.7				3.6				
46	3.9		5.0	4.3			4.2				4.0				
47	4.3	4.1[a]	3.8	4.2[a]			3.9				3.5[a]	3.4	3.9[a]		
Placebo arm															
1	3.9	4.4	4.2	4.3			4.1		4.1		3.9			4.1	4.2
2	4.1	4.0	4.1	4.5			4.3		4.0		4.3			4.0	4.8
3	4.1	3.9	3.6	4.0			3.9		3.9		4.1			3.5	
4	4.3	4.1	4.2	4.0			4.2		4.0		4.1			4.1	
5	4.0[a]	3.9	3.7	4.1			3.7		3.8		4.1				
6	3.8	3.8	4.0	4.1			4.0		3.9		4.2				
7	4.0	4.1	4.1	4.4			4.3		3.9		4.2				
8	4.0	4.1	4.0	4.5			4.1				4.3				
9	3.8	4.2	4.1	4.0			4.1		3.7		3.7				

	BL	D4	D7	D14	Extra[1]	Extra[2]	D28	Extra[3]	D35	Extra[4]	D56	Extra[5]	Extra[6]	D91	D119
10	3.7	3.6	3.9	3.7			3.7				3.7				
11	3.9	4.0	3.8	4.1			4.0				4.1				
12	3.8	4.1	4.1	4.3			4.2				4.2				
13	3.5	4.0	4.0	3.9			3.8				4.1				
14	4.0	3.8	4.0	3.8			3.7				3.8				
15	4.3	4.0	4.0	4.1			4.0				4.0				
16	3.7	4.1	4.2	4.0			4.2				4.1				
17	4.1	3.6	3.9	4.3			4.3				3.7				
18	4.4	3.9	4.1	4.4			4.1				3.8				
19	3.7	3.9	4.0	4.0			4.6[a]				4.2				
20	4.3	4.1	3.9	4.1			4.2				5.0				
21	3.9		4.8[a]	4.0			4.4				4.5[a]				
22	3.6	3.8	4.3	4.1			3.9				3.9				
23	3.7	4.1	4.2	4.3			4.3				4.0				
24	4.0	4.1	4.2	4.2			4.3				4.4				
25	4.1	3.9	4.0	4.0			4.2				3.9				
26	4.0	4.0	4.5	4.3			4.1				4.3				
27	3.8	4.3	4.4	4.1			4.3				4.6				
28	4.6	4.1	4.3	4.6			4.4				4.2				
29	3.7	3.9	4.1	4.1			4.0				4.0				
30	4.1	3.9	4.3	4.5[a]			4.4				4.3				
31	3.5	3.8	3.8	3.9			3.6				3.8				
32	4.1		5.1												
33	3.9	4.2	4.2	4.0		4.0	4.4								
34	3.9		4.1	4.2			4.5				4.1				
35	3.7	3.8	4.0	4.2			4.0				4.1				
36	4.3		4.1	4.7[a]			4.3				4.0				
37	4.3	4.2	3.9	4.0			4.3				4.2				
38	4.0	4.3	4.0	3.8			4.1[a]				4.0				
39	3.9	4.3	3.8	3.8			4.2				4.2				

	BL	D4	D7	D14	Extra[1]	Extra[2]	D28	Extra[3]	D35	Extra[4]	D56	Extra[5]	Extra[6]	D91	D119
40	4.3		4.3	4.2			4.4				4.1				
41	3.9	3.5	3.8	4.4			3.9				3.8				
42	4.8[a]	4.6	4.3	4.2			3.9				4.3				
43	4.0	4.6[a]	4.3	5.5[a]			4.3				4.2				
44	3.9		4.2	4.2			4.1				4.1				
45	4.1	4.1	3.8	4.2			4.1				4.4				

Note: Data are mmol/L; BL=baseline. [a]= haemolytic sample

Figure S2. Correlation Between Change in SRS-2 and RBS-R between D91 and D0



Note: Colors indicate placebo (orange) and bumetanide (blue) treatment. Correlations are tested with Pearson correlation and significance level $p = <.05$.

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"It's what we think we know that keeps us from learning." — Claude Bernard

CHAPTER 4

Effects of Bumetanide on Neurocognitive Functioning in Patients with Autism Spectrum Disorder: Secondary Analysis of a Randomized Placebo-Controlled Trial

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ABSTRACT

Background Neurocognitive deficits have repeatedly been associated with autism spectrum disorder (ASD), yet neurocognitive tests are seldom included in ASD medication trial designs. The aim of this study is to test the effect of bumetanide treatment on neurocognitive functioning in children with ASD by using analyses that consider the complexity and interdependence of neurocognitive functions.

Methods A secondary analysis of the 'Bumetanide in Autism Medication and Biomarker' (BAMBI) study, a randomized double-blind placebo-controlled (1:1) trial testing the effect of 3-months bumetanide treatment (up to 1mg twice-daily) in unmedicated children aged 7-15 years with ASD. Children with IQ<70 were excluded from analyses. Children were assessed at baseline and after 3 months with neurocognitive tests and questionnaires. Baseline performance was tested for deficits. Treatment effects were analysed on the intention-to-treat population with generalized linear models on neurocognitive domains resulting from principal component analysis (PCA) and network analysis parameters. Questionnaires were tested with linear mixed-models.

Results 92 children were allocated to treatment and 83 were eligible for analyses. Heterogeneous impairments in neurocognitive functioning were present at baseline. PCA revealed eight neurocognitive domains on which bumetanide did not show improvements, nor (sedative) side effects. Network analysis showed higher modularity after treatment (mean difference -.165, 95%CI -.317 to -.013, p=.034) and changes in the relative importance of response inhibition in the neurocognitive network (mean difference -.037, 95%CI -.073 to -.001, p=.042).

Conclusions This study showed neurocognitive impairments in children with ASD at baseline and relative changes in neurocognitive network organization through 3 months of bumetanide treatment.

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

Keywords ASD, bumetanide, RCT, children, cognition, neurocognitive functioning, network analysis

INTRODUCTION

Autism spectrum disorder (ASD) is classified by dysfunctions in social communication and restricted, repetitive behavior[1]. Consistent with this definition, ASD trial studies generally focus on behavioral outcomes as endpoint measures of efficacy. Yet, neurocognitive functioning deficits have systematically and repeatedly been reported in ASD, but neurocognitive outcome measures are very rarely included in trial designs. Both impairments in social and non-social neurocognitive functioning have been found in children and adults with ASD, suggesting a pervasive pattern of dysfunction in neurocognitive functioning[2,3]. Impairments in executive functioning[2,4,5] and working memory[6,7] are among the most prominent and consistent findings. Together, these findings have led to the suggestion that neurocognitive deficits underlie ASD core symptomatology, and that improvements in neurocognitive functioning may cascade to reduced core behavioral symptomatology[8-10]. This identifies neurocognitive function as an important treatment target, which is supported by evidence that neurocognitive functioning is predictive of adaptive functioning (i.e. how well one can perform daily activities and meet every day environmental demands), school attainment and professional career opportunities[11,12].

Since (non-social) neurocognitive tasks are rarely incorporated in ASD medication trials, potential medication effects on neurocognitive functioning are generally unknown. Studies in children with epilepsy have shown that anti-epileptic drugs (AEDs) can have neurocognitive (side) effects, both being able to improve and deteriorate neurocognitive functioning[13-18]. These studies do indicate feasibility of psychoactive medication to capture neurocognitive effects in a trial design, although wide variety in neurocognitive task methodologies precludes comparisons between trials[19]. Moreover, many neurocognitive tasks tap into overlapping constructs, thereby obscuring the detection of more specific neurocognitive effects. Furthermore, neurocognitive assessments are typically analyzed using a univariate approach (i.e. the measurement and analysis of isolated neurocognitive functions), while neurocognitive functions are known to have complex inter-dependency (for a review, see [20]). The conventional univariate approach to neurocognitive assessment therefore provides an oversimplified view of neurocognitive functioning, disregarding the complex interplay in a network of neurocognitive functions, with the risk of missing treatment (side) effects. These observations highlight the challenges and opportunities for neurocognitive assessment in trial designs. In the context of ASD trials, additional challenges are posited by extensive heterogeneity in neurocognitive profiles between and within children with ASD[21]. As a consequence, neurocognitive treatment effects in ASD are expected to be complex and not straightforward to detect at the group

level. This directly emphasizes the importance of more advanced approaches to neurocognitive assessment, which may allow for inter-individual differences in the neurocognitive profile and have the potential to detect more subtle, yet clinically important effects on neurocognitive functioning.

Several new treatment options have emerged that may improve neurocognitive functioning in ASD. These candidates have been derived from animal model studies and are regarded as mechanism-based treatments, for instance in their capacity to restore elements of synaptic plasticity and neuronal transmission. One example of such a treatment candidate is bumetanide, a diuretic that is being repurposed for ASD treatment. In several animal models of ASD, bumetanide has shown to reinstate GABAergic inhibition and enhance neuronal oscillations through correction of neuronal chloride homeostasis[22,23]. Following these observations, a number of randomized controlled trials (RCTs) in children with ASD have shown improvement in ASD symptomatology[24-28]. In these RCTs, the effect of bumetanide on neurocognitive functioning was not investigated. An open label study with TSC patients included neurocognitive tests, but found no treatment effects[29]. However, several lines of evidence suggest that bumetanide could affect neurocognitive functioning. A pilot study using fMRI and eye-tracking in children with ASD showed that 10 months of bumetanide treatment resulted in improved social neurocognitive function (i.e. emotional face processing)[30,31]. In patients with drug-resistant epilepsy, 6 months of bumetanide treatment improved performance on spatial memory tests[32]. In animal studies, bumetanide showed improvements in predominantly memory functioning in a valproate induced rat model[33] and in Down syndrome[34] and Huntington's disease mouse models[35]. These disease models are tightly linked to disturbances in chloride regulation and/or GABA polarity[36-40] and therefore suggest that bumetanide may restore certain aspects of neurocognitive functioning related to neuronal chloride homeostasis.

The aim of this study is to test the effect of bumetanide treatment on neurocognitive functioning in children with ASD. This study is performed as secondary analyses of the 'Bumetanide in Autism Medication and Biomarker' (BAMBI) study in which the effects of 3 months of bumetanide treatment on behaviour and electroencephalography were tested as well. We included a broad test battery to cover a range of important neurocognitive domains. As children with ASD show highly variable configuration of neurocognitive profiles, we expect complex multifactorial effects of bumetanide on cognitive functioning, and neurocognitive functions to show complex interplay. We therefore undertook an analysis strategy utilizing principal component analysis to

cluster neurocognitive domains and additionally applying network analysis to capture treatment effects on the organization of neurocognitive functions in a network.

METHODS

This study is part of the BAMBI study, of which the primary behavioral outcome has been previously reported[28]. The BAMBI trial was a mono-center, parallel-group, patient-randomized, double-blind, placebo-controlled phase-2 superiority trial testing the effect of bumetanide treatment during 91 days, followed by 28-day wash-out. Detailed information on the study design, sample selection procedures and sample characteristics can be found in Sprengers et al.[28]. In this paper we elaborate on details of the neurocognitive outcomes. The trial was conducted at the UMC Utrecht, the Netherlands, a nation-wide tertiary out-patient center, approved by the medical ethical committee of the UMC Utrecht and conducted in accordance with the provisions of the declaration of Helsinki and Good Clinical Practice. All participants or their legal representatives signed informed consent.

Participants

In brief, inclusion criteria were children aged 7-15 years with an expert confirmed ASD diagnosis according to DSM-IV-TR[41] (i.e. autism, Asperger syndrome or PDD-NOS) or DSM-5[1] criteria and either an ADOS≥6, SRS-2 T-score ≥60 or a confirmed diagnosis by an independent in-house child-psychiatrist. Exclusion criteria were an IQ<55 (and IQ<70 for neurocognitive analyses in this paper); psychoactive medication use less than eight weeks prior to screening visit (except chronic melatonin treatment); start of any new therapy for developmental disorder problems (e.g. cognitive behavioral therapy); comorbid neurological disorders; chronic renal disease; unstable serious illness; NSAID treatment; and/or documented history of hypersensitivity reaction to sulphonamide derivatives. Furthermore, children were allowed to receive care as usual.

Study design

Eligible participants were allocated to receive bumetanide or placebo treatment. Patients, parents, healthcare providers and outcome assessors were masked for randomization. Participants received bumetanide liquid formulation (0.5mg/ml) or placebo formulation matched for taste, smell and viscosity, albeit without diuretic properties. The formulation was twice-daily administered orally with minimally 6 hours between the gifts. Children <30kg started with twice-daily 0.015mg/kg bumetanide or an equivalent volume of the placebo formulation. Children ≥30kg

received twice-daily 0.5mg bumetanide or placebo (i.e. 1ml). When blood analysis showed no abnormalities at D7, the dosage was doubled. All participating children were supplemented with 0.5mmol/kg potassium chloride <30kg, or twice-daily 8mmol potassium chloride ≥30kg.

During the first visit, baseline clinical outcomes were assessed (e.g. questionnaire) and during subsequent baseline assessment, neurocognitive measurements were performed. Participants returned for outcome evaluations at the end of the 91-day medication phase (with neurocognitive assessment) and at the end of the 28-day wash-out period (without neurocognitive assessment).

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

Outcomes

Neurocognitive measures

An abbreviated WISC-III intelligence test was conducted to screen intelligence for study exclusion (when no IQ test was performed in the previous two years). Since we could not formulate a priori hypothesis on the nature and extent of neurocognitive effects of bumetanide in ASD, we composed a test battery measuring a broad range of neurocognitive functions. The following domains were selected: information processing speed and attention, memory and executive functioning using a balanced battery assessing both auditory and visual processing. The neurocognitive tasks, the corresponding functions they intend to measure and variables used in the analyses are depicted in Table 1. The duration of the complete neurocognitive battery was approximately 120 minutes, which was performed in the morning in a quiet room with one trained psychological assistant supervised by a clinical psychologist. Basic processing speed, response inhibition and attentional flexibility were measured with subtasks of the Amsterdam Neuropsychological Task battery (ANT)[42]. Working memory for auditory and visual information were assessed with respectively the digit span of the Wechsler Intelligence Scale for Children-III (WISC-III-DS)[43] and spatial span of the Wechsler Nonverbal Scale of Ability (WNV-SS)[44,45]. Verbal and visual learning and memory were examined with the Rey Auditory Verbal Learning Test (RAVLT)[46] and the Rey Visual Design Learning Test (RVDLT)[47]. The 'post-response interval' between the different trials of the ANT tasks was event-driven. Different versions of the RAVLT were used to minimize practice effects.

Table 1. Description of neurocognitive tasks and functions

Task	Stimulus	Reference in paper	Short task description	Variables for PCA
RAVLT[46]	Auditory	Verbal memory	Immediate recall of 15 verbalized words. Repeated 5 times. Assesses immediate memory span (after each presentation), delayed recall, and recognition memory for verbal information.	RAVLT imprinting; RAVLT consolidation*; RAVLT retrieval*
RDVLT[47]	Visual	Visual memory	Immediate recall of 15 figures on a computer screen. Repeated 5 times. Assesses immediate memory span (after each presentation), delayed recall, and recognition memory for non-verbal information.	RVDLT imprinting; RVDLT consolidation*; RVDLT retrieval*
WNV-SS[44,45]	Visual	Visual working memory	Immediate repetition of a series of blocks being tapped, in the original and reversed order.	WNV-SS ce*; WNV-SS forward
WISC-III DS[43]	Auditory	Verbal working memory	Immediate repetition of verbalized digits, in the original and reversed order.	DS ce*; DS forward
Go No-Go (ANT)[42]	Visual	Response inhibition	"Go" and "No-Go"-stimuli are presented (either in equal proportion or with more Go-stimuli in the 'biased' trials, which is more difficult). A keyboard response has to be inhibited on the No-Go stimuli. The response time and the percentage of false alarms are measured.	GNG RT; GNG false alarms; GNG_B RT; GNG_B false alarms
Baseline speed (ANT)[42]	Auditory	Baseline speed and stability	Basic RT task, requiring an immediate keyboard response when seeing a square on the computer screen.	BS RT; BS SD
SSA part 1 (ANT)[42]	Auditory	Auditory baseline speed	Auditory baseline speed task. In this fixed compatible condition prepotent responses are required by keyboard reactions to auditory stimuli.	SSA_1 RT
SSA part 2 (ANT)[42]	Auditory	Auditory prepotent response inhibition	Prepotent response inhibition: In this fixed incompatible condition, prepotent responses to the auditory stimuli should be inhibited and replaced by an incongruent response. Response inhibition is calculated from the difference between the incompatible trials (part 2) and compatible trials (part 1).	SSA_2-1
SSA part 3 (ANT)[42]	Auditory	Auditory attentional flexibility	Attentional flexibility: the compatible and incompatible trials are now randomly presented. Congruent or incongruent responses should be accurately given depending on the stimulus. Attentional flexibility is calculated from the difference between the compatible trials in the random condition from part 3 and the fixed condition in part 1.	SSA_3C-1

Task	Stimulus	Reference in paper	Short task description	Variables for PCA
SSV part 1 (ANT)[42]	Visual	Auditory baseline speed	Visual baseline speed task. Comparable with SSA-task but presenting visual stimuli.	SSV_1 RT
SSV part 2 (ANT)[42]	Visual	Visual prepotent response inhibition	Comparable with SSA-task part 2 but presenting visual stimuli. Response inhibition is calculated from the difference between the incompatible trials (part 2) and compatible trials (part 1).	SSV_2-1
SSV part 3 (ANT)[42]	Visual	Visual attentional flexibility	Comparable with SSA-task part 3 but presenting visual stimuli. Attentional flexibility is calculated from the difference between the compatible trials in the random condition from part 3 and the fixed condition in part 1.	SSV_3C-1

*Calculations of the variables are shown in supplementary information 1. *Abbreviations*. ANT: Amsterdam neuropsychological tasks; -B: biased trials; BS: baseline speed; -C: compatible trials; ce: central executive; PCA: principal component analysis; RAVLT: Rey Auditory Verbal Learning Test; RT: reaction time; RVDLT: Rey Visual Design Learning Test; SD: standard deviation; SSA: Shifting Attentional Set Auditory; SSV: Shifting Attentional Set Visual; WISC-III DS: Wechsler Intelligence Scale for Children-III digit span; WNV-SS: Wechsler Nonverbal Scale of Ability – subtask spatial span.

Behavioral questionnaires

In addition to the neurocognitive task battery, a behavioral equivalent of neurocognitive processes was measured. The Behavior Rating Inventory of Executive Functioning (BRIEF; both parent and teacher reported)[48] was included to measure executive functioning behaviors in school and home environments. Core symptom behavior was measured with the Social Responsiveness Scale 2 (SRS-2)[49] and the Repetitive Behaviors Scale – Revised (RBS-R)[50].

Statistical analysis

These secondary analyses were part of the statistical plan of the BAMBI study, except for the network analysis. First, we defined neurocognitive deficits at baseline (i.e. z-scores ≤-1) including only the tasks for which norm-data (based on typically developing children as presented in the test-manuals) were available (i.e. no norm-data were available for computed, difference or domain scores). It is important to note that norm-data were predominantly unavailable for participants aged >12 years, which might cause bias. Significance was tested with one sample chi-square tests assuming 16% deviation in the norm population (i.e. z-score ≤-1). Further analyses of treatment effect therefore included raw data in order to include all participants and all tasks. Second, contrast scores were computed to isolate specific neurocognitive

functions (see Supplementary Information 1). Third, domain scores were extracted using data-driven principal component analysis (PCA) with varimax rotation from the Psych package in R[51]. The number of components (i.e. neurocognitive domains) to extract was determined using the elbow method and the cumulative variance explained from the eigenvalues histogram (R^2 ~ 70%). Subsequently, each component was labeled as a neurocognitive domain based on the set of test scores that made the strongest contribution to the components, as measured in terms of η^2. The boundary for this set of test scores was set at the largest drop in n^2 between two subsequent variables in the scree plot. The eight neurocognitive domains resulting from this procedure were used in subsequent analyses (see the Results section). Fourth, we tested treatment effect on the neurocognitive domains using generalized linear models (GLMs), whereas the BRIEF-questionnaires also included wash-out data and were analyzed with linear mixed models (LMMs). The models included baseline measurement, age and sex to correct for potential confounding factors. Assumptions were tested by residual plots. Estimated means per treatment group and mean differences after treatment were calculated with 95% confidence intervals and p-values. Treatment interactions with sex and age were tested with likelihood ratio rests. Finally, we explored treatment effects on neurocognitive network organization using neurocognitive network analysis. This is an innovative approach that uses network analysis on an individual's neurocognitive data from a single assessment. Dispersion in one's neurocognitive profile is used as the measure of connectivity, which is calculated between each possible pair of neurocognitive functions by the intra-individual difference in relevant test scores (z-score)[52]. This was used to construct connectivity matrices, from which we calculated global network parameters (strength, modularity, assortativity, characteristic path length, transitivity, and smallworldness; reflecting organization of neurocognitive functions in the network as a whole) and local network parameters (hubness score, reflecting the relative importance of each neurocognitive function in the network)[52]. To limit the number of comparisons, we selected the analysis regarding local network parameters to the 20% most influential neurocognitive functions in the network. For more information regarding the neurocognitive network analysis and global and local network parameters, see Königs et al.[52]. We tested global and local network parameters for treatment effects with the same GLMs as described for the analysis of neurocognitive domains. All GLMs analyses were performed with SPSS v25 (IBM, Corp., Armonk, NY), LMMs with SAS v9.4 (SAS Institute, Cary, NC) and PCA and network analyses were performed with the 'igraph' and 'qgraph' packages[53,54] in R v3.6[55].

RESULTS

Participant characteristics

Participants were enrolled between June 21st 2016 and December 6th 2018 and 92 participants were randomly allocated to treatment. 88 participants completed the study of which 6 were excluded for neurocognitive analyses because their IQ was below 70 and 3 due to unreliable measurements. The resulting sample that is analyzed in this paper is depicted in Table 2.

Table 2. Baseline characteristics of the BAMBI population

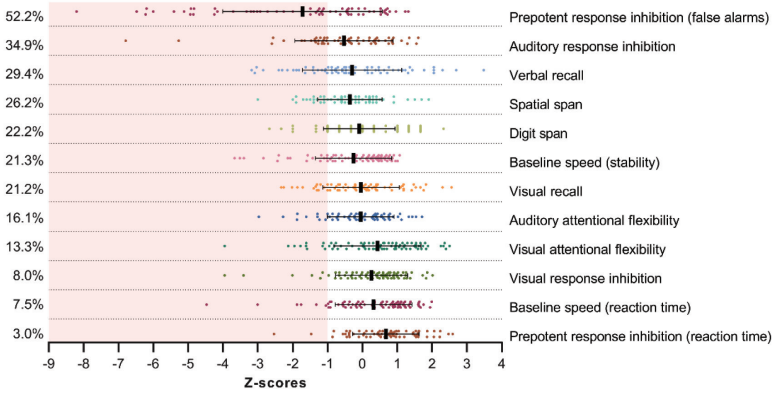
	Placebo group (*n*=41)	**Bumetanide group (*n*=42)**	**Total (*n*=83)**
Age (y, SD)	10.5 (2.4)	10.5 (2.4)	10.5 (2.4)
Male (%)	29 (70.7)	32 (76.2)	61 (73.5)
Female (%)	12 (29.3)	10 (23.8)	22 (26.5)
IQ (SD)	104.9 (18.4)	103.3 (17.7)	104.1 (18.0)
SRS-2 (SD)	89.4 (18.8)	88.7 (21.0)	89.0 (19.8)
ADHD (%)	7 (17.1)	10 (23.8)	17 (20.5)

Note. Data are mean (SD) or N (%). Y = year; ADHD = Attention-Deficit/Hyperactivity Disorder; SRS-2 = Social Responsiveness Scale (range 0-195; higher score is more affected).

Outcomes

Figure 1 presents an overview of neurocognitive deficits at baseline (i.e. z-scores ≤-1 based on available norm-data) arranged in order from highest to lowest percentage deviance in z-scores. The most prevalent deficits were observed in the number of false alarms (in 52.2% of the participants) on the GNG task, indicative of deficits in response inhibition (χ^2 [1, n=68] = 74.65, p=.000), followed by deficits on SSA task 2 measuring auditory prepotent response inhibition (34.9%, χ^2 [1, n=65] = 18.17, p=.000) and RAVLT measuring verbal recall (number of items: 29.4%, χ^2 [1, n=68] = 9.10, p=.003). The least frequently observed deficits were observed in reaction time on the GNG task (3.0%, χ^2 [1, n=68] = 8.63, p=.003) and the baseline speed task (7.5%, χ^2 [1, n=82] = 4.6, p=.032). These baseline results confirm extensive variability in neurocognitive profiles among participants and deficits in (prepotent) response inhibition.

Figure 1. Deviations in neurocognitive performance at baseline compared to typically developing children



Notes. Baseline score of neurocognitive tasks (non-computed scores) ≤-1 standard deviation are depicted in the shaded area. Domains are arranged in order from highest to lowest percentage of deviant z-scores (percentages shown on the left).

The PCA yielded eight components that together explained 76% of the variance of all neurocognitive tasks. We named these components according to the common neurocognitive domains they represented: information processing and control, memory imprinting, visual memory, verbal memory, visual working memory, verbal working memory, attentional flexibility, and motor inhibition. Supplementary Table 1 shows the concomitant factor loadings.

Analyses of treatment effect after 3 months treatment with generalized linear models showed no superior effect of bumetanide versus placebo on any of the neurocognitive domains (p>.275; see Table 3). Secondary analyses with sex and age showed no interaction effects (p>0.07, data not shown).

Table 3. Treatment effect after 91 days for the bumetanide and placebo group

	Placebo group		**Bumetanide group**		**Treatment effect**	**p-value**
	Baseline	**D91**	**Baseline**	**D91**		
n	41	41	42	42		
Domain 1: Information Processing and Control						
Mean	.03 (.94)	-.13 (1.09)	.13 (1.01)	-.03 (.85)	-.62 (-3.25 to 0.201)	.646
Domain 2: Memory Imprinting						
Mean	-.01 (1.04)	.22 (.87)	-.26 (.91)	.06 (1.03)	.030 (-0.302 to 0.361)	.861
Domain 3: Visual Memory						
Mean	.00 (1.02)	.05 (1.34)	.11 (.84)	.16 (.97)	.248 (-.229 to 0.725)	.308

	Placebo group		Bumetanide group		Treatment effect	p-value
	Baseline	D91	Baseline	D91		
Domain 4: Verbal Memory						
Mean	-.01 (.88)	-.08 (.97)	.02 (1.0)	.07 (1.03)	-.168 (-0.586 to 0.249)	.429
Domain 5: Visual Working Memory						
Mean	.01 (1.27)	.02 (.85)	-.06 (.85)	.03 (.64)	-.025 (-0.324 to 0.274)	.868
Domain 6: Verbal Working Memory						
Mean	.05 (1.07)	.21 (.85)	-.24 (1.12)	-.02 (.80)	.190 (-0.151 to 0.530)	.275
Domain 7: Attentional Flexibility						
Mean	-.37 (1.22)	.09 (.90)	.03 (.85)	.24 (1.01)	.010 (-0.350 to 0.369)	.957
Domain 8: Motor Inhibition						
Mean	-.15 (1.03)	.31 (.63)	-.31 (1.20)	.15 (.83)	.103 (-0.161 to 0.367)	.444

Notes. Generalized linear models on neurocognitive components after 91 days of bumetanide or placebo treatment. Data are means (SD). Data is shown for the participants intention-to-treat population. Treatment effects are measured with factors: Treatment and Sex; covariates: baseline score and Age; and shown with (95% CI). Significance level is $p<0.05$.

To test whether parent and teacher-reported questionnaires of neurocognitive functioning showed treatment effects, the BRIEF was analyzed. Based on total scores we found no difference in symptom severity after bumetanide treatment (Supplementary Table 2). Supplementary Tables 3 and 4 show changes in all subscales after treatment and wash-out.

Next, the complex inter-dependency between neurocognitive functions was investigated using network analysis. The average neurocognitive network is displayed in Figure 2. Analyses of treatment effect on neurocognitive network organization (Table 4) revealed a significant effect on the global network parameter modularity ($p = .034$), while no effects were found for strength, assortativity, characteristic path length, transitivity, and smallworldness. The bumetanide group showed higher modularity after treatment compared to the placebo group, indicating a stronger degree of subdivision of the neurocognitive network into specialized modules. In contrast, no effects were observed in terms of total connectivity in the network (strength), hierarchy (assorativity), integration (characteristic path length), clustering (transitivity) or balance between integration and clustering (smallworldness).

Considering local network parameters, the following neurocognitive functions had the highest hubness score, indicating that these functions have high relative importance in the neurocognitive network: visual baseline speed (SSV 1 RT), baseline speed (BS RT), stability of baseline speed (BS SD), auditory baseline speed (SSA 1 RT), visual recall (RVDLT imprinting) and response inhibition (GNG biased RT).

Table 4. Treatment effect on neurocognitive network organization after 91 days

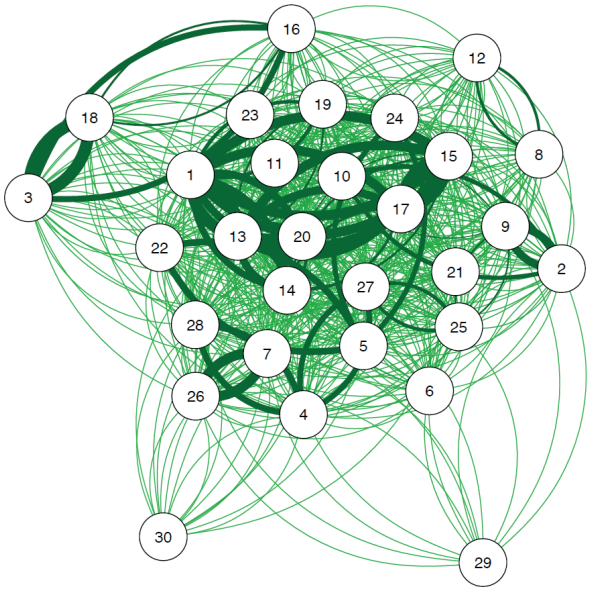
	Placebo group		Bumetanide group		Treatment effect	p-value
	Baseline	**D91**	**Baseline**	**D91**		
Global network parameters						
n	41	41	42	42		
Characteristic path length						
Mean	.75 (.02)	.75 (.02)	.75 (.02)	.75 (.02)	.002 (-.006 to .009)	.660
Assortativity						
Mean	.04 (.04)	.04 (.05)	.04 (.04)	.03 (.05)	.008 (-.011 to .027)	.398
Strength						
Mean	24.88 (9.16)	22.60 (9.73)	24.17 (8.1)	21.65 (8.74)	.678 (-3.111 to 4.466)	.726
Transitivity						
Mean	.35 (.01)	.35 (.01)	.35 (.01)	.35 (.01)	-.002 (-.007 to .003)	.376
Modularity						
Mean	1.56 (.40)	1.43 (.30)	1.56 (.39)	1.59 (.43)	-.165 (-.317 to -.013)	.034*
Smallworldness						
Mean	.54 (.02)	.54 (.04)	.53 (.02)	.54 (.02)	-.002 (-.015 to .011)	.752
Local network parameters						
n	41	41	42	42		
SSV 1 RT						
Mean	.41 (.10)	.42 (.08)	.42 (.11)	.44 (.09)	-.019 (-.056 to .017)	.299
BS RT						
Mean	.43 (.06)	.41 (.09)	.41 (.09)	.39 (.11)	.016 (-.026 to .059)	.452
BS SD						
Mean	.42 (.06)	.39 (.09)	.39 (.11)	.37 (.11)	.024 (-.017 to .066)	.246
SSA 1 RT						
Mean	.39 (.10)	.39 (.10)	.40 (.10)	.38 (.10)	.000 (-.041 to .041)	.998
RVDLT imprinting						
Mean	.42 (.09)	.38 (.11)	.39 (.11)	.37 (.13)	-.005 (-.051 to .042)	.844
GNG biased RT						
Mean	.37 (.09)	.36 (.10)	.39 (.08)	.40 (.06)	-.037 (-.073 to -.001)	.042*

Notes. Generalized linear models on global and local (top 6 hubness) network parameters after 91 days of bumetanide or placebo treatment. Data are means (SD). Data is shown for the intention-to-treat population. Treatment effects are measured with factors: Treatment and Sex; covariates: baseline score and Age; and shown with (95% CI). Significance level is $p<0.05$. Abbreviations. BS: baseline speed; GNG: Go No-Go; RT: reaction time; RVDLT: Rey Visual Design Learning Test; SD: stability; SSA: Shifting Attentional Set Auditory; SSV: Shifting Attentional Set Visual.

We found a treatment effect on response inhibition (GNG biased RT; p = .042), while no effects were found regarding the other network hubs. Regarding response inhibition, the bumetanide group showed increased hubness after treatment whereas the placebo group showed decreased hubness after treatment. This finding suggests that bumetanide treatment may increase the relative importance of response inhibition in the neurocognitive network.

In the children allocated to bumetanide, both change in modularity and change in response inhibition hubness showed no correlation with change in ASD behavior measured by the SRS-2 (respectively ρ=.239, p=.154; ρ=.032, p=.849) or the RBS-R (respectively ρ=.114, ρ=.508; ρ=.111, ρ=.519).

Figure 2. Schematic representation of the average neurocognitive network



Notes. The circles represent the individual test parameters, the lines the associations between them, with thicker lines representing larger associations. Neurocognitive domains: 1 = information processing and control; 2 = memory encoding; 3 = motor inhibition; 4 = visual memory; 5 = attentional flexibility; 6 = visual working memory; 7 = verbal working memory; 8 = verbal memory. Other variables: 9 = RAVLT imprinting; 10 = RVDLT imprinting; 11 = WNV-SS forward; 12 = DS forward; 13 = BS RT; 14 = BS SD; 15 = GNG RT; 16 = GNG false alarms; 17 = GNG_B RT; 18 = GNG_B false alarms; 19 = SSV_1 RT; 20 = SSA_1 RT; 21 = SSV_2-1; 22 = SSV_3C-1; 23 = SSA_2-1; 24 = SSA_3C-1; 25 = RAVLT retrieval; 26 = RAVLT consolidation; 27 = RVDLT retrieval; 28 = RDVLT consolidation; 29 = DS ce; 30 = WNV-SS ce.

DISCUSSION

We presented the secondary analyses of the neurocognitive data in the BAMBI trial. We found that the cohort manifested with mild heterogeneous impairments in neurocognitive functioning at baseline. Treatment did not result in (sedative) side effects, other than mild diuretic effects. Principle component analysis (PCA) revealed eight neurocognitive domains, which were not improved through bumetanide treatment. Network analysis showed subtle treatment effects by increased specialization in the neurocognitive network (i.e. higher modularity) and opposite effects of bumetanide and placebo on the relative importance of response inhibition in the neurocognitive network, both of which showed no linear correlations with clinical improvement. These results are in line with our preconceived notion that neurocognitive effects of bumetanide would show non-linear relations to clinical effects due to the complexity of neurocognitive networks and heterogeneity within ASD.

A comprehensive neurocognitive test battery was composed since a-priori effects of bumetanide on neurocognitive functioning were not available. The test battery consisted of equivalent auditory and visually processed tests covering information processing speed and attention, memory and executive functioning, as these domains have shown to be affected in ASD populations[3,56-60]. The primary analysis of the BAMBI trial focused on evaluating behavioral effects of bumetanide for which it used an ASD symptom thresholds as the inclusion criterion. For these secondary analyses, no inclusion criterion of neurocognitive deficits (apart from an IQ>70 for reason of task comprehension) was used to stratify for a more affected population, which would have potentially increased the likelihood of finding larger effect sizes. To control for multiple testing with the elaborate test battery, test scores were first assembled with PCA. The PCA showed rational clustering of tasks in eight separate domains in line with the test designs. The test battery in this sample showed heterogeneous baseline neurocognitive impairments with significant deficits in verbal recall, response inhibition and auditory prepotent response inhibition compared to typically developing age-matched children. In contrast, this ASD cohort seemed to excel in their reaction times in the response inhibition task, in which they were faster compared to typically developing children. However, this concurred with high rates of false alarms, which illustrates the difficulty when analyzing neurocognitive tasks when the outcomes show an intricate trade-off.

Absolute treatment effects

This study addressed neurocognitive treatment effects at two levels of statistical analysis. First it adhered to common randomized controlled trial methodology by regarding the *absolute effects* on the test outcomes. One important finding is that,

as is expected from its mechanism of actions, bumetanide treatment does not harm neurocognitive processes, which has been shown for several AED or antipsychotic drug treatments[61,62]. Indirectly, this shows that bumetanide probably has no sedative effects since no impairments in neurocognitive performance, nor slowing of motor response were observed. This is in line with clinical observations of the study participants and anecdotal reports of their parents that frequently described them as being "more aware" or "more attentive". The absence of treatment effects on individual neurocognitive tasks maybe due to the limited duration of treatment (i.e. 3 months) for improvements to be established, or the study being underpowered to detect small treatment effect sizes in this non-stratified study population.

Relative treatment effects

Second, we performed network analysis to analyze whether *relative effects* were evident. With this term we imply the organization of the individual neurocognitive network, without this depending on specific functions. Hereby, we consider the pre-existing plurality of neurocognitive strengths and weaknesses and focus on the question whether neurocognitive functions will relate differently to each other after treatment. With this novel strategy to analyze neurocognitive outcomes, we identified significant changes in the global network parameter modularity and the local network parameter (i.e. hubness score) of response inhibition compared to placebo-induced changes. A sensitivity analysis was run to assess the effects of imputation used for the intention-to-treat analysis, which replicated the reported treatment effects, although the significance for the hubness score of response inhibition just escaped significance (-.036 [-.75 to .002], p = .062). At this stage, it is difficult to relate these connectivity changes to clinical or real-world changes, since they can no longer be directly pinpointed to single tasks or brain processes and changes showed no linear correlations with clinical improvement. Nevertheless, the effect on modularity reflects that the neurocognitive network adapts to a more specialized organization in response to bumetanide, while the relative importance of response inhibition decreased, the latter being impaired in 50% of the children at baseline. These changes did not show a linear association with improvement in social behavior, stereotype and repetitive behavior (which did show significant improvement in the BAMBI-trial) or in executive behavior. Nevertheless, the findings support the idea that bumetanide has influence on neurocognitive functioning, which warrants further research into the subsequent (non-linear) impact on daily life functioning. This may also support the hypothesis that bumetanide effects are diluted and not discovered in unselected ASD populations. These tentative relative effects and a lack of absolute effects make neurocognitive benefits of bumetanide treatment, at this point, uncertain.

Although speculative, these subtle neurocognitive changes may fit the suggested mechanism of action of bumetanide on excitatory and inhibitory (E/I) balance in neuronal networks. It may be conceivable that the frequently observed neurocognitive deficits in ASD are in part a reflection of E/I imbalances[63-66]. Furthermore, studies in computational psychology and electrophysiology provide evidence for tight regulation of the balance between excitation and inhibition in order to maintain efficient neurocognitive information processing[67-70]. Small deviations are speculated to influence neurocognitive functioning[67] and dysregulation of this balance has been implicated in several neurological (Alzheimer, Parkinson, Huntington, epilepsy) and psychiatric (ASD, Down syndrome, schizophrenia) diseases[36-40], all of which are characterized by neurocognitive deficits.

To our knowledge this was one of the first ASD trials with children to include neurocognitive measurements in addition to behavioral outcomes[71]. During this trial we encountered multiple limitations and recommendations for future studies. First, 3-months of treatment might have been too short to establish profound neurocognitive changes. Parents consistently reported gradual treatment effects in terms such as "being able to catch up developmental processes" or "being more aware", which may need more time to become detectable in neurocognitive tests. Indeed, other psychoactive drugs, such as antidepressant, antipsychotic and anti-epileptic drugs also show gradual effects taking several months to become apparent. In comparison, studies with stimulants are by definition associated with more immediate neurocognitive effect[72,73]. It is conceivable that bumetanide has a more delayed onset of effects on neurocognitive functioning due to the time course of plasticity alterations in neurocognitive networks with altered GABAergic transmission[31]. Second, the variability of neurocognitive profiles at baseline might obfuscate effects, although this is inherently related to the unselected sample design. Third, there is a large variation in neurocognitive tasks and scripts, of which the majority was developed to characterize neurocognitive impairments rather than to detect treatment effects. As a consequence, most test are performed under ideal circumstances, minimalizing the chance of false positive findings at the expense of ecological validity. Moreover, longitudinal testing would be preferred at multiple time-points rather than the two in the current design. Finally, initiatives to harmonize neurocognitive test batteries are evidently warranted for comparability of treatment effects between trials.

In conclusion, this study showed baseline neurocognitive impairments in children with ASD with small changes in neurocognitive network organization after treatment, which could imply neurocognitive treatment effects of bumetanide. This proof-of-

concept trial showed that inclusion of neurocognitive tests can be important for understanding mechanism-based treatment effects and to comprehend how they can have beneficial effects in children with ASD. Extended treatment duration and adaptations to the test battery and its analysis e.g. network analysis come forward as important considerations for future trial study designs in these heterogeneous populations.

SUPPLEMENTARY MATERIAL

Supplementary Information 1. Computed contrast scores for specific neurocognitive functions

Verbal Memory Retrieval

et_scaled$WT_ret <- (et_scaled$WT_RECOG - et_scaled$WT_DELAY) * -1

Verbal Memory Consolidation

et_scaled$WT_cons <- (et_scaled$WT_DELAY - et_scaled$WT_IMPRINT)

Visual Memory Retrieval

et_scaled$RVDLT_ret <- (et_scaled$RVDLT_RECOG - et_scaled$RVDLT_DELAY) * -1

Visual Memory Consolidation

et_scaled$RDVLT_cons <- (et_scaled$RVDLT_DELAY - et_scaled$RVDLT_IMPRINT)

Verbal Working Memory Central Executive

et_scaled$CR_ce <- et_scaled$CR_BACKW - et_scaled$CR_FORW

Visual Working Memory Central Executive

et_scaled$WNVRO_ce <- et_scaled$WNVRO_BACKW -et_scaled$ WNVRO_FORW

SUPPLEMENTARY TABLE 1. Principal component analysis on neurocognitive tasks

	Domain 1	**Domain 2**	**Domain 3**	**Domain 4**	**Domain 5**	**Domain 6**	**Domain 7**	**Domain 8**
	Information processing speed and control	**Memory imprinting**	**Visual memory**	**Verbal memory**	**Visual working memory**	**Verbal working memory**	**Attentional flexibility**	**Motor inhibition**
	Loadings	Loadings	Loadings	Loadings	Loadings	Loadings	Loadings	Loadings
BS RT	0.889	0.144	NA	-0.110	NA	0.140	NA	NA
GNG RT	0.875	0.177	NA	NA	0.195	NA	NA	NA
GNG_B RT	0.852	0.151	NA	NA	0.164	NA	0.200	NA
SSA_1 RT	0.764	NA	NA	0.222	-0.139	NA	NA	NA
BS - SD	0.726	0.199	0.219	-0.101	-0.278	NA	NA	0.121
SSV_1 RT	0.630	NA	NA	0.178	NA	0.185	0.167	NA
RAVLT imprinting	0.196	0.823	NA	-0.192	0.113	NA	NA	NA
RVLDT imprinting	0.327	0.664	-0.112	0.114	NA	NA	0.182	0.217
RVDLT consolidation	0.282	0.236	0.740	NA	NA	-0.102	NA	NA
RVDLT retrieval	NA	0.302	0.730	0.269	0.194	NA	NA	-0.128
RAVLT consolidation	0.101	-0.108	0.110	0.873	-0.105	NA	NA	-0.166
RAVLT retrieval	NA	0.497	0.393	0.521	0.304	NA	-0.158	-0.193
WNV-SS ce	NA	NA	NA	0.107	-0.893	NA	NA	NA
WNV-SS forward	0.512	0.349	NA	0.193	0.542	NA	NA	0.244
DS ce	-0.134	0.118	NA	0.102	NA	-0.917	NA	NA
DS forward	0.355	0.463	-0.162	NA	NA	0.662	NA	NA
SSV_3C-1	NA	-0.186	NA	0.109	NA	0.119	0.761	0.166
SSA_3C-1	0.282	0.199	-0.181	-0.149	NA	NA	0.706	NA
SSV_2-1	0.259	NA	NA	NA	-0.385	-0.218	0.526	NA
GNG_B false alarms	NA	NA	NA	NA	NA	NA	NA	0.926
GNG false alarms	0.149	0.316	NA	-0.300	NA	NA	NA	0.778
SSA_2-1	0.458	NA	-0.539	NA	0.140	0.117	0.311	NA

Notes. [a]PCA with varimax rotation. *Abbreviations*. 2-1: part 2 – part 1; 3C-1: part 3 with compatible trials – part 1; BS: baseline speed; -C: compatible trials; Ce: central executive;; DS: Wechsler Intelligence Scale for Children-III digit span; GNG: Go No-Go; GNG_B: Go No-Go Biased; NA: Not applicable; RAVLT: Rey Auditory Verbal Learning Test; RT = reaction time; RVDLT: Rey Visual Design Learning Test; SSA: Shifting Attentional Set Auditory; SD: standard deviation; SSV: Shifting Attentional Set Visual; WNV-SS: Wechsler Nonverbal Scale of Ability – subtask spatial span. NA = variance explained <10%.

SUPPLEMENTARY TABLE 2. Treatment effect on BRIEF total score after 91 days

	Placebo group			Bumetanide group			Treatment effect	p-value
	Baseline	**D91**	**D119**	**Baseline**	**D91**	**D119**		
BRIEF-parent total								
n	30	30	30	31	31	31		
Mean	166.37 (18.43)	160.30 (22.43)	160.77 (22.76)	161.81 (20.13)	152.23 (21.22)	153.68 (20.37)	-1.576 (-7.57 to 4.42)	.602
BRIEF-teacher total								
n	11	11	11	22	22	22		
Mean	145.91 (26.15)	136.82 (20.08)	139.45 (28.01)	146.32 (34.73)	141.91 (28.06)	140.82 (31.13)	3.565 (-6.32 to 13.45)	.470

Notes. Linear mixed model after 91 days of bumetanide or placebo treatment. Data are means (SD). Treatment effects are measured with linear mixed models (model including visit, baseline score, sex and age) and shown with (95% CI). *Abbreviations:* BRIEF: The Behavior Rating Inventory of Executive Functioning.

SUPPLEMENTARY TABLE 3. Means of BRIEF-parent subscales after treatment and wash-out

	Placebo group			Bumetanide group		
	Baseline	**D91**	**D119**	**Baseline**	**D91**	**D119**
Inhibit						
n	40	36	34	42	38	38
Mean	21.8 (4.8)	21.1 (4.5)	21.8 (4.5)	21.1 (5.1)	19.7 (4.9)	19.5 (4.9)
Shift						
n	40	36	34	42	38	38
Mean	18.2 (3.7)	17.4 (3.7)	18.0 (3.9)	18.3 (2.9)	17.3 (3.2)	17.3 (3.6)
Emotional control						
n	40	36	34	42	38	38
Mean	18.2 (3.7)	22.8 (4.0)	23.2 (4.4)	18.3 (2.9)	20.7 (5.6)	20.8 (5.5)
Initiate						
n	40	36	34	42	38	38
Mean	17.9 (3.4)	17.1 (4.2)	17.9 (4.2)	18.7 (3.5)	17.8 (3.5)	18.2 (3.7)
Working memory						
n	40	36	34	42	38	38
Mean	23.9 (4.6)	22.7 (5.0)	23.4 (4.2)	23.4 (4.3)	22.3 (4.3)	22.3 (3.8)
Plan/organize						
n	40	36	34	42	38	38
Mean	25.0 (5.4)	24.5 (5.5)	24.6 (5.6)	24.6 (4.7)	24.0 (4.9)	23.8 (4.2)
Organization of materials						
n	40	36	34	42	38	38
Mean	14.0 (3.1)	13.7 (3.1)	14.1 (3.3)	13.6 (3.4)	13.1 (3.8)	13.6 (3.7)
Monitor						
n	40	36	34	42	38	38
Mean	19.5 (3.1)	19.1 (3.3)	18.9 (3.1)	19.3 (3.1)	18.8 (3.3)	18.9 (3.2)

Note. Mean scores with standard deviations after 91 days of bumetanide or placebo treatment and 28 days wash-out (D119). *Abbreviations:* BRIEF: The Behavior Rating Inventory of Executive Functioning.

SUPPLEMENTARY TABLE 4. Means of BRIEF-teacher subscales after treatment and wash-out

	Placebo group			Bumetanide group		
	Baseline	**D91**	**D119**	**Baseline**	**D91**	**D119**
Inhibit						
n	33	25	21	35	35	31
Mean	17.3 (5.4)	17.6 (5.3)	19.0 (5.8)	19.5 (7.3)	19.1 (6.1)	18.8 (6.6)
Shift						
n	33	25	21	35	35	31
Mean	21.3 (4.4)	20.0 (4.8)	19.2 (4.5)	22.0 (4.7)	20.7 (5.0)	20.3 (4.8)
Emotional control						
n	33	25	21	35	35	31
Mean	16.9 (4.4)	16.4 (3.8)	16.2 (4.9)	17.5 (5.2)	17.3 (4.5)	17.0 (5.3)
Initiate						
n	33	25	21	35	35	31
Mean	14.5 (3.9)	13.8 (3.4)	14.1 (4.1)	15.5 (3.8)	14.8 (3.3)	15.0 (3.1)
Working memory						
n	33	25	21	35	35	31
Mean	19.9 (5.8)	19.3 (5.3)	19.7 (6.4)	21.1 (5.3)	20.5 (4.6)	20.2 (4.9)
Plan/organize						
n	33	25	21	35	35	31
Mean	18.7 (4.5)	17.4 (3.9)	17.9 (4.8)	19.6 (4.9)	18.7 (4.5)	18.9 (4.2)
Organization of materials						
n	33	25	21	35	35	31
Mean	11.7 (4.3)	11.2 (3.8)	12.3 (4.2)	12.1 (4.4)	11.1 (4.0)	10.5 (4.0)
Monitor						
n	33	25	21	35	35	31
Mean	20.5 (5.0)	20.4 (4.7)	21.3 (5.0)	21.4 (5.6)	21.7 (4.8)	20.5 (5.7)

Note. Mean scores with standard deviations after 91 days of bumetanide or placebo treatment and 28 days wash-out (D119). *Abbreviations:* BRIEF: The Behavior Rating Inventory of Executive Functioning.

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"I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood and I-
I took the one less traveled by,
And that has made all the difference."
- Robert Frost

CHAPTER 5

Bumetanide for Irritability in Children with Sensory Processing Problems across Neurodevelopmental Disorders: a Pilot Randomized Controlled Trial

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ABSTRACT

Abstract Symptom traits such as aberrant sensory reactivity are present across neurodevelopmental disorders and might reflect common mechanistic targets for drug development. In this pilot RCT, we tested effectiveness of 91 days bumetanide treatment on irritable behavior in a cross-disorder neurodevelopmental cohort (autism spectrum disorder, attention-deficit/hyperactivity disorder and/or epilepsy) aged 5-15 years defined by the presence of sensory reactivity problems (Sensory Profile-NL). 19 children were allocated to bumetanide and 19 to placebo. Bumetanide was superior on the primary outcome, the ABC-irritability (MD: -4.78, 95%CI: -8.43 to -1.13, p = .0125). Side effects were reversible and as expected: hypokalemia (p = .046) and increased diuresis (p = .020). Despite the results being underpowered, this study raises important recommendations for future cross-diagnostic trial designs.

Trial registration Bumetanide for the Autism Spectrum Clinical Effectiveness Trial (BASCET); EU Clinical Trial Register, EudraCT 2016-002875-81. Registered 25 October 2016.

Keywords ASD, ADHD, epilepsy, bumetanide, RCT, irritability

INTRODUCTION

Neurodevelopmental disorders (NDDs) manifest in early childhood and are thought to result from atypical brain development, maturation or function. The most common NDD classes denoted by the current Diagnostic and Statistical Manual of Mental Disorders (DSM-5)[1] are autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), intellectual disability and learning disorders[2]. They are classified according to distinctive symptoms and behaviors, but in clinical reality show a high degree of overlap and comorbidity. Furthermore, there is shared heritability between different NDDs and causal genetic risk variants are mostly not restricted to one NDD class[3, 4]. The clinical validity of NDD DSM-5 classes is further complicated due to increasing recognition of extreme variability in severity and symptomatology in clinical manifestation between individuals of the same NDD class[2].

Drug development for NDD, however, is still largely focused on DSM-5 classifications disregarding heterogeneity, which may underlie the multitude of failed trials[5]. In particular in the field of ASD, diagnosis centered trials have yielded highly variable treatment responses resulting in non-significant group effects. An alternative is to stratify trial cohorts on the basis of traits that are present across different neurodevelopmental disorders. These cross-disorder traits may reflect a degree of shared developmental trajectories and common mechanistic pathways and enhance efficacy and reduce variability in treatment response in stratified trial designs[6].

Examples of cross-disorder traits in NDD are attention problems and altered sensory reactivity or also referred to as sensory processing difficulties (SPDs). SPD is a highly frequently occurring symptom in ASD, ADHD and epilepsy[7-9] described both as behavioral over- or under-responsiveness to singular or multiple types of sensory stimuli. An important suggested mechanism in the development and maintenance of adequate sensory processing is the regulation of the balance between excitatory and inhibitory inputs (E/I) in neuronal networks[10]. Perturbations to E/I balance at the synaptic level have been shown to affect sensory processing mechanisms and may cascade to SPD[10-13]. Indeed, E/I dysregulation is increasingly associated with NDD pathophysiology as a final common pathway, linking synaptic dysregulation to disturbance in mass-neuronal activity. Many existing compounds influence components of E/I regulation and may have purpose as rational treatments in NDD. Here, we hypothesized that stratification of SPD in NDD might be a strategy to enhance effectiveness of E/I targeting compounds.

Bumetanide is an example of an E/I influencing drug repurposing candidate for ASD. This drug has been used for decades as a diuretic drug with a mild-profile of

adverse effects mostly due to its effect on fluid and electrolyte homeostasis. The ASD rationale for bumetanide, a chloride importer (NKCC1) antagonist, is to shift the polarity of GABAergic signaling through modulation of intraneuronal chloride levels. Chloride concentrations in developed neurons are maintained low after birth through inactivation of NKCC1, which shifts the polarity of GABAergic signaling from depolarizing to hyperpolarizing[14, 15]. Persistent NKCC1 activity and depolarizing GABA activity has been shown in several animal models of NDD to contribute to neuronal hyperexcitability[16-18]. In these models, bumetanide normalized hyper excitability and NDD-related traits[16, 19].

These studies fueled human trials in ASD[20] and epilepsy[21]. A significant effect of bumetanide on core symptoms of ASD (i.e., social behavior) was shown in three placebo-controlled trials, which used the childhood autism rating scale (CARS) as the primary outcome[22-24]. A fourth trial from our lab did not find an effect on the primary outcome of the Social Responsiveness Scale but showed an improvement on a more specific core symptom scale of repetitive behavior[25]. To date, no RCT has tested bumetanide in ADHD and a single study in children with epilepsy was prematurely terminated precluding conclusions on the effect on seizures[26]. Overall, most ASD bumetanide trials showed variability in treatment responses between children, most likely due to etiological heterogeneity. Given the burden of frequent blood checks needed for surveillance of diuretic effects and other potential side effects, these results warrant improved trial designs in pre-stratified NDD populations.

Cross-disorder trials face certain challenges, such as the choice of inclusion measures, concomitant medication use and outcome selection. There are several characterization questionnaires for SPD, but we lack consensus regarding diagnostic features. For a large number of children with NDD, care as usual includes medication use to ameliorate behavioral problems. Thus, to attain a representative sample of the NDD population, it is important to allow concomitant medication use. Lastly, within NDD research, there is no golden standard for outcome measures, let alone for cross-disorder sensory reactivity outcomes. In this study we selected the aberrant behavioral scale-irritability (ABC-I) as the primary endpoint as this might overlap with behavioral sensory tolerance, is a reasonable outcome measure to detect change and is a frequently used behavioral scale in various NDD trials making it suitable for cross-disorder trial designs[27, 28]. Here, we present the results of the effectiveness of bumetanide in a pilot stratified cross-NDD RCT design.

METHODS

Study Design and Participants

The trial was designed as multicenter, patient-randomized, double-blind placebo-controlled phase-2 superiority trial testing effectiveness of 91 days bumetanide treatment followed by a 28-day wash-out period. The trial was initiated and conducted by the UMC Utrecht in the Netherlands with Jonx Groningen as participating center. Participants were recruited through outpatient clinics and advertisement on websites of the Dutch ASD parent association (NVA), epilepsy expert association (SEIN) and the Dutch ADHD parent association (Balans). The medical ethical committee of the UMC Utrecht approved the trial protocol (19/10/2016) and the study was conducted according to the principles of the Declaration of Helsinki and Good Clinical Practice (ICH-GCP). Written informed consent was obtained from all parents or legal representatives and participants received no financial compensation. The trial was registered on 25/10/2016 with registration number 2016-002875-81 in the EU Clinical Trials Register (www.clinicaltrialsregister.eu/ctr-search/trial/2016-002875-81/NL/). The full trial protocol is available at www.umcutrecht.nl/nl/ziekenhuis/wetenschappelijk-onderzoek/de-bascet-studie.

Children with a current ASD, ADHD (according to DSM-IV-TR or DSM-5 criteria) and/or epilepsy diagnosis, aged 5-15 years and IQ≥55 were eligible as participant. Children were enrolled when a diagnosis was accompanied by altered sensory reactivity, defined as a deviant score (>1 SD deviant) on the Sensory Profile for parents or teachers (SP-NL or SP-SC)[29, 30]. Use of concomitant psychoactive and antiepileptic drugs (AED) was allowed, when being taken on an unadjusted dosage at least 2 months prior to baseline measures. Exclusion criteria were renal or liver insufficiency, serious unstable illnesses (including gastroenterological, respiratory, cardiovascular, endocrinologic, immunologic, hematologic disease, dehydration or hypotension, electrolyte disturbances), treatment with NSAIDS, aminoglycosides, digitalis, antihypertensive agents, indomethacin, probenecid, acetazolamide, lithium, other diuretics, stimulants (like methylphenidate and dexamphetamine, due to it assumed diametrical effects) and drugs known to have a nephrotoxic potential. Children were allowed to receive care as usual when it was initiated minimally two months prior to baseline measures. Amendments to eligibility criteria were made to further include patients with ADHD and/or epilepsy besides patients with ASD with or without epilepsy. Consequently, the SP-NL was used as inclusion criteria to select patients based on sensory reactivity problems rather than diagnosis.

Randomization and Masking

Detailed descriptions of randomization and masking practice are described in an earlier RCT with similar study design[25]. In brief, participants were randomly allocated (1:1) to receive bumetanide or placebo treatment, which was provided by Tiofarma. Sequence generation, concealment and treatment allocation was overseen by a third-party not involved in the study (i.e. Julius Centre, a consultant support agency for clinical research and trials located in the UMC Utrecht). Restricted randomization was used with permuted block design randomly varying between two, four and six participants. Treatment allocation was done automatically using minimization with a probability of 0.75 on the participant factors active epilepsy (y/n), IQ (55-75; 76-110; >110) and study center (UMC/Jonx). Participants, parents, healthcare providers and outcome assessors were masked for randomization, by organizing safety checks at the pediatric nephrology department of the nearby Wilhelmina Children's Hospital.

Procedures

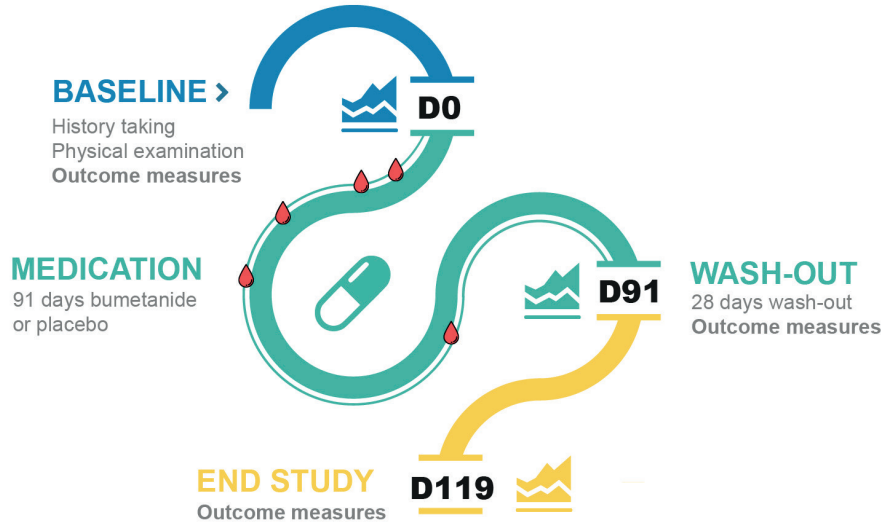
The study procedures are equal to the study procedures described by Sprengers et al.[31] and to which we refer for details on the procedures. The first study visit included clinical history taking by a medical doctor or psychologist, the administration of an abbreviated WISC-III intelligence test and medical screening by a medical doctor. Besides, study outcomes were measured at baseline (D0) and repeated after treatment (D91) and 28-day wash-out (D119).

Within 45 days of the baseline visit participants were randomized (D0) and received bumetanide or placebo tablets (0.5mg) matched for taste, smell and viscosity, albeit without diuretic properties. The tablets were taken orally twice-daily with minimally 6 hours between the administrations (e.g. typically with breakfast and dinner). Children ≤33kg started with halved tablets (i.e. twice-daily 0.25mg). Children >33kg received twice-daily 1 tablet (0.5mg). When blood analysis showed no abnormalities at D7, the dosage was doubled. All participating children were supplemented with 0.5mmol/kg potassium chloride when <30kg, or twice-daily 8mmol potassium chloride when ≥30kg.

Safety visits were scheduled at D4, D7, D14, D28, D56, D91 and D119 at the department of pediatric nephrology of the Wilhelmina Children's Hospital, with the purpose of masking the researchers, and included blood analysis (D4, D7, D14, D28 and D56), medical evaluation and documentation of adverse events (adhering to NCI-CTCAE and MedDRA methodologies). Participants returned for outcome evaluations at the end of the 91-day medication phase and at the end of the 28-day wash-out period.

Parents were interviewed at the last study visit (D119) about their experiences (i.e. treatment, adverse event and wash-out evaluations) and were asked to predict which treatment their child had received. A schematic overview of the trial is depicted in Figure 1.

Figure 1. Overview study visits of the trial



Outcomes

The primary outcome was severity of irritable behaviors measured by the ABC-I (range 0-45; higher score is more affected) after 91 days of treatment. This measure was chosen because it is a commonly used outcome scale in (neuro)behavioral trials [32] and because we hypothesized that a beneficial clinical effect of bumetanide in this population would become evident by reducing behavioral reactivity to sensory stimuli. The SP-NL was also added as secondary outcome although this questionnaire was primarily developed as a screening and characterization scale (range 125-625, lower score is more affected). Other secondary outcomes were chosen to cover broad NDD core symptom domains; severity of restricted and repetitive behaviors, measured by the Repetitive Behavior Scale-Revised (RBS-R; range 0-129, higher score is more affected), symptom severity of social communication and social interaction, measured by the SRS-2 (range 0-195; higher score is more affected) and severity of behavioral executive functioning, measured by the Behavior Rating Inventory of Executive Function (BRIEF-parent; range 72-216; higher score is more affected) total scores at D91. To assess executive function and sensory behaviors in the school

environment, the BRIEF-teacher (range 73-219; higher score is more affected) total score and domain scores of the SP-School Companion (SP-SC) were included, respectively. When participants were diagnosed with epilepsy, frequency and type of seizures were registered with an epilepsy diary. Adverse events were passively (spontaneous report) and actively (evaluation of known side effects) collected. Incomplete individual clinical questionnaires were imputed as 'no change' when less than four questions were missing (RBS-R: n=1; SP-NL: n=3; SP-SC: n=2; BRIEF-T: n=4). When four or more questions were missing, the outcome measures were excluded from analysis (n=4).

Statistical analysis

This study was initially powered at 90% to detect an effect size of 0.5 on the primary outcome measure (ABC-I) with a standard deviation of 9.3 (i.e., mean change difference of 4.6 points), assuming two-sided alpha level of 0.05. Allowing for 10% attrition rate, 190 participants had to be randomized. Due to lower-than-expected inclusion rates, the sample size was reevaluated allowing for 80% power resulting in an intended sample size of 124 participants.

Due to the explorative nature, we analyzed outcomes by modified intention-to-treat on allocated participants (see results section for details). Screening differences between randomized and non-randomized participants were analyzed with appropriate t-statistics or Fisher's exact tests for dichotomized variables.

Primary and secondary outcomes at all available time points were analyzed with a linear mixed model. A random intercept was included to correct for multiple follow-up measurements per participant. Treatment and treatment by time interaction were included to assess the difference between placebo and bumetanide. In a second step, sex, age and baseline measurement of the corresponding outcome measures were included to correct for potential confounding and optimize the statistical analysis for power[33, 34]. Statistical assumptions of the models (i.e. distributional assumptions, homoscedasticity) were assessed by examining residuals[35]. From these models, we derived estimated means for each treatment arm as well as a mean difference between treatment groups at 91 days with 95% confidence intervals (CI) and p-values. Additional analyses were performed for treatment interactions with sex, age and total IQ and were evaluated with likelihood ratio tests (LRT). Safety was analyzed in all allocated subjects (i.e., ITT) with Fisher's exacts tests. Agreement of predictions by parents of the allocated treatment arm versus the actual treatment allocated to children was analyzed with Cohen's kappa. All analyses were performed with SPSS v25 (IBM, Corp., Armonk, NY) and SAS v9.4 (SAS, Cary, NC).

Study safety was overseen twice a year by the Data Safety Monitoring Board (DSMB) of the UMC Utrecht. This study was registered with the EudraCT trial registry (2016-002875-81) and Dutch trial registry (NL6178).

RESULTS

Participant characteristics

Participants were enrolled between June 20th 2017 and June 26th 2019, the end of planned recruitment and funding. The study was finished without meeting the intended study population, since inclusion rates were not met. Multiple attempts were undertaken to increase recruitment, including adding research staff, advertisements on Dutch ASD, ADHD and epilepsy parent associations and presentations during their meetings. However, various strategies to increase inclusions failed, which rendered extension of the trial to meet the sample size not feasible. The participating center in Groningen was unable to follow the study procedures and the few participants randomized at this site (n=5) could not be included for analysis in the study due to incomplete questionnaires. No serious adverse events were reported in this participating center. As a consequence, the study is reported as a single center trial.

As shown in the CONSORT diagram in Figure 2, a total of 158 caregivers contacted the research team and obtained a study information folder. After information was sent, 53 potential participants gave informed consent and 52 were assessed for eligibility. 14 participants did not progress to randomization for reasons of non-eligibility (n=7), requirement of immediate other therapy (n=4), inability to adhere to study protocol (n=1), resistance to blood withdrawal (n=1) and participation in another study (n=1), resulting in 38 participants that were randomly allocated to bumetanide or placebo treatment (Additional File 1). There was no difference in baseline characteristics and outcomes between eligible participants who did and who did not advance to randomization ($p \geq .153$). Based on the initial power calculation the actual power of the study reached 27%.

Of the 38 randomized participants, 19 (4 female participants) were allocated to bumetanide and 19 (6 female participants) to placebo. Five participants discontinued treatment prior to collecting outcomes. Two were allocated to placebo: one required immediate psychiatric intervention with other therapy and one withdrew consent because of the high burden, absence of benefits and perseverance of mild potential side effects (i.e. dermal abnormalities). The other three discontinued treatments had been allocated to bumetanide: one required immediate psychiatric intervention

with other therapy, one due to repeating hypoglycemia and one due to palpitations. During the trial nobody had to be unmasked. On completion of the trial and before unmasking, one participant was excluded from analyses as questionnaires were not reliable (i.e., were filled out by a different parent; placebo) and for two participants multiple D91 questionnaires were missing (1 placebo, 1 bumetanide).

Figure 2. CONSORT diagram of the trial

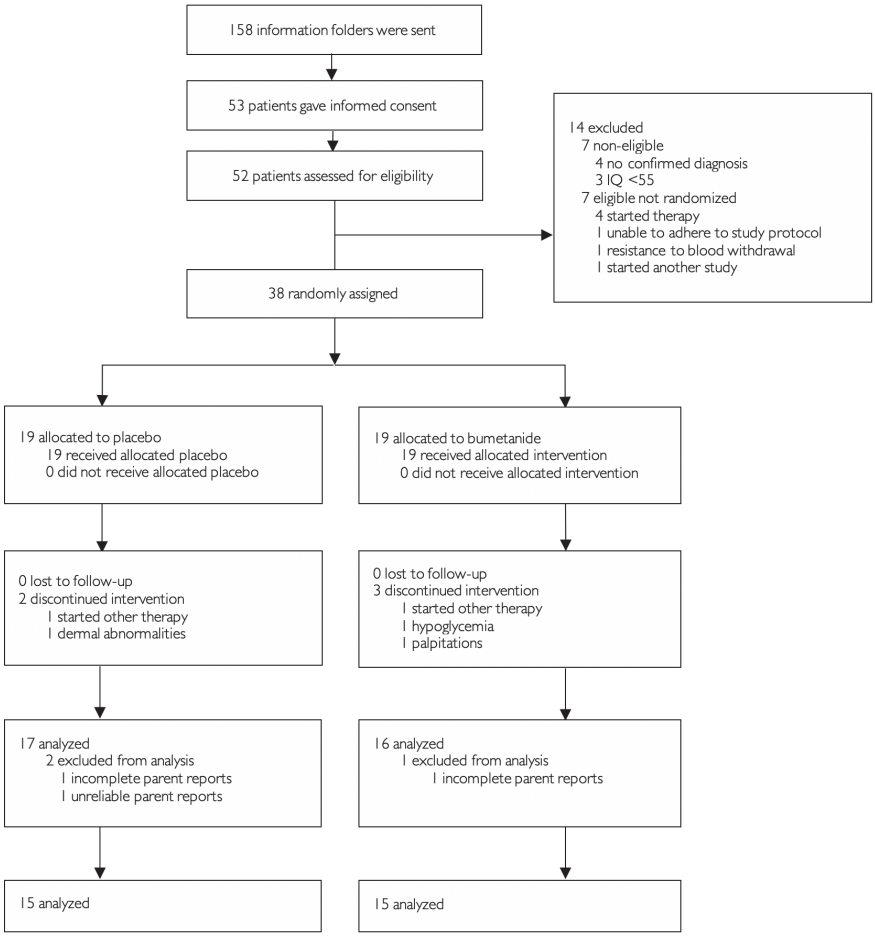


Table 1 depicts baseline characteristics of the analyzed sample including (previous) medication use and diagnoses.

Table 1. Baseline characteristics of the analyzed population

		Placebo group (*n*=15)		Bumetanide group (*n*=15)		Total (*n*=30)	
Age (y, SD)		8.7 (3.1)		10.9 (2.5)		9.8 (3.0)	
Sex (%)	Male	10 (66.7)		12 (80.0)		22 (73.3)	
	Female	5 (33.3)		3 (20.0)		8 (26.7)	
IQ (SD)		99.5 (25.3)		98.9 (24.0)		99.2 (24.2)	
Medication use (%)		**Prior**	**During trial**	**Prior**	**During trial**	**Prior**	**During trial**
None		7 (46.7)	11 (73.3)	9 (60.0)	12 (80.0)	16 (53.3)	23 (76.7)
AP		1 (6.7)	1 (6.7)	1 (6.7)	2 (13.3)	2 (6.7)	3 (10.0)
AED		5 (33.3)	2 (13.3)	1 (6.7)	0 (0)	6 (20.0)	2 (6.7)
SSRI		0 (0)	0 (0)	1 (6.7)	1 (6.7)	1 (3.3)	1 (3.3)
AP + benzo		0 (0)	1 (6.7)	0 (0)	0 (0)	0 (0)	1 (3.3)
Benzo		0 (0)	0 (0)	1 (6.7)	0 (0)	1 (3.3)	0 (0)
Stimulant		3 (20.0)	0 (0)	4 (26.7)	0 (0)	7 (23.3)	0 (0)
Alpha2		2 (13.3)	0 (0)	0 (0)	0 (0)	2 (6.7)	0 (0)
Diagnoses (%)							
ASD		11 (73.3)		11 (73.3)		22 (73.3)	
	ASD only	7 (46.7)		8 (53.3)		15 (50.0)	
	ASD + ADHD	3 (20.0)		3 (20.0)		6 (20.0)	
	ASD + epilepsy	1 (6.7)		0 (0)		1 (3.3)	
ADHD		2 (13.3)		4 (26.7)		6 (20.0)	
Epilepsy		2 (13.3)		0 (0)		2 (6.7)	

Note: Data are mean (SD) or N (%). ADHD = attention deficit hyperactivity disorder; AED = antiepileptic drug; AP = antipsychotics; ASD = autism spectrum disorder; Benzo = benzodiazepine; Prior = medication history up to 8 weeks before trial start; SSRI = selective serotonin reuptake inhibitor; Y = years.

Of all analyzed participants, 22 (73.3%) were classified with ASD with or without comorbidities, 6 (20%) with ADHD and two (6.7%) had epilepsy (Table 1). A total of 16 (53.3%) participants were naïve for the use of psychoactive medication. At the start of (and during) the trial three participants (10%) were taking antipsychotics, two (6.7%) were taking AEDs, one participant used a selective serotonin reuptake inhibitor (SSRI) (3.3%) and one antipsychotics together with benzodiazepines. Twenty-three (76.7%) were not taking any medication.

Medication adherence was monitored via several approaches: interview, inspection of returned medication packages and a drug diary. We found no evidence of non-adherence in either treatment group. The mean provided bumetanide dosage was

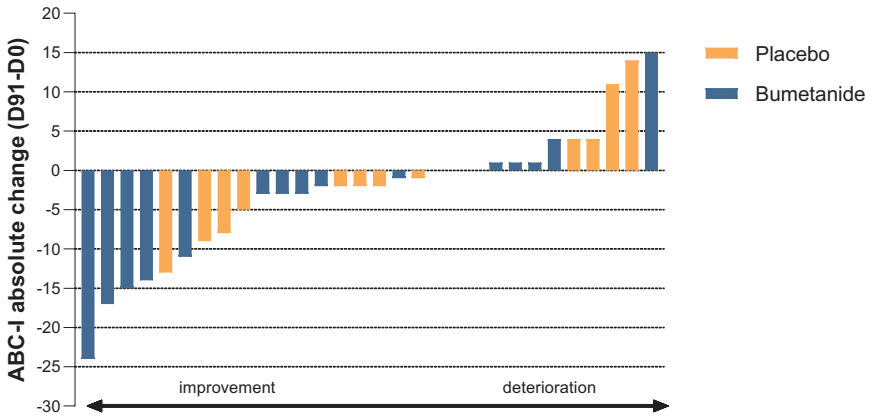
0.0430mg/kg/day (range: 0.0182-0.0637). Treatment dose was increased at D7 in all but two participants (due to hypokalemia and a postponed safety visit and which were both increased at D14). In three participants, the target dose had to be temporarily halved for 11, 12 and 13 days respectively due to hypokalemia (n=3, bumetanide).

We documented parent predictions of the treatment their child had received once the last study visit for the participant was completed. In the bumetanide group (n=15), 12 parents expected allocation to bumetanide and three parents expected allocation to placebo. In the placebo group (n=15), one parent expected allocation to bumetanide, 14 parents expected allocation to placebo, and one parent was not assessed. A substantial accordance between expected and actual treatment allocation was found (κ=.737 [with 0 indicative of effective masking and 1 indicative of a potential failure of masking], p = .000).

Outcomes

Bumetanide showed a superior treatment effect on severity of irritability symptoms, the primary outcome, (ABC-I mean difference [MD]: -4.78, 95%CI: -8.43 to -1.13, p = .0125; Figure 3 and Table 2)

Figure 3. Individual treatment effect on the primary outcome



Note. Absolute change on the Aberrant Behavior Checklist-Irritability subscale (ABC-I) after 91 days of treatment (D91 minus D0). The primary endpoint shows a significant treatment effect (p = .0125) favoring the bumetanide group.

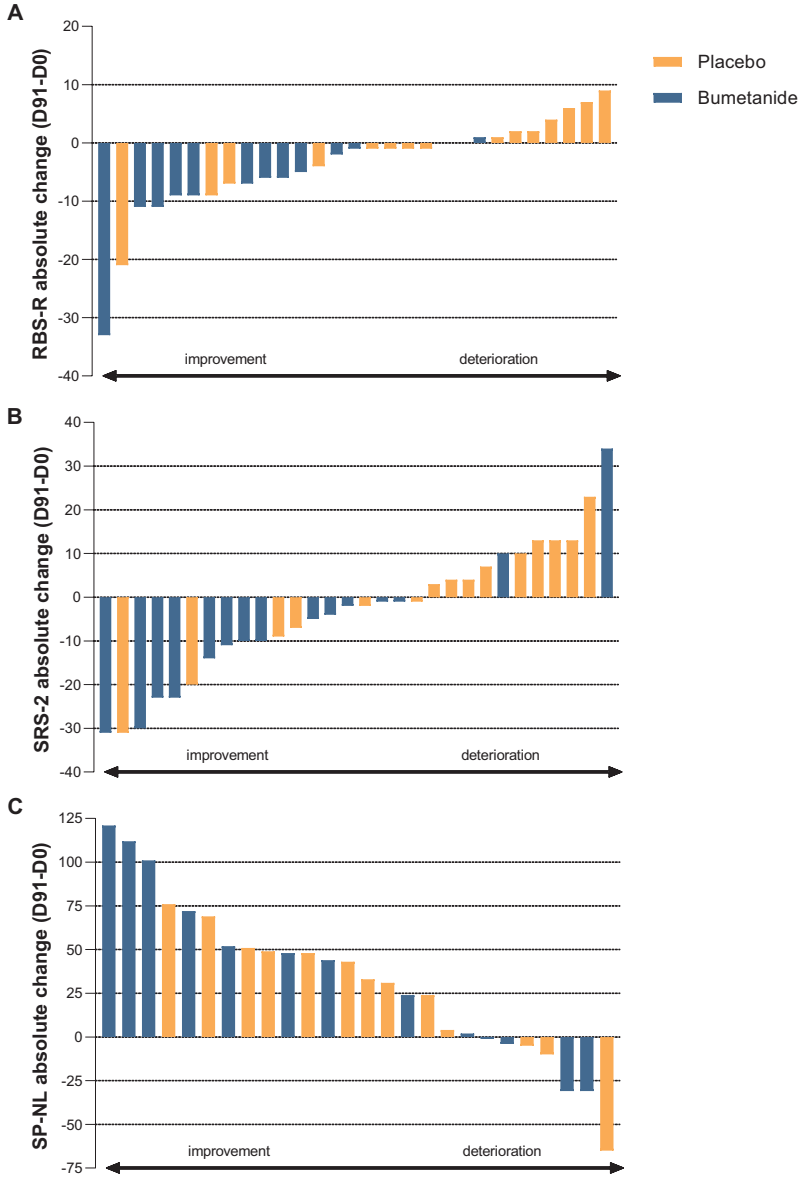
No superior effect of bumetanide was found on the secondary core ASD outcome measures RBS-R (model adjusted for heteroscedasticity, MD: -4.90, 95%CI: -10.97 to 1.17, p = .109) and SRS-2 (MD: -6.61, 95%CI: -16.51 to 3.28, p = .181), indicating no effect of bumetanide on repetitive behaviors and social communication and social interaction (Figure 4 and Table 2). No wash-out effects were observed on any outcome. Descriptive results of the subscales are presented in Additional File 2 as the study was not sufficiently powered to statistically test subscales.

Table 2. Changes in primary and secondary outcome measures after treatment and wash-out

	Placebo group			**Bumetanide group**			**Treatment effect**	**p-value**
	Baseline	D91	D119	Baseline	D91	D119		
ABC-I subscale								
n	15	15	11	14	14	14		
Mean	17.1 (9.1)	16.5 (8.6)	13.2 (8.8)	13.1 (10.3)	6.9 (4.3)	7.9 (6.7)	-4.78 (-8.4 to -1.1)	.0125
SRS-2 total								
n	15	15	11	15	15	15		
Mean	80.0 (32.9)	81.3 (33.1)	88.5 (25.5)	78.9 (26.1)	70.9 (23.8)	73.9 (24.3)	-6.61 (-16.5 to 3.3)	.181
RBS-R total								
n	15	15	11	14	14	14		
Mean	17.8 (13.0)	16.9 (12.5)	19.4 (18.0)	17.4 (16.6)	10.4 (9.4)	15.3 (14.1)	-4.90 (-11.0 to 1.2)	.109
SP-NL total								
n	14	14	10	15	15	15		
Mean	452.7 (55.9)	477.8 (66.3)	473.8 (64.6)	442.0 (55.7)	482.3 (47.1)	472.8 (59.6)	3.14 (-29.3 to 35.6)	.844
BRIEF-parent total								
n	15	15	11	15	15	14		
Mean	164.3 (20.0)	162.2 (19.3)	160.1 (20.7)	159.5 (21.5)	150.9 (20.0)	153.8 (17.1)	-7.88 (-17.6 to 1.8)	.105
BRIEF-teacher total								
n	13	13	8	11	11	9		
Mean	148.8 (23.3)	145.7 (25.8)	149.0 (19.2)	148.6 (17.2)	143.3 (27.8)	141.9 (31.7)	-3.08 (-19.7 to 13.5)	.698

Note: Data are means (SD). Data is shown for those participants that completed D91. Treatment effects are measured with linear mixed models (including age, gender and baseline measurements) and shown with (95% CI). ABC-I = Aberrant Behavior Checklist – Irritability (range 0-45; higher score is more affected); BRIEF = Behavior Rating Inventory of Executive Function (Parent range 72-216 and Teacher range 73-219; higher score is more affected); RBS-R = Repetitive Behaviors Scale-Revised (range 0-129; higher score is more affected). SP-NL = Sensory Profile-Dutch version (range 125-625; lower score is more affected); SRS-2 = Social Responsiveness Scale-2 (range 0-195; higher score is more affected). Significance level is $p<0.05$.

Figure 4. Individual treatment effect on secondary outcomes



Note. Absolute change on the Repetitive Behavior Checklist-Revised (RBS-R) after 91 days of treatment showing no superior treatment effect (p = .109). B) Absolute change on the Social Responsiveness Scale-2 (SRS-2) total score showing no superior treatment effect (p = .181). C) Absolute change on the Sensory Profile-NL (SP-NL) total score showing no superior treatment effect (p = .844)

Sub-analyses on treatment-by-sex, age and IQ interaction showed only a significant treatment-by-IQ interaction effect on the BRIEF-teacher (MD: -.65, 95%CI: -1.33 to .02, LRT = 3.9, p = .0483), indicating that within the bumetanide group, a higher IQ was associated with higher scores after treatment, whereas in the placebo group a higher IQ was associated with lower scores.

Mean treatment dose showed no association with change in ABC-I in the bumetanide group (ρ=.157, p=.591) i.e., there was no dose-response relationship. Lastly, change in ABC-I showed no association with baseline SP-NL total scores in the bumetanide group (respectively r=.438; p = .117).

Tolerability and Adverse Effects

Adverse events (AEs) that occurred in more than 5% of participants are shown in Table 3. All events were mild to moderate according to CTCAE rating-scale and all resolved. Three serious AEs occurred in two patients (both bumetanide group): anaphylactic reaction to incidental cow milk ingestion in a child with preexisting cow milk allergy, blood loss after elective adenoidectomy/tonsillectomy requiring prolonged hospital observation; and exaggeration of preexisting palpitations by sinus tachycardia. Cardiac evaluations showed no abnormalities. The serious AEs were registered as probably unrelated to study treatment, except for palpitations, which was possibly related due to hypovolemia, although no signs of hypovolemia were found on echocardiography. Common cold, myalgia, orthostatic hypotension and hypokalemia were the most frequently occurring AEs. A total of 32% of participants in the bumetanide group experienced increased diuresis compared to none in the placebo group (p = .020). In addition, 26% of participants in the bumetanide group developed hypokalemia against none in the placebo group (p = .046). Hypokalemia occurred in one patient at D10. In all other patients hypokalemia occurred only after D14 and potassium levels did not drop below 3.0mmol/L and normalized with increased oral potassium chloride (Additional File 3).

Table 3. Adverse Events Occurring in >5% of Participants Classified in MedDRA Categories

	Bumetanide group (n=19)				Placebo group (n=19)			
Symptom	# of AEs	# of part.	Severity	IR[a]	# of AEs	# of part.	Severity	p-value
Total AE	100	19			61	17		
Metabolism and nutrition disorders								
Hypokalemia	9	5	Moderate	1				0.0463
Dehydration	3	3	Moderate	1				0.230
Hypoglycemia	2	1	Mild	3				1.000
Hyponatremia	2	1	Moderate	2				1.000
Gastrointestinal disorders								
Vomiting	3	3	Mild	2	3	3	Mild	1.000
Nausea	7	6	Mild	2	3	2	Mild	0.232
Abdominal pain	7	6	Mild	3	3	3	Mild	0.447
Obstipation					2	2	Moderate	0.487
Gastroenteritis	3	3	Mild	3	4	4	Mild	1.000
Vascular disorder								
Orthostatic hypotension	9	8	Mild	1	3	3	Mild	0.151
Infections and infestations								
Common cold	3	3	Mild	3	14	10	Mild	0.0382
Musculoskeletal and connective tissue disorders								
Myalgia	10	7	Mild	2	3	3	Mild	0.269
Muscle cramp	2	2	Mild	2	2	1	Mild	1.000
Renal and urinary disorders								
Dysuria	2	2	Mild	2				1.000
Enuresis[b]	1	1	Mild	1				1.000
Increased diuresis	6	6	Mild	1				0.0197

	Bumetanide group (n=19)				Placebo group (n=19)			
Nervous system disorders								
Headache	6	4	Mild	3	7	7	Moderate	0.476
Dizziness	2	2	Mild	3	2	2	Mild	1.000
Psychiatric disorders								
Insomnia	1	1	Mild	3	4	4	Mild	0.340
General disorders and administration site conditions								
Fatigue	4	3	Mild	2	2	2	Mild	1.000
Skin and subcutaneous tissue disorders								
Dermal abnormalities	3	3	Moderate	3	2	2	Moderate	1.000
Injury, poisoning and procedural complications								
Injury	4	4	Moderate	3				0.105

Note: Data are n. Differences were tested with Fisher Exact tests. # = number; AE = adverse event; IR = intervention relationship; Part = participants;[a] 1: definitely related; 2: possibly related; 3: not related; [b]Occurring in <5% of participants, but listed as important expected AE. Significance level is $p<0.05$.

DISCUSSION

NDD diagnoses allow extensive etiological and clinical heterogeneity and lack effective treatment options, which highlights the need for innovative trial designs. The effectiveness of trials might be augmented in cohorts ascertained by traits that may reflect an enhanced degree of shared pathophysiology, shifting from diagnosis-centered to trait-centered inclusions. In this context, we hypothesized that SPD marks an important cross-disorder trait and tested whether bumetanide may improve sensory induced irritable behavior. Albeit the limited sample size, we found a superior effect of bumetanide on this primary endpoint. Bumetanide was well tolerated with only mild to moderate, expected (i.e., hypokalemia and diuresis) and reversible side effects.

The observed treatment effect in this pre-stratified sample is encouraging. Existing treatment options to reduce irritable behavior in NDDs are limited to antipsychotics such as risperidone and aripiprazole that may owe their effect to sedative, symptomatic properties with detrimental side effects. Bumetanide is an attractive alternative due to its rational mechanism suggested from a large body of experimental research[36].

We encountered several challenges during this pilot trial that may be improved when future studies consider a similar design. We expected that a trial design with recruitment based on traits would be more appealing for participants than trials following classical inclusion based upon ASD and ADHD diagnoses. Hence, the inclusion difficulties were contrary to our expectations. The study failed (n=38) to meet the recruitment target (n=124), resulting in a power of 27% which is undesirable but not uncommon: a review estimated the median achieved power of studies in neurosciences between 8 and 31%[37]. Several aspects seem to have contributed to problematic recruitment. First, healthcare support, access to special education, and referral systems in the Netherlands are still organized along DSM-classifications, which might render certain patients and caregivers reluctant to participate. Second, the placebo-controlled trial design was frequently mentioned as reason to decline participation. Some children requiring drug intervention were expected not to endure a period of placebo allocation. Indeed, caregivers of children on psychostimulants, an exclusion criterion due to its expected diametrical effect to bumetanide, were eager to participate due to experienced side effects (e.g., rebound, sleeping problems, and emotional blunting). For them, bumetanide was appealing as a safe alternative, although they were hesitant to stop medication for the duration of the trial and risk deterioration in school performance and family stability. Third, we suspect that the limited participation of children with epilepsy was due to the treatment focus on

seizure management instead of behavioral problems by parents, pediatricians and neurologists. Taken together, it seems that several reasons may have hampered the readiness for cross-disorder trait approaches, which may be improved by including for instance comparative trial designs.

Another evident challenge is the development of more appropriate clinical outcomes. Although there are excellent assessment tools to characterize SPD, the most prominent being the Sensory Profile-NL these have limited applicability to detect treatment effect. As a consequence, the assumption that improvements in irritable behavior are mediated by improvements in sensory behavior could not be tested. For now, the ABC-I, however, is a reasonable outcome measure to detect change and is a frequently used behavioral scale in NDD trials[27, 28]. Still, there is a great need for suitable outcomes that can more directly measure certain expected mechanistic effects, preferably scales that can be individually adapted[38] as treatment response variability between subjects varies greatly in trials. Future trials may benefit by personalizing instead of specifying clinical outcome measures. In this way, improvement in debilitating behaviors can be evaluated which results in more notable and valuable improvements in daily life. The inclusion of diagnostic companions (e.g., electroencephalography) to bridge clinical to assumed mechanistic effects offers another opportunity. In this trial, it may have demonstrated mechanistic insights on central nervous system effects given the limited brain availability of bumetanide[21]

In addition to these challenges we highlight several limitations that obstruct interpretation of our findings. An underpowered study is problematic as it reduces the chance of detecting true effects (in outcomes but also in adverse events) and also reduces the likelihood that the significant treatment effect on irritable behavior reflects a true, replicable effect. The small sample size did not allow for subgroup analyses precluding recommendations for specific NDD classifications. Further, as all participants with epilepsy were allocated to placebo, we have no record of the potential effect of bumetanide on seizure frequency. To improve this in future trials it would be recommended to perform trials across diagnosis specific expertise centers in order to balance recruitment per diagnosis.

Another limitation is that functional unmasking may have interfered with the results. There was substantial agreement between expected and actual treatment allocation as reported by parents. In addition, expected diuretic side effects were restricted to the bumetanide group. While several parents (7 out of 14) claim that their prediction was based on clinical improvement, we should adopt a more conservative interpretation as adverse effects may have contributed to unmasking. Indeed, diuretic

side effects (i.e., increased diuresis, hypokalemia, enuresis and dysuria) occurred in 10 out of 19 participants treated with bumetanide. Functional unmasking is a concern in bumetanide RCTs due to its renal effects. To prevent unmasking, we followed the same rigorous procedures that were used in our previous bumetanide RCT in ASD, where no indication of insufficient masking was found[25]. All participants started with potassium chloride supplementation and the researchers were masked for safety controls and side effects. Despite our rigorous efforts, adequate masking remains a challenge in bumetanide RCTs that not include comparatives with diuretic properties.

Fortunately, there is a growing awareness to develop new trial approaches moving from traditional medicine to personalized or stratified approaches, such as the Research Domain Criteria initiative (RdoC)[39]. A primary assumption of RDoC is that interventions are more effective if heterogeneity within and amongst disorders is reduced, for instance by symptom stratification. Future trial designs may adopt these approaches and start to move away from one-size-fits-all to more stratified therapy and increase the benefit-risk ratio for patients.

Here, we have presented a pilot RCT based on a stratified trial design in which we found a superior effect (modified ITT analysis) of bumetanide on irritable behavior in children with NDD and SPD. Although the small sample size and potential functional unmasking do not allow firm conclusions or generalizability of treatment effect, these results encourage future studies that implement SPD stratification in testing bumetanide or similar agents. Our recommendations for future trials include dedicated expertise centers for balanced recruitment across diagnoses, consideration of using comparatives with diuretic properties to reduce the risk of functional unmasking (in the specific case of bumetanide) and improvement of cross-disorder inclusion and outcome measures.

SUPPLEMENTAL MATERIAL

ADDITIONAL FILE 1

Table S1. Baseline characteristics of the intention to treat population

		Placebo group (n=19)		Bumetanide group (n=19)		Total (n=38)	
Age (y, SD)		9.0 (3.3)		11.2 (2.6)		10.1 (3.1)	
Sex (%)	Male	13 (68)		15 (79)		28 (74)	
	Female	6 (32)		4 (21)		10 (26)	
IQ (SD)		98.3 (22.6)		97.1 (22.9)		97.7 (22.4)	
Medication use (%)		**Prior**	**During trial**	**Prior**	**During trial**	**Prior**	**During trial**
None		9 (47.4)	13 (68.4)	9 (47.4)	13 (68.4)	18 (47.4)	26 (68.4)
AP		2 (10.5)	1 (5.3)	4 (21.1)	3 (15.8)	6 (15.8)	4 (10.5)
AP + benzo		0 (0)	1 (5.3)	0 (0)	0 (0)	0 (0)	1 (2.6)
Benzo		1 (5.3)	0 (0)	1 (5.3)	0 (0)	2 (5.3)	0 (0)
AED		6 (31.6)	3 (15.8)	1 (5.3)	0 (0)	7 (18.4)	3 (7.9)
SSRI		1 (5.3)	0 (0)	2 (10.5)	2 (10.5)	3 (7.9)	2 (5.3)
SSRI + AP		0 (0)	1 (5.3)	0 (0)	1 (5.3)	0 (0)	2 (5.3)
Stimulant		5 (26.3)	0 (0)	6 (31.6)	(0)	11 (28.9)	(0)
Alpha2		2 (10.5)	0 (0)	1 (5.3)	(0)	3 (7.9)	(0)
Diagnoses (%)							
ASD		15 (78.9)		15 (78.9)		30	
	ASD only	10 (52.6)		12 (63.2)		22 (57.9)	
	ASD + ADHD	4 (21.1)		3 (15.8)		7(18.4)	
	ASD + epilepsy	1 (5.3)		0 (0)		1 (2.6)	
ADHD		2 (10.5)		4 (21.1)		6 (15.8)	
Epilepsy		2 (10.5)		0 (0)		2 (5.3)	

Note: Data are mean (SD) or N (%). ADHD = attention deficit hyperactivity disorder; AED = antiepileptic drug; AP = antipsychotics; ASD = autism spectrum disorder; Benzo = benzodiazepine; Prior = medication history up to 8 weeks before trial start; SSRI = selective serotonin reuptake inhibitor; Y = years.

ADDITIONAL FILE 2

Table S2. Mean and standard deviations of subscales at different time points

	Placebo group			**Bumetanide group**		
	Baseline	**D91**	**D119**	**Baseline**	**D91**	**D119**
SRS-2 subscales	n=15	n=15	n=11	n=15	n=15	n=15
Social Awareness	10.9 (4.5)	11.6 (4.5)	12.6 (3.4)	10.9 (4.0)	9.9 (3.9)	9.9 (3.5)
Social Cognition	15.6 (6.2)	15.1 (8.0)	16.0 (4.9)	14.2 (5.5)	13.9 (4.9)	14.7 (5.5)
Social Communication	26.8 (11.4)	26.2 (9.8)	28.5 (8.7)	25.7 (8.9)	22.9 (8.7)	23.7 (8.6)
Social Motivation	14.1 (7.6)	15.1 (6.3)	15.6 (6.7)	13.9 (5.2)	12.3 (4.6)	12.7 (5.5)
Autistic Preoccupations	12.7 (7.7)	13.4 (8.1)	15.9 (7.2)	14.2 (6.9)	11.9 (5.9)	12.9 (5.8)
RBS-R subscales	n=15	n=15	n=11	n=14	n=14	n=14
Stereotypic Behavior	2.6 (2.5)	2.6 (2.9)	2.9 (2.9)	3.7 (2.9)	2.8 (3.2)	3.6 (3.4)
Self-injurious Behavior	1.0 (1.2)	1.3 (2.1)	.9 (1.1)	2.4 (5.6)	1.0 (1.8)	1.4 (3.0)
Compulsive Behavior	1.9 (2.4)	2.1 (2.5)	2.8 (4.5)	1.8 (1.8)	1.2 (1.5)	2.1 (2.8)
Ritualistic Behavior	3.5 (2.8)	3.9 (3.1)	3.6 (3.7)	3.0 (3.2)	1.5 (2.0)	2.6 (2.5)
Sameness Behavior	6.9 (4.6)	5.2 (4.2)	7.2 (7.0)	4.9 (5.0)	3.2 (3.6)	4.4 (4.4)
Restricted Interests	1.9 (2.1)	1.7 (1.8)	1.9 (2.1)	1.7 (1.6)	.6 (.9)	1.3 (1.7)

	Placebo group			Bumetanide group		
	Baseline	**D91**	**D119**	**Baseline**	**D91**	**D119**
SP-NL quadrants	n=14	n=14	n=10	n=15	n=15	n=15
Low Registration	57.1 (9.5)	60.4 (10.0)	59.0 (11.6)	52.5 (10.5)	58.5 (9.6)	52.3 (8.7)
Sensation Seeking	96.7 (16.1)	102.5 (15.8)	103.7 (19.2)	97.8 (17.2)	108.7 (14.5)	106.1 (16.9)
Sensory Sensitivity	74.2 (10.4)	77.2 (11.6)	77.3 (11.9)	71.7 (10.1)	77.8 (7.2)	76.4 (9.8)
Sensation Avoiding	100.6 (14.8)	107.0 (15.7)	103.4 (12.3)	100.0 (15.3)	107.5 (15.7)	103.4 (19.6)
SP-SC quadrants	n=12	n=12	n=7	n=11	n=11	n=10
Low Registration	57.4 (13.6)	60.0 (9.2)	54.9 (5.5)	57.2 (12.5)	58.6 (10.9)	57.6 (13.5)
Sensation Seeking	35.6 (9.8)	36.5 (8.4)	36.1 (6.7)	37.3 (9.6)	41.6 (9.9)	40.7 (11.2)
Sensory Sensitivity	49.4 (10.2)	50.3 (7.9)	47.6 (8.6)	48.3 (6.1)	52.3 (9.5)	49.8 (8.7)
Sensation Avoiding	64.0 (15.7)	65.4 (12.9)	60.7 (10.0)	60.8 (9.0)	63.7 (9.4)	62.9 (11.9)
ABC subscales	n=15	n=15	n=11	n=14	n=14	n=14
ABC Total	55.3 (23.9)	51.7 (22.8)	42.6 (24.1)	50.0 (26.9)	32.4 (16.0)	35.1 (18.7)
Irritability	17.1 (9.1)	16.5 (8.6)	13.2 (8.8)	13.1 (10.3)	6.9 (4.3)	7.9 (6.7)
Lethargy	7.1 (8.7)	5.5 (5.0)	5.9 (4.3)	9.6 (8.7)	7.0 (7.0)	7.4 (8.1)
Stereotypic Behavior	3.9 (5.2)	3.6 (4.2)	2.3 (2.1)	5.8 (6.7)	2.9 (3.4)	4.5 (5.3)
Hyperactivity	23.7 (10.9)	22.6 (11.1)	16.8 (12.3)	18.4 (8.2)	12.9 (6.3)	13.1 (7.7)
Inappropriate Speech	3.5 (2.4)	3.5 (2.4)	4.4 (3.4)	3.2 (2.1)	2.6 (2.4)	2.2 (1.8)

	Placebo group			Bumetanide group		
	Baseline	**D91**	**D119**	**Baseline**	**D91**	**D119**
BRIEF-parent subscales	n=15	n=15	n=11	n=15	n=15	n=14
Inhibit	23.3 (4.6)	23.2 (4.1)	22.2 (5.6)	21.8 (4.6)	19.5 (4.4)	20.0 (4.5)
Shift	17.9 (3.3)	17.3 (3.4)	18.9 (2.7)	17.2 (3.4)	15.5 (3.6)	16.4 (3.3)
Emotional Control	23.8 (3.9)	23.6 (4.9)	23.4 (5.2)	22.1 (5.5)	19.3 (4.2)	19.6 (4.0)
Initiate	18.5 (4.0)	18.0 (4.3)	18.6 (3.5)	17.9 (3.3)	17.3 (3.6)	17.1 (3.4)
Working Memory	24.4 (4.3)	23.9 (4.3)	21.9 (4.4)	24.1 (3.8)	22.7 (3.8)	23.6 (3.7)
Plan/Organize	23.2 (4.3)	22.9 (4.7)	22.7 (5.0)	24.7 (3.7)	24.7 (4.8)	25.0 (4.3)
Organization of Materials	13.5 (3.5)	14.1 (3.7)	13.4 (3.9)	12.7 (3.8)	12.8 (2.9)	12.8 (2.9)
Monitor	19.7 (3.4)	19.3 (3.4)	19.0 (3.6)	18.9 (3.2)	18.9 (2.9)	19.2 (3.2)
BRIEF-teacher subscales	n=13	n=13	n=8	n=11	n=11	n=9
Inhibit	23.5 (4.9)	21.4 (5.7)	22.4 (4.0)	19.8 (5.1)	18.0 (6.5)	19.6 (6.2)
Shift	19.6 (5.7)	19.3 (4.6)	20.4 (4.5)	21.3 (3.3)	20.9 (5.3)	20.2 (4.6)
Emotional Control	17.5 (5.3)	17.9 (4.9)	17.3 (4.6)	17.3 (4.6)	16.3 (4.5)	16.6 (4.1)
Initiate	14.2 (3.8)	13.4 (3.9)	13.8 (3.3)	14.7 (1.8)	15.2 (3.3)	14.0 (3.4)
Working Memory	20.4 (5.8)	20.9 (5.9)	21.6 (5.4)	23.0 (2.1)	21.7 (2.8)	20.8 (3.5)
Plan/Organize	17.6 (4.6)	18.0 (3.8)	17.3 (4.1)	19.1 (1.8)	19.4 (4.2)	19.4 (4.2)
Organization of Materials	12.6 (3.7)	12.6 (4.3)	13.4 (4.1)	10.7 (2.6)	10.9 (3.2)	10.8 (2.7)
Monitor	23.3 (4.0)	22.2 (4.8)	23.0 (4.0)	22.7 (4.1)	20.9 (5.6)	20.6 (5.9)

Note: Data are mean (SD). ABC = Aberrant Behavior Checklist; BRIEF = Behavior Rating Inventory of Executive Function; RBS-R = Repetitive Behaviors Scale-Revised; SP-NL = Sensory Profile-2; SP-SC = Sensory Profile School Companion; SRS-2 = Social Responsiveness Scale-2.

ADDITIONAL FILE 3

Table S3. Individual potassium levels split out per treatment group

BL	D4	D7	Extra[1]	D14	Extra[2]	Extra[3]	D28	Extra[4]	Extra[5]	Extra[6]	D56	Extra[7]
Bumetanide group												
4.4	4.3	4.2		4.1			4.4				DR	
3.5	4.3	3.9		4.0			3.8				3.4	3.6
4.3	4.1	4.0		3.5	3.9		DR					
4.2	4.1	4.0		4.2			3.7				3.9	
4.1	4.7	4.2		4.0			4.1				4.0	
3.9	3.9	4.3		4.0			3.6				4.0	
3.9	3.9	4.1		4.2			4.1				4.1	
3.8	4.0	4.1		4.2			3.9				3.8	
4.1	3.9	3.4[b]	3.7	3.3			3.5				3.6	
4.2	3.9	4.2		3.9			4.0				3.8	
3.9	4.1	4.3		3.9			3.9				3.6	
4.1[a]	4.4	3.6		3.9			3.5				3.6	
3.9	3.6	3.6		3.3	3.7		3.2	3.2	3.8	3.5	3.5	
4.1		3.7		3.5			3.6				3.5	
3.8		3.5			3.0	3.9	3.4				3.4	
4,1	4.0	3.9		3.7			3.7				3.7	
4.0		5.0		3.5			4.1				3.5	
4.0	3.9	3.7		3.4	3.9		4.0				4.1	
4.0	3.9	3.8		3.3			DR					
Placebo group												
4.1	4.3	4.0		4.1			4.0				4.4	DR
3.6	4.3	4.0		5.3			4.4				4.0	
4.0	4.7	4.5		4.7			4.3				4.3	
4.1	4.1	4.2		5.3			4.5				4.3	
4.0		3.8		4.7			DR					
4.2	3.9	3.9		4.1			4.0				3.8	
4.0	4.3	4.2		3.9			4.1				4.2	
4.4	4.7	4.6		4.0			4.4				4.2	
3.6	3.7	3.9		3.8			3.9				3.9	
4.3		4.1		4.0			5.1				5.4	

BL	D4	D7	Extra[1]	D14	Extra[2]	Extra[3]	D28	Extra[4]	Extra[5]	Extra[6]	D56	Extra[7]
4.3		4.2		4.6			4.3				3.7	
4.3		4.3		3.8			4.1				4.2	
4.1		4.6		4.7			5.5				4.1	
3.8	4.3	4.1		4.0			3.9				4.1	
3.9	4.2	4.2		3.9			3.9				4.0	
3.9	4.5	4.0		4.0			4.2				4.5	
3.8		4.1		4.3			4.5				4.3	
4.5		4.3		4.0			4.5				4.1	
4.0		4.2		4.2			4.2				4.5	

Note: Data are mmol/L; BL = baseline; DR = drop out. [a]haemolytic sample [b]visit at D10

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"Everything that can be counted does not necessarily count; everything that counts cannot necessarily be counted." — Albert Einstein

CHAPTER 6

The Sensory-Reactivity PROM Set: Identification of a Parent Reported Outcome Measure Set for Autism Spectrum Disorder

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ABSTRACT

Background Most children with autism spectrum disorder (ASD) suffer from aberrant responses to sensory stimuli that significantly impact the quality of life. To develop sensory interventions, individually tailored outcome measures are crucially needed for the domain of sensory reactivity problems. Here, we describe the identification of relevant sensory themes according to caregivers of children with ASD according to the guidelines for developing a (parent proxy) patient-reported outcome measure set. Subsequently, we identify parallels between these themes and a well-validated and supported PROMIS® portal to facilitate implementation. Interviews with clinicians and focus groups and interviews with parents of children with ASD were used in the initial phase for concept elicitation. Codes and themes were generated by qualitative thematic data analysis on the transcripts and cognitive interviews with different parents were used for revisions. The resulting themes were compared to existing generic PROMIS-item banks and other existing questionnaires.

Results A total of 11 parent-reported outcomes were identified that could be either classified as directly or indirectly related to sensory reactivity. Directly related themes comprised of: 1) sensory stimulation tolerance and 2) sensitivity to sensory stimuli. Indirectly related themes were: 3) irritable behavior 4) anxiety problems 5) mood problems 6) sleep problems 7) fatigue 8) physical complaints 9) daily functioning and participation 10) routines, structure and dealing with change and 11) problems in social interaction and communication. Seven out of 11 themes could be measured with generic PROMIS item banks. The four remaining outcomes (sensory stimulation tolerance; irritable behaviour; routines, structure and dealing with change; and sensitivity to sensory stimuli) were found suitable to be inventoried by existing PROMs.

Conclusion The majority of parent-reported problems seemed related to *indirect* consequences of sensory reactivity, which are suitable to be measured with generic item banks. In sum, we identified a sensory-reactivity PROM (parent-proxy) set consisting of PROMIS® item banks and additional domains that together form a comprehensive and readily available outcome set for sensory reactivity problems in children with ASD.

Keywords ASD, sensory reactivity problems, PROM, PROMIS, trials

BACKGROUND

The majority of children with autism spectrum disorder (ASD) display aberrant responses to sensory experiences compared to their typically developing peers[1]. These responses are commonly referred to as sensory reactivity problems (SRPs) and are estimated to occur in 69-95% of patients with ASD[2, 3]. SRPs often hamper opportunities to participate in daily activities that promote learning[4, 5] and have a significant impact on quality of life for both children and caregivers[6, 7]. Intensive behavioral therapies such as the Early Start Denver Model[8] and Pivotal Response Treatment[9] partly rely on improving SRPs. In addition, novel mechanism-based medications are being developed to ameliorate SRPs through effects on neuronal activity[10-12]. Overall, interventions for SRPs are seen as a top priority in the ASD community[13].

To study the effect of existing and future interventions for SRPs, reliable and relevant outcome measures are mandatory, preferably from the patient or parent perspective to assess meaningful effects in daily life. Several existing instruments characterize sensory sensitivity behaviors such as the Sensory Profile[14] and the Evaluation of Sensory Processing[15] that have great utility for diagnostic profiling especially in typically developing populations. For repeated outcome measurements in clinical intervention trials, such in ASD, the questionnaires are rather lengthy and not well suited to detect change[16]. In addition, these questionnaires were mostly developed for typically developing populations and may be less suitable to address specific SRPs in clinical populations. Indeed, sensory reactivity problems in these populations can extend into problematic behavior or affective dysregulation that in turn may lead to some of the core symptoms of ASD[16]. In sum, there is a great need for appropriate outcome measures for treatments targeting SRPs in ASD.

To address this need, we set out to identify a comprehensive set of sensory reactivity related outcomes relevant to ASD. We first followed the steps of developing a (parent proxy) patient reported outcome measure (PROM) to be used as endpoint to establish efficacy in clinical trials[17, 18]. Our aim was to understand concepts that are relevant for patients and their caregivers. Our questions of interest therefore focused on what caregivers find relevant about sensory reactivity difficulties; which aspects have the most impact on their child's and their own life; and how they would notice improvement when an intervention targeting sensory reactivity would be successful. To inventory these issues, we initiated focus groups and interviews with caregivers of children with ASD and sensory reactivity problems. We then assessed face validity of these concepts against currently existing instruments, which would

facilitate their implementation in clinical trials. Therefore, we chose to compare our patient/caregiver relevant concepts to the items from the child and parent-proxy item banks from the *Patient-Reported Outcomes Measurement Information System*® (PROMIS)[19]. The PROMIS was initiated by the "NIH Roadmap Initiative" and has developed large item banks, based on Item Response Theory (IRT) with the possibility to use Computerized Adaptive Testing (CAT)[20] to develop meaningful, reliable and precise outcome measures which can be used internationally and across disorders. These properties enable easy and direct implementation of PROMIS CATs in clinical trials.

In this report we describe the identification of patient/caregiver relevant concepts and the development of a Sensory Reactivity-PROM set using PROMIS as a first milestone in establishing ecologically valid outcome measures for sensory reactivity.

METHODS

Selection of PRO by concept elicitation

Ethical approval was granted by the Medical Ethical Committee of the University Medical Center (UMC) Utrecht. We selected parents/caregivers as respondents to be able to assess children from the age of 5 years. Parents of children with ASD were invited via advertisement on the Dutch ASD parent association (NVA) website to attend a focus group or interview over the phone. The inclusion criteria were parents of patients (boys and girls) with a confirmed ASD diagnosis (based on the DSM-IV or DSM-5) varying between 5-17 years of age. To obtain a heterogeneous and representative sample, no exclusion criterion based on intellectual functioning of patients and levels of parental education were selected. During the first steps of the qualitative phase, the aims and logistics of the PROM were developed to aid *concept elicitation*. Concept elicitation is a process by which concepts deemed important to patients and parents (i.e. symptoms as well as the impact of symptoms) emerge spontaneously through open-ended questions in interview settings, for instance in focus groups. First, symptoms of and impact of symptoms on children with ASD and their parents visiting the outpatient Psychiatry department for consultation or participating in scientific research at the 'Care and Research program Sensory Processing' between September 2016 and March 2019 (n=200) were reviewed (case files). Second, structured expert brainstorms with 5 child psychiatrists from the department of Psychiatry of the UMC Utrecht were conducted to gather information from clinicians that worked with children with ASD. Lastly, a focus group (n=8) and interviews over the phone (n=10) with caregivers of children with ASD were conducted by following a structured interview guide. These conversations were transcribed and

entered into Nvivo once the phone interviews reached data saturation (i.e. the point where no new themes or topics were obtained from further interviews).

A qualitative thematic data analysis[21], based on the method of Boeije[22] was performed on the transcription of phone interviews. A total of 181 codes were generated by DA and scored by an independent second rater (GT) and these were conceptually grouped into 29 (sub)themes. As a result, eleven overarching themes were extracted. Subsequently, these themes were presented to 21 new participants (i.e., other parents of children with ASD) to evaluate whether themes were missing or irrelevant. The eleven themes were confirmed and therefore maintained.

RESULTS

The study population that was interviewed and used for concept elicitation consisted of 38 caregivers of 37 children (age M = 11.5; SD = 3.0) with an ASD diagnosis. The population was balanced with regard to older and younger children (age 5-11: n = 19; age 12-17: n = 18) and boys (n = 19) and girls (n = 18). Intellectual functioning ranged from total intelligence quotient (TIQ) 50 to 145 (M = 98.9; SD = 28.2) and was proportionally divided into children with below average (TIQ 50-84: n = 10), average (TIQ 85-115: n = 10), and above average (TIQ 116-145: n = 8) intellectual functioning. Caregivers were most likely to have followed higher education: 56% completed higher professional education and 11% completed research-oriented education. Vocational education was completed by 30% and 4% had followed pre-vocational education.

The following eleven concepts were identified through concept elicitation and item generation: 1) Sensory stimulation tolerance 2) Sensitivity to sensory stimuli 3) Irritable behavior 4) Anxiety 5) Mood problems 6) Sleep problems 7) Fatigue 8) Physical complaints 9) Daily functioning and participation 10) Routines, structure and dealing with change and 11) Problems in social interaction and communication. Thus, the majority of these concepts reflected what we would refer to as 'indirect' consequences of altered sensory reactivity. For instance, many caregivers regarded their children's fatigue and lack of energy to be closely related to aberrant sensory reactivity and to have a profound impact on the quality of life. Another example of indirect consequences were irritable behaviours, especially when their child experienced "sensory overload" leading to anger, temper tantrums, crying, yelling or short-temperedness. Only two themes - Sensory stimulation tolerance and Sensitivity to sensory stimuli – entailed direct behavioral responses to sensory stimuli, e.g., covering their ears, adjusting their daily routines or contacts to avoid sensory stimuli, or finding it difficult to differentiate between relevant and irrelevant stimuli.

PROM selection

Next, we identified which reliable existing PROMs can be used to represent the identified sensory concepts. We chose to use PROMIS item banks, measured by CATs. Seven out of eleven concepts were found to be covered by PROMIS item banks (see Table 1). Importantly, each of these PROMIS item banks are available as parent proxies and pediatric self-report versions. Four concepts were not measurable by PROMIS: Sensory Stimulation Tolerance; Irritable Behaviour; Routines, Structure and Dealing with Change and Sensitivity to Sensory Stimuli. The domain Daily Functioning and Participation was only partly covered by the PROMIS items bank 'Cognitive function' and therefore an additional questionnaire would be needed to cover all codes that parents reported. The identification of relevant sensory reactivity PROs and PROMs resulted in a Sensory Reactivity-PROM set

The Sensory Reactivity-PROM set

To measure Sensory stimulation tolerance and Sensitivity to sensory stimuli, we advise to use the following two questionnaires:

Short Sensory Profile (SSP)

The SSP is a shortened form of Dunn's SP caregiver questionnaire[14] and contains 38 items, arranged into 7 subscales, aimed at measuring abnormal responses to sensory stimuli[23]. It has a reliability of .90 and discriminate validity >95% to identify children with and without sensory processing difficulties[24]. Although the total score is reliable for youth with ASD (α=0.89), the structural validity of the SSP subscales shows poor fit[25, 26] and some researchers have recommended against the use of the SSP total score due to the measure's multidimensionality[25].

Sensory Experiences Questionnaire Version 3.0 (SEQ-3.0)

Unlike other instruments that are often used in sensory processing research (e.g., SP-NL[14] and Sensory Processing measure[27], the SEQ-3.0 [28] was developed and standardized in ASD populations. Earlier versions of the SEQ have demonstrated its reliability and validity (version 1: Internal consistency α = .80; test–retest reliability total score ICC = .92[29]).

To measure Irritable behavior, we advise to use the following subscale:

Aberrant Behavior Checklist-Irritability (ABC-I)

The ABC measures problematic behavior and contains 58 items organized into 5 subscales[30]. The ABC is developed as a scale to assess treatment effects[30] and has

very good internal consistency (α= ranging from low .80s to the middle .90s) and test-retest reliability (mid-.60s to highs in the .90s)[31, 32]. Studies have further given psychometric support for the use of the ABC in ASD [33]. The Irritability subscale consists of 15 items and measures agitated/irritable behavior.

To measure Routines, structure and dealing with change, we advise to use the following subscale:

Repetitive Behavior Scale-Revised (RBS-R)
The RBS-R is a measure of the presence and severity of restricted and repetitive behaviors and contains 43 items organized into 6 subscales[34]. The subscales Ritualistic Behavior (i.e., performing activities of daily living in a similar manner) and Sameness Behavior (i.e., resistance to change, insisting things stay the same) have been selected. Internal consistency of these subscales in an ASD sample was α = .71 and .88, respectively[35].

To measure Daily Functioning and Participation, the following two questionnaires can be used:

Child and Adolescent Scale of Participation (CASP)
The CASP is a 20-items caregiver questionnaire measuring the extent of participation and restriction of children (3-22 years) in home, school and community life situations and activities[36]. The questionnaire has shown high internal consistency (Cronbach's α = .98), test-retest reliability (ICC = .94) and construct validity[37, 38]

Participation and Environment Measure for Children and Youth (PEM-CY)
The PEM-CY is a 25-items caregiver questionnaire that measures participation across life situations at home, school and community settings in children (5-17 years) with and without disabilities[39]. The questionnaire has both moderate to good internal consistency (Cronbach's α = .59 and above) and test-retest reliability (.58 and above)[40].

Table 1. Final Sensory Reactivity-PROM set

Relevant outcomes	PROMIS item banks	Validated PROM (sub) scales
Directly related to sensory reactivity		
1. Sensory stimulation tolerance		- SSP or SEQ-3.0
2. Sensitivity to sensory stimuli		- SSP or SEQ-3.0
Indirectly related to sensory reactivity		
3. Irritable behavior		- ABC-Irritability
4. Anxiety	- Anxiety (v2.0) - Psychological stress experiences (v1.0)	
5. Mood problems	- Depressive symptoms (v2.0) - Life satisfaction (v1.0)	
6. Sleep problems	- Sleep-related impairment (v1.0) - Sleep disturbance (v1.0)	
7. Fatigue	- Fatigue (v2.0)	
8. Physical complaints	- Physical stress experiences (v1.0)	
9. Daily functioning and participation	- Cognitive function (v.1.1)	- CASP or PEM-CY
10. Routines, structure and dealing with change		- RBS-R Ritualistic Behavior - RBS-R Sameness Behavior
11. Problems in social interaction and communication	- Peer relationships (v2.0) - Family relationships (v1.0)	

Abbreviations. ABC: Aberrant Behavior Checklist; CASP: Child and Adolescent Scale of Participation; PEM-CY: Participation and Environment Measure for Children and Youth; RBS-R: Repetitive Behavior Scale-Revised; SSP: Short Sensory Profile.

DISCUSSION

This study aimed to derive a valid outcome measure set for sensory reactivity targeting interventions in children with ASD elicited by parent interviews. The parent interviews and focus groups revealed a total of eleven concepts relating to SRPs. We can classify the most frequently mentioned problems as indirect consequences of sensory reactivity, such as fatigue or behavioral irritability. Interestingly, these kinds of symptoms are often recognized as comorbid features, but were here identified by parents as indirect consequences of altered sensory processing functions associated with ASD[16, 41]. In contrast, other behavioral responses were noted that were directly related to the sensory environment such as immediate distress or an exaggerated avoidance to sensory stimuli. In all, we classified nine out of 11 concepts as indirect versus two direct consequences of sensory reactivity. Such a distinction has not yet been explicated in the field[16, 41], but may be important to fully appreciate the effect of SRP targeting treatments.

The majority of the identified concepts (7 out of 11) can be measured with generic item banks provided by PROMIS. It would be recommended to validate these item banks in the target population (children with ASD). This set can be administered by CATs with both parent proxy reports (ages 5-17) and pediatric self-reports (ages 8-17) available. These CATs are an important method to reduce the time to complete questionnaires and to render them more individually tailored. CATs follow decision trees by each time choosing tailored selections based upon previous answers of the respondent, e.g. if a patient is unable to walk then all questions on mobility are deemed irrelevant. CAT algorithms hereby maximize the efficiency of number of questioned items (usually 4 to 12 items in PROMIS) and reduces the burden for respondents whilst allowing more domains to be measured. Another advantage of CAT in clinical trials is that smaller sample sizes are needed to achieve the same statistical power in comparison to conventional instruments. Lastly, there is less floor or ceiling effect with T-scores and different domains can be compared on the same scale. Thus, using PROMIS item banks for ASD is an important way forward since it is more reliable and reduces administration time for respondents and researchers. Hence, they are suitable to be implemented in clinical trials where multiple and repeated assessments are often desirable. In addition, using these generic item banks allow for comparisons between different (rare) disorders, typically developing children as well as comparisons across different countries.

A number of other concepts are not covered by existing PROMIS item banks at present: tolerance and sensitivity to sensory stimuli, behavioral irritability and

problems with structure and dealing with change. To assemble an all-encompassing outcome measure set, these outcomes need to be added and (for the time-being) to be measured with other validated PROMs. To this end, we propose to add six additional, existing PROMs to cover the above-mentioned missing outcomes: the ABC-I subscale to assess irritable behavior, the SSP or SEQ-3.0 to assess items in the missing sensory-specific outcomes, the RBS-R Ritualistic Behavior and Sameness Behavior-subscales to address dealing with routine and change and the CASP or PEM-CY to cover the daily functioning and participation outcome. We do acknowledge that some of these measures have been developed more than 40 years ago and may not be in line with regulatory qualification demands in order to be implemented (immediately) in registrational clinical trials. Unfortunately, there are currently only few measures that have been validated and cover the identified relevant concepts. Indeed, a limitation of this study is the extensive variety and number of instruments that are needed to cover all relevant concepts identified in this study, which poses a significant burden on respondents. These results therefore highlight the need for the development of PRO instruments in the ASD population, that capture relevant concepts and have established clinically meaningful thresholds. Ultimately, the goal would be to fully rely on PROMs administered with CAT to comprise all identified concepts in the sensory reactivity domain.

In conclusion, we bring forward a sensory reactivity-PROM set that addresses the main concepts relevant to parents of children with ASD. Through parent concept elicitation, we emphasize the need to measure both direct and indirect consequences of altered sensory reactivity. We hope that this report inspires ASD researchers to implement relevant outcome measures in their clinical trials with instruments that are user-friendly, less time-consuming and measure patient-relevant outcomes relating to sensory reactivity. In the short term, we suggest clinical trials in ASD focusing on SRPs to include this hybrid PROM set of PROMIS items banks and existing questionnaires. For the longer term, we propose to complete the PROM set by transforming conventional scales to reliable PROMs administered by CATs.

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"Tempora mutantur, nos et mutamur in illis."
[The times change, and we change with them.]

CHAPTER 7

Discussion

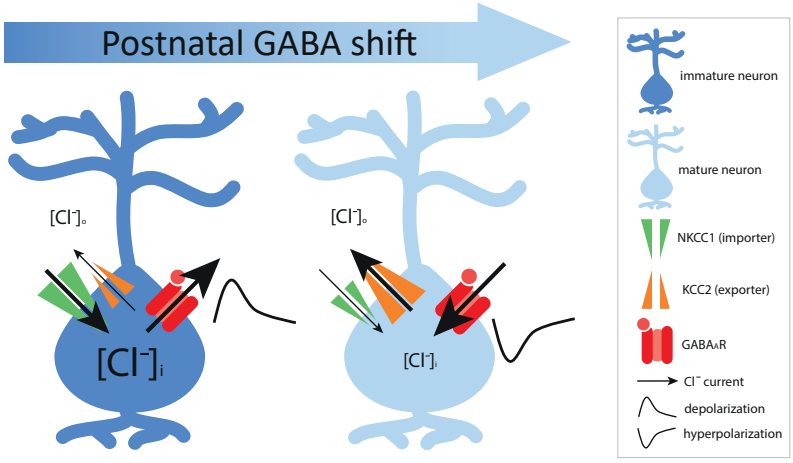
DISCUSSION

For years, we have neglected the overwhelming pre-clinical evidence that each child with a neurodevelopmental disorder (NDD) has unique etiological features and thus will require, at least to a certain extent, a tailored approach to serve his or her needs and challenges. In this context, the research in this thesis was dedicated to ultimately improve effective and tailored treatment for NDDs. As the first part of the title indicates "*Crossing borders in NDDs*", we adopted a flexible, transdiagnostic approach to acknowledge the extensive heterogeneity of etiologies and clinical manifestations of NDDs. The overarching hypothesis of the thesis was that increased phenotypic and/or etiological homogeneity translates to reduced variability in treatment response, if measured with appropriate endpoints. The results of the trials and endpoint studies in this thesis partly support this hypothesis but also show many challenges ahead.

The studies tested different stratification options in the context of a novel and mechanism-based treatment and investigated existing and novel endpoints to detect improvements for NDDs. In terms of treatment, the choice to focus on bumetanide, a chloride importer antagonist, fitted the transdiagnostic scope: the developmental downregulation of chloride is associated with many different developmental and neurological conditions and was therefore a suitable mechanism-based candidate to embark on this innovative research path (Figure 1).

Hence, the clinical trials that were conducted showed a variety of stratification strategies from stratification based on behavior and neurocognitive functioning in ASD (**chapter 3 and 4**), to genetic disorder ascertainment (**chapter 2**) and cross-disorder trait stratification (**chapter 5**). Finally, the sensory-PROM study (**chapter 6**) identified relevant parent-reported outcomes that can be readily implemented in clinical trials. In this discussion chapter, the main findings from the different studies will be briefly summarized and discussed in light of the road to more personalized child psychiatry and trial design.

Figure 1. The GABA shift



This figure illustrates the so-called *GABA shift*, a developmental change in which GABA transmission through chloride-permeable GABA receptors reverses from depolarizing to hyperpolarizing[1]. This GABA shift is the result of a reduction of intracellular chloride concentrations, caused by a change in the expression of two chloride co-transporters: the NKCC1 importer and KCC2 exporter[2, 3]. The immature neuron on the left shows higher intracellular chloride concentrations compared to the mature neuron on the right. It is proposed that in some pathological states, neurons remain in (or return to) this immature status, which may be restored with bumetanide treatment, which impairs the chloride influx through NKCC1 antagonism.
Figure adapted from Peerboom & Wierenga (2021)[40] with permission from the authors

I. Summary of the main findings

Chapter 2: Effects of bumetanide on behavior, cognition, and EEG in TSC: the BATSCH trial

This chapter pursued a genetic stratification approach by testing the effect of bumetanide in patients (age 8-21 years) with Tuberous Sclerosis Complex (TSC) using an open-label design with behavioral, cognitive, and electroencephalography (EEG) outcome measures. The rationale to study this monogenetic disorder was that altered neuronal chloride homeostasis (i.e., molecular disruptions in NKCC1 and KCC2 expression) has been associated with the consequences of the *TSC1* and *TSC2* gene mutation. Indeed, we found that bumetanide treatment improved irritable, social and hyperactive behavior. Moreover, patients rated their health-related quality of life significantly higher after bumetanide treatment. Another asset of the TSC-trial was the incorporation of event-related potential (ERP) measurements. Initial analyses revealed atypical ERPs compared to typically developing peers and bumetanide treatment had a normalizing effect on a measure of auditory processing in children with TSC. No evidence of changes in cognitive functioning were found. Although not

all children were able to conduct the EEG and cognitive test batteries, we concluded that bumetanide has potential as a rational treatment to improve behavioral problems and quality of life in TSC.

Chapter 3: Effects of bumetanide on ASD core symptoms: the BAMBI trial
This bumetanide randomized controlled trial (RCT) focused on the development of cognitive and EEG stratification markers for application of bumetanide in ASD as well as to replicate previously shown positive behavioral effects on core symptoms. Of note, the BAMBI trial was the first randomized medication trial in ASD to include EEG and cognitive stratification measures. In 92 children with ASD (age 7-15) we found no superior treatment effect of bumetanide on social, irritable and sensory behaviors. We did observe a significant treatment effect on repetitive behavior, another core ASD symptom domain. This effect seemed more explicit in female participants and we observed a potential age effect, with more efficacy on social responsiveness symptoms in younger children with ASD. These nuances may also (partly) explain the lack of significant social improvements compared to other bumetanide trials where on average younger children were included[4, 5]. A more pronounced effect in younger children may also be consistent with its assumed effect on immature GABAergic transmission. With this initial clinical report of the BAMBI trial, we highlighted that the advent of repurposing treatments targeting specific mechanisms requires stratified trial designs or post-hoc analyses to understand treatment response variability

Chapter 4: Effects of bumetanide on neurocognitive functioning in ASD: secondary analyses of the BAMBI trial
To explore the feasibility of a cognitive stratification marker in trial designs, the next chapter tested the effect of bumetanide on neurocognitive tests and neurocognitive network organization in the BAMBI trial (secondary analyses of chapter 3). We hypothesized that a fine-tuned balance between neuronal E/I balance is critical for adequate information processing and therefore, an E/I influencing agent such as bumetanide might improve neurocognitive deficits. A range of tests were used to cover important neurocognitive domains. We utilized principal component analysis to cluster neurocognitive domains and network analysis to measure relative and absolute effects. At baseline, we found extensive variability in neurocognitive readouts and profiles, with response inhibition and auditory prepotent response inhibition being most frequently affected. After treatment, we found no superior treatment effect on the main neurocognitive components and no signs of cognitive deterioration. Interestingly, analysis of treatment effect on neurocognitive network organization revealed a significant effect on global and local network parameters. This indicates

that the effects of bumetanide may be subtle, variable and only detectable when inspecting the organization of various cognitive components instead of focusing on merely individual neurocognitive parameters.

Chapter 5: Effects of bumetanide on behavior in NDDs with sensory processing difficulties: the BASCET trial

We hypothesized that an alternative stratification strategy to overcome variability in treatment response is to pre-select traits that may reflect a degree of shared developmental trajectories and common mechanistic pathways. The pilot RCT with bumetanide described in this chapter included 38 children that were stratified based on the symptom trait aberrant sensory processing across NDDs (ASD, ADHD, and epilepsy). Almost three-quarter of included patients were classified with ASD; 20% with ADHD, and 2 patients had epilepsy. The study failed to recruit the intended number of subjects, hence the results were underpowered. The study may be interpreted as proof-of-principle study with recommendations for future similar approached rather than as generalizable findings. Surprisingly, in this underpowered sample, we did find a superior treatment effect on the primary endpoint of irritable behavior but not on the other included endpoints. Furthermore, we observed no association between clinical improvement on irritability and sensory processing difficulties at baseline, which served as the inclusion criteria. As we encountered several challenges in recruitment and trial management with this novel design, we recommend future researchers and clinicians to increase the success of cross-disorder trait approaches by including dedicated expertise centers for balanced recruitment across diagnoses and to focus on relevant outcome measures that capture cross-disorder traits rather than diagnosis-specific traits.

Chapter 6: Identification of a Sensory-Reactivity outcome measure set for ASD trials: the sensory-PROM study

Following our interest in sensory processing as a cross-disorder trait and mechanism-based treatment target, individually tailored outcome measures are crucially needed for the domain of sensory reactivity problems as relevant and psychometrically-sound outcome measures are currently lacking. In this chapter, we describe the identification of relevant sensory themes according to caregivers of children with ASD according to the guidelines for developing a (parent proxy) patient-reported outcome measure (PROM) set. This qualitative study utilized interviews and focus groups to first identify relevant parent-reported themes, which led to a total of 11 outcomes that were directly or indirectly related to sensory reactivity. We then identified parallels between these outcomes and existing generic item banks to facilitate implementation in

clinical trials. Seven out of 11 outcomes could be measured with generic PROMIS item banks and four with existing PROMs. Interestingly, the majority of reported themes were indirect consequences of sensory reactivity, a distinction that has not yet been explicated in the field but may be important for targeted treatment development. Also, these outcomes can efficiently be measured with generic item banks that are user-friendly, less time-consuming, and measure patient-relevant outcomes relating to sensory reactivity.

II. General discussion

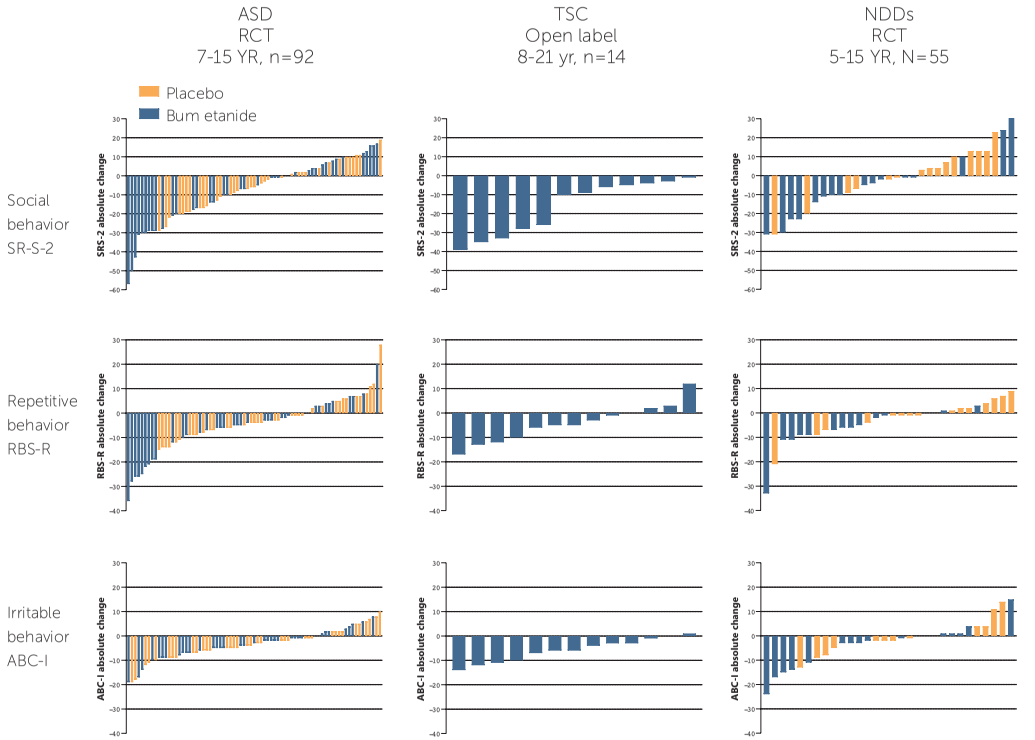
Insights and implications

We have taken some encouraging steps to move from symptom-based treatment in broad, unselected NDD populations to mechanism-based treatment in selected subgroups. Over the past five years, several challenges, opportunities and hurdles encountered our path. Below, I will discuss some of the main lessons learned that are crucial to take forward in precision medicine.

1. Bumetanide effects and adverse effects in neurodevelopmental disorders

The following figure shows an overview of the main behavioral outcomes in the three clinical trials.

Figure 2. The absolute change after 91 days bumetanide or placebo treatment on social, repetitive and irritable behavior in the BAMBI, BATSCH and BASCET trials



Note. Orange indicates placebo and blue indicates bumetanide. ASD = BAMBI trial; TSC = BATSCH trial; NDDs = BASCET trial.

Behavioral effects

Across the three conducted trials in three different NDD populations, we found evidence for positive effects on conventional endpoint questionnaires of irritable and repetitive behavior. The most consistent clinical effects were observed in the BATSCH-trial (TSC) trial, which may suggest that 'etiological' stratification is most successful in reducing response variability. However, this was an open-label design while the other two involved randomized placebo-controlled designs. The results of these RCTs indicated a subgroup of most responsive individuals delineated from that of placebo-treated individuals for repetitive behavior (RBS-R) in the BAMBI-trial and irritable behavior (ABC-I) in the BASCET-trial. It is plausible that these subgroups share an etiological mechanism leading to symptomatology, which is alleviated by the proposed E/I balance shift induced by bumetanide. In these most responsive individuals, where treatment effect seems less interleaved with placebo-treated individuals, neurophysiological and cognitive measures may capture more genuinely the relationship between bumetanide's neurological effect and the associated clinical improvement.

Seizure comorbidity and susceptibility

E/I imbalances and neuronal hyperexcitability have been hypothesized to explain shared common pathways between epilepsy and ASD. However, we found no evidence that bumetanide has an effect on seizure frequency neither in the BATSCH-trial (**chapter 2**), nor in the BASCET-trial (**chapter 5**), where all three patients with epilepsy were unfortunately allocated to the placebo group despite the block randomized design. We cannot draw conclusions due to the limited sample size, but our observations seem to be consistent with other research that failed to show anti-seizure efficacy of bumetanide in neonatal seizures[6] (for a review see [7]), although a pilot study showed a potential effect for temporal lobe epilepsy[8]. We may however view bumetanide as a pharmacological agent that may exerts effects on E/I balance, with the potential to enhance previously ineffective GABAergic treatment for seizures – or have a disease-modifying effect on behavioral consequences of epilepsy[9]. Yet, it is complicating that, apparently, chloride dysregulation does not seem to contribute to epileptic seizures, and in a similar manner that epilepsy does not arise simply by increased neuronal excitation and/or decreased inhibition. All kinds of different mechanisms may contribute to the seizure threshold and these may in turn trigger different compensatory mechanisms[10]. Thus, we cannot perceive epilepsy and ASD simply as disorders of "hyperexcitability", albeit they often seem to co-occur. The relationship between different sources of E/I balance (dys)regulation and clinical symptoms needs to be further tested in innovative trial studies.

Side effects

Pharmacological agents usually come with unwanted side effects. The safety profile of bumetanide was favorable over those of conventional medications such as stimulants and anti-psychotic medication. In all three clinical trials, bumetanide treatment was well-tolerated by the participating children and adolescents. No serious adverse events related to the study medication were reported and all adverse events were reversible and resolved (see Table 1). As expected from its diuretic effect, hypokalemia was the most commonly observed adverse event (in almost half of the bumetanide treated participants), but could be safely mitigated by oral potassium-chloride supplements. Orthostatic hypotension, increased diuresis, and myalgia were also frequently occurring adverse events in participants treated with bumetanide. Taking the three trials together, hypokalemia did not occur before the safety visit on day 14 in all but two patients. Potassium levels did not drop below 3.0mmol/L and were normalized with increased oral potassium-chloride. These blood safety analyses suggest that blood checks can be reduced from 5 to 3 times by omitting visits on days 4 and 7, which reduces the burden for patients in future bumetanide trials. It is important to note that for some children however, the necessary blood checks for monitoring bumetanide treatment are too demanding. Although hypokalemia was well manageable, when left unmonitored it is potentially dangerous. Thus, dedicated expertise is needed when monitoring bumetanide treatment. Needless to say, it is important to continuously reflect whether the advantages of a treatment outweigh the disadvantages. Enhancing the likelihood of treatment response by improved treatment prediction would influence this consideration.

Table 1. Most common adverse events as a result of bumetanide treatment

Adverse events	BATSCH-trial	BASCET-trial	BAMBI-trial	Total
Hypokalemia	7/13	5/15	24/47	48.0 %
Orthostatic hypotension	1/13	8/15	17/47	34.7 %
Increased diuresis	1/13	6/15	14/47	28.0 %
Myalgia	0/13	7/15	12/47	25.3 %
Dehydration	1/13	3/15	8/47	16.0 %
Obstipation	2/13	0/15	5/47	9.3 %

Subjective experiences

In addition to the clinical endpoints employed in the trials we learned from patients' and caregivers' personal perspectives on treatment effects in the almost hundred children treated with bumetanide over the last years. Overall, we identified several recurrent

themes in these patient and parent-reported observations: 1) having more cognitive 'capacity' (for example, a teacher had commented that a child seemed to absorb more information and contemplated to a greater extend before answering questions which led to better formulated answers), 2) showing less anger and/or frustration and 3) being more 'present' or attentive both in learning and social context. Parents consistently reported that these three themes resulted in developmental progress, either in school or home environments. This accords with earlier observations from other researchers showing comments from parents stating i.e., *"increased 'presence' with family and friends at school"* (p. 5[4]) after bumetanide treatment[11]. Also consistent with previous studies[4, 11], a large number of parents requested to continue bumetanide treatment for their child after the trials, reflecting positive experiences with bumetanide treatment.

The TAND-checklist - a characterization measure and used for clinical check-ups – that was used in the BATSCH-trial (**chapter 2**) further showed the importance to include the patient and caregiver perspective on clinical effects. This measure showed that the number and duration of temper tantrums and aggressive outbursts decreased. Importantly, the familial situations also improved, which was quantified as a reduction of familial stress and improved relationships between parents. Although the TAND checklist was included as an exploratory measure - it showed to be highly valuable in describing in which areas parents and patients desired and noticed improvements and how these improve daily life and functioning.

Methodological considerations

Unfortunately, not all measurements are obtainable in all clinical populations. Although it is intriguing to study brain activity with EEG in patients with severe NDDs (such as TSC) due to the proposed aberrations in excitatory and inhibitory neurotransmissions, this may be unrealistic in clinical reality. As many children with an NDD also have intellectual disability, it is difficult to detect a change of treatment on tests of cognitive functioning or ERP paradigms with instructions that may be too complex. We experienced this in the TSC-trial, which turned out to be too demanding for participants with intellectual disability. It is important that future studies that assess EEG parameters or neurocognitive functioning, include tests that can adapt to the level of the participant.

Another recommendation for future bumetanide trial designs is to prevent potential unmasking in RCTs due to the diuretic properties of bumetanide. There was substantial agreement between parent-reported expected and actual treatment allocation in the BASCET-trial (**chapter 5**). Although treatment and masking regimes

in the BASCET and BAMBI trial (**chapter 3**) were identical, no indication of insufficient masking was found in the latter trial. Adverse diuretic effects may have contributed to unmasking, as diuretic side effects were restricted to the bumetanide groups. Future trials may circumvent this general concern in bumetanide trials by considering the use of comparatives with diuretic properties. Another avenue is the discovery of novel NKCC1-selective inhibitors, without inhibition of kidney Cl^- transporter NKCC2 and subsequent diuresis. Fortunately, such a small molecule has recently been discovered and was able to restore aberrant neuronal chloride *in vitro* and ameliorate core behaviors in ASD mice without diuretic effects[12].

2. What researchers monitor versus what caregivers hope to improve

Clinical questionnaires as endpoint measures

Throughout the trials, we observed an important discrepancy between what clinical trial guidelines prescribe as endpoints and what parents hope to improve. Parents further criticized the large number of questionnaires they had to fill out repeatedly during the trial. Their main concerns were the amount of questions (e.g., 125 items for the SP-NL), the time it took to complete them, the redundancy of questions between questionnaires, not being able to fill in "not applicable", the relevance for their child or situation and most importantly, a difficulty to indicate change during the trial based on the available questions and answer options. This is not surprising, since most included questionnaires were developed as screening or 'characterization' tool, instead of outcome measure. Besides, there is no consensus about which questionnaire to use for specific interventions or patient populations and how to approach multiple missing or "not applicable" items in questionnaires.

As mentioned before, in the BATSCH-trial (**chapter 2**) we used a checklist to evaluate which problems, according to caregivers, are most prominent and need the most attention. This is an important step since we need to focus on measuring those symptoms that are of importance to patients and parents. For instance, a main parental concern was the manifestation of temper tantrums and aggressive outbursts. We used the checklist to count the number of occasions, the duration, and in which context these behaviors manifested. We could hereby evaluate whether this particular behavior indeed improved (or worsened), and whether this varied depending on specific contexts. The advantage of the TSC-trial being a small open-label trial was that we could attain this more detailed information from all participants and parents. However, we noted that for (large) RCTs, there are currently no techniques or methods available to obtain reliable outcomes from the patient and parent perspective.

Patient-report outcome measures (PROMs)

To address this need, we progressed to develop these more relevant outcomes in the sensory-PROM study (**chapter 6**). The sensory-PROM study was fueled by feedback from parents that participated in the different trials. We aimed to develop an outcome measure in the sensory domain generated by relevant sensory themes according to caregivers of children with ASD. Analysis of the interviews with these caregivers showed that sensory behaviors were as unique as fingerprints and often dependent on specific settings (e.g., loud music may be problematic for a child in public transport but at the same time preferred when in their own room), that target symptoms varied considerably between parents and that they often indicated indirect symptoms related to sensory processing. Thus, instead of adding to the pile of new questionnaires that are being developed and subsequently need to be psychometrically tested, we adjusted our scope and searched for a more satisfactory alternative.

We found this in the use of a dynamic system of psychometrically sound patient-reported outcomes. This universally accepted system is the Patient-Reported Outcomes Measurement Information System (PROMIS®), which contains a variety of domains consisting of PRO measures[13]. The PROMIS has developed large item banks by using modern psychometric methods such as Item Response Theory (IRT) that enable the use of Computerized Adaptive Testing (CAT)[14] to develop meaningful and precise outcome measures which can be used across disorders and internationally. When compared against conventional outcomes, PROMIS domains are found to be more relevant, more reliable, and have a reduced respondent burden[15-17]. In addition, the use of IRT-based scales results in improved sensitivity to change, an important advantage for clinical trials. We compared the parent-reported outcomes identified from our study with item banks from PROMIS.

In retrospect, our trials would have benefited from using PROMIS CATs as (primary) outcome measures. Due to the improved sensitivity to detect change with IRT-based scales, this could have resulted in larger treatment effects and consequently to less participants needed for adequate statistical power which circumvents (our) underpowered trials. As PROMIS CATs can be used across different disorders, it is also possible to use this single system with relevant questions for each patient and comparable T-scores across different disorders, such as TSC, epilepsy, ASD and ADHD. Besides, the often-voiced concern of redundant and lengthy questionnaires will be substantially improved. Potential biases associated with the use of PROMs and thus PROMIS should be acknowledged[18], although these may be circumvented by having a placebo-controlled design. Indeed, the EMA and FDA state that PROMs are

not recommended as primary outcome in open-label studies and that they should be combined with functional or objective outcomes[19, 20].

Although the use of the TAND-checklist was a good starting point to assess more context-specific and relevant behavioral changes, we believe that we should progress to the use of innovative, reliable and psychometrically sound item banks in future trials. In addition, using PROMIS CATs in future trials will improve power and comparability of results between patient groups and countries.

3. Does the mechanism-based treatment treat the mechanism?

The study of a pharmacological agent with a mechanism-based approach inherently involves questions about the existence of the mechanism, in addition to whether the mechanism is being treated by the intervention. Studies have claimed that bumetanide poorly crosses the blood-brain barrier after systemic administration[21]. A major challenge is that bumetanide levels in the brain, or local chloride concentrations, cannot be measured in human subjects (yet). Therefore, doubts have been raised on the nature of bumetanide effects and whether for instance these are not be attributed to systemic effects. We are inclined to interpret that bumetanide enters the brain sufficiently to alter clinical correlates of network functioning. Other studies have indicated functional brain effects of bumetanide in ASD using magnetic resonance spectroscopy and eye tracking, albeit in open-label trial designs[22, 23]. EEG effects of bumetanide in ASD and TSC have been described in case reports[24, 25] and the TSC-trial in this thesis showed changes in ERPs. It could be possible that brain availability of bumetanide is higher in ASD and epilepsy due to different pharmacokinetics, a more permeable blood-brain barrier (BBB)[26, 27], or effects through BBB-free areas (e.g., the median eminence)[7]. Fortunately, strategies to improve the brain delivery of bumetanide, for instance, with lipophilic analogs of bumetanide derivatives that more easily penetrate the brain, are being developed and studied[28, 29].

Perhaps the most attractive framework to explain behavioural effects of bumetanide is via changes in the regulation of brain activity, and the balance between excitatory and inhibitory signals, the so-called Excitation-Inhibition (E/I) ratio. Administration of bumetanide may lower chloride levels and reinstate GABAergic transmission, resulting in a more balanced E/I essential for adequate sensory and information processing. As discussed, parents reported improvements in cognitive 'capacity' and their children being more 'present', which tentatively may be conceived as behavioural consequences of enhanced signal transmission.

We realize and acknowledge that E/I balance is a very broad and multi-layered construct. The umbrella term ranges from molecular changes to network level measurements of average E/I including billions of neurons. However, although we currently lack consensus on a measure of E/I balance, there are studies experimenting with exploratory measures that indirectly quantify E/I balance[30-32], which may progress to better markers in future studies. We view E/I balance as a useful concept for understanding how well-known pre-clinical mechanisms in animal models, as illustrated by the TSC-trial, may translate to and advance human studies.

The next question will be, if we know which mechanism we treat, how can we identify children that will benefit most from targeting different elements of E/I regulating mechanisms?

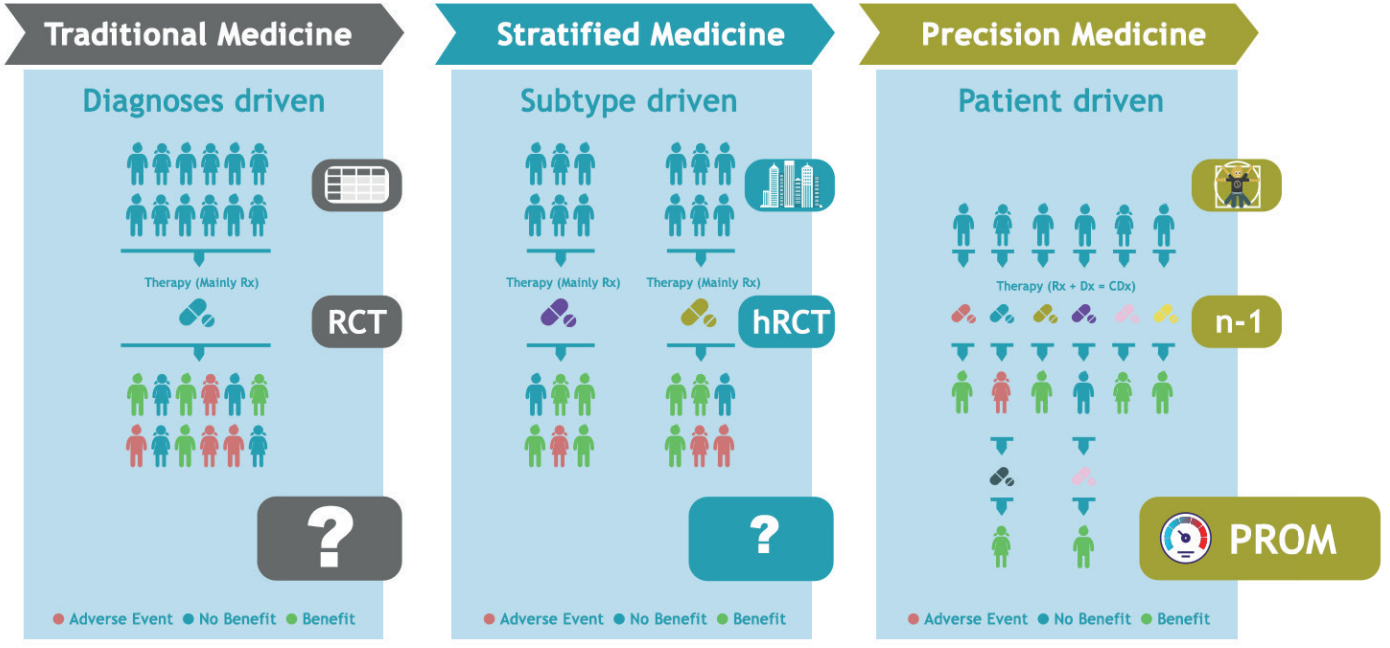
4. From traditional to stratified psychiatry

From the BAMBI trial (**chapter 3**), we concluded that our findings do not support broad bumetanide applicability in ASD, but do indicate effectiveness for a *subset* of patients. This statement was met with resistance from some researchers. Ben-Ari and colleagues responded to our article[33], stating that the discrepancy between our studies can be explained by an "*important placebo response that made the drug unable to reveal its efficacy on the primary criterion*". However, this claim could not be substantiated since the standardized placebo effect in the BAMBI-trial (standardized mean change [SMC] = -.27) was comparable to their studies (SMC = -.43[4]; SMC = -.32[5]) and lower than the average of other reviewed ASD trials[34]. In our reply[35], we argued that our results did not discourage bumetanide trials in the future, but that stratified trial designs or post hoc analyses may shine more light on treatment response variability, which is clearly observed in the currently available bumetanide trials and cannot solely be explained by different trial designs. The ongoing incentive of Ben-Ari and colleagues to register bumetanide for the entire ASD population (Phase III: https://clinicaltrials.gov/ct2/show/NCT03715153), may be supported by the pharmaceutical industry that pursues DSM-diagnosis based-application. Such desire may lead to millions of people taking pharmacological agents without benefit (or that are even harmful) which is sometimes called 'imprecision medicine'[36, 37]. It may be clear by now, that we don't share this desire. It seems more plausible and likely that a mechanism-based pharmacological agent is at best effective for a yet-to-be-defined subgroup within the heterogeneous ASD population.

The utility of neurocognitive and EEG tests as stratification marker appeared to be complex for bumetanide. Although our trials showed clear bumetanide effects on

a variety of behavioral manifestations, no linear effects were found on the broad neurocognitive and EEG test batteries we included in the TSC and ASD trials. The findings rather implicate that bumetanide may have effects that lead to subtle, relative shifts in neurocognitive networks, but are not detectable when measuring individual neurocognitive parameters. For future trials, it is important to realize that neurocognitive functioning can be perceived both as clinical endpoint and as functional measure for stratification purposes to predict treatment response. Whether to include it as stratification or outcome measure may depend on the proposed mechanism of treatment and remains a complex question that should be further elucidated. Additional resting-state EEG analyses of the BATSCH and BAMBI-trials will be published shortly. Indeed, provisional EEG analyses from the BAMBI-trial showed a clear effect of bumetanide on neuronal oscillations and suggest that resting-state EEG biomarkers may aid treatment response stratification.

Figure 3. Approaches in psychiatry: from a diagnosis-driven approach to a patient-driven approach



New horizons: N-of-1 trials and adding a fifth 'P' to P4 medicine

Our trial results showed that bumetanide can ameliorate behavioral manifestations in different NDDs. The success of follow-up studies depends on the approach researcher adopt. For instance, in the BATSCH-trial, we concluded that more elaborate trials are needed and justifiable given the promising results on behavior and quality of life associated with TSC in this open-label study. A traditional next step would be to opt for a large RCT to determine the application and effect size of bumetanide for the TSC population. However, we observed subtle but potentially important differences in treatment response that may be explained by individual differences, which are difficult to incorporate in large RCTs. For instance, patients with higher IQ's showed better treatment response, and we partly expect this to be a consequence of our measurements not being sensitive enough to detect change in lower IQs (or severely affected children may possibly need more time to display a treatment response). It would therefore be helpful to create individually appropriate outcome measures. We may also want to adapt our dosing regimen depending on the specific patient, based on for instance previous medication use or expected adverse events. This is also difficult to achieve in RCTs that rely on standardized measurements and treatment regimes. Overall, it is time to move away from large RCTs as our gold standard and refine our approaches to focus on individual, instead of average, responses to treatment (Figure 3). Patients do not receive individualized feedback in RCTs as researchers only obtain an average of the group and large sample sizes and long durations are needed to establish efficacy. Recruitment is often challenging as patients are reluctant to participate due to a 50% chance of receiving placebo treatment for the entire trial duration. An important alternative is to be found in N-of-1 trial designs, which aim to provide a definitive answer to the following question: does this *specific* treatment works for this *particular* person (Figure 4)?

Imagine...

A patient goes to the psychologist or psychiatrist and asks about a certain treatment he heard about, to deal with his extreme fatigue. The doctor replies *"Yes! We observed a* ***significant effect*** *of the treatment* ***on average****, which is great!"* The patient asks: *"But will it work for me?"* to which the doctor responds: *"Oh* ***I don't know if it works for you****, but the results of the RCT were very positive!".*

To date, this is reality.

N-of-1 trial designs are personalized to the patient, from choosing outcome measures to providing certain treatments, to individualized feedback. Of interest to researchers, data from multiple N-of-1 trials can be aggregated to obtain an average treatment response on population-level. N-of-1 trials may not only be desirable in research, but may become the norm in general when starting (or discontinuing) treatment for children with NDDs, to monitor and evaluate the best possible treatment. In the long haul, we could expand our (diagnostic) system into one in which labels do not dominate, but individual problems of the child are central and are the starting point for the most optimal treatment and guidance for clinicians.

Figure 4. An example of N-of-1 trials and P4 medicine

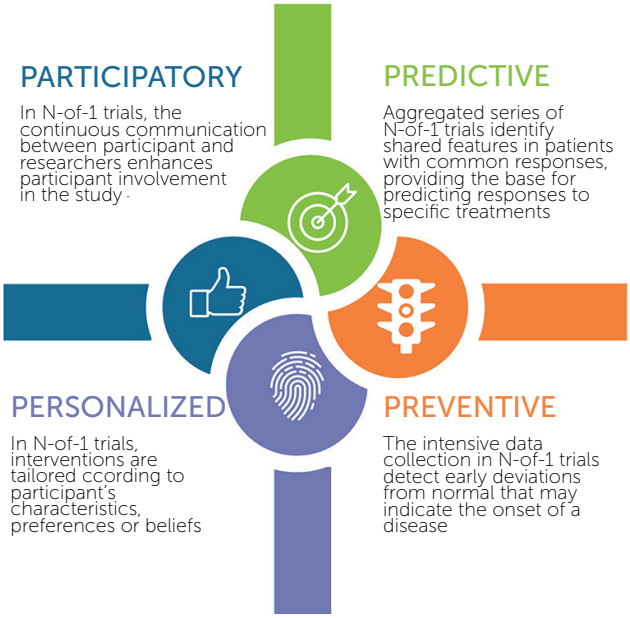


Figure adjusted from Soldevila-Domenech et al., 2019 (Frontiers in Nutrition)[39]

The use of N-of-1 trials in research and clinical settings is also a useful approach in the vision that is coined as *P4 medicine*: predictive, preventive, personalized and participatory[38]. **Predictive** symbolizes the possibility to develop prediction models, to answer the question whether a certain type of treatment is suitable for a specific patient, for instance based on shared features and markers identified from aggregated series of N-of-1 trials. **Preventative** stands for the ability to detect deviations from normal or side effects much earlier due to the repeated and in-dept data collection

in N-of-1 trials. As already mentioned, treatment in N-of-1 trials can be individually tailored with stratification based on a combination of several individual characteristics and preferences, such as age, gender, history of disease and/or the molecular profile, which is termed **personalized**. Lastly, the involvement of patients in N-of-1 trials will be enhanced due to continuous communication between researcher and patient and since the patient feels that the trial is about him or her, and not about a group in general, which is coined **participatory**. This enhanced involvement will certainly benefit recruitment in future trials. Thus, N-of-1 trials and P4 medicine aim to move from reactive to proactive medicine. When looking back at the conclusions based on this dissertation, we might add a fifth "P" to this concept: to always use **PROMs** to assess only reliable and relevant outcomes, for all patients.

III. Conclusion

The aim of this dissertation was to improve rational, mechanism-based treatment options for NDDs by testing whether increased phenotypic and/or etiological homogeneity translates to reduced variability in treatment response. The results described in this thesis show:

- Bumetanide may have a positive effect on behavioral manifestations in different NDDs, and specifically may improve irritable and repetitive behavior. The neuronal mechanisms that drive these behavioral effects need to be further elucidated.

- We emphasize the need for more stratified psychiatry aimed at providing specific treatment options only for those children that are likely to benefit from it and to use better outcome measures. Stratification on the basis of genetic disorders is a promising approach along with stratification based on a potential shared developmental pathway, such as the existence of sensory processing difficulties, which may have more value in clinical trials compared to unselected samples.

- N-of-1 trial designs may be a powerful option to overcome important drawbacks of RCT designs in heterogeneous NDD populations.

- The use of PROMIS item banks in clinical trials may be a solution to assess both direct and indirect consequences of sensory issues across NDDs and improve the development of sensory interventions, which is seen as top priority in the ASD community. Future studies are encouraged to refine and evaluate their value as primary outcomes measures.

In conclusion, these findings highlight the need of a shift from a traditional diagnosis-driven approach in trial design with no incorporation of biological knowledge to a more stratified and subtype-driven approach. It is of utmost importance to evaluate the severity and precise nature of symptoms on the daily life of the patient and their social and family life and refining treatment options based on this. Ultimately, we need to advance to patient-driven medicine, which integrates a variety of clinical and biological information to indicate the most effective and least harmful treatment for the individual child with NDD problems.

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NEDERLANDSE SAMENVATTING

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ACHTERGROND

De afgelopen vijf jaar heb ik onderzoek mogen doen naar het brede scala aan problemen en verstoringen waar kinderen met een ontwikkelingsstoornis mee te maken krijgen. Op dit moment worden ongeveer 1 op de 6 kinderen ergens tijdens hun jeugd gediagnosticeerd met een ontwikkelingsstoornis en de meesten hebben hulp nodig voor de bijkomende gedrags- en ontwikkelingsproblemen. Psychologen en psychiaters kunnen een kind classificeren met een specifieke ontwikkelingsstoornis op basis van een vooraf gedefinieerde set van zichtbare gedragscriteria: een klinische classificatie, vaak ook een *diagnose* of *label* genoemd. Bekende en veelvoorkomende klinische classificaties zijn autisme spectrum stoornis (ASS) en 'attention deficit hyperactivity disorder' (ADHD). Er is echter een groeiend besef dat verschillende diagnoses geassocieerd zijn met dezelfde mechanismen en andersom is er veel overlap in symptomen tussen kinderen met verschillende klinische classificaties, tezamen ook wel met klinische heterogeniteit aangeduid, oftewel de mate van verscheidenheid. Mede dankzij genetische en neurowetenschappelijke studies is het bewustzijn van heterogeniteit van ontwikkelingsstoornissen versterkt en is er een trend zichtbaar die afstand neemt van het idee dat ze onder te verdelen zijn in aparte en welomlijnde classificaties of categorieën. We beschouwen ontwikkelingsstoornissen nu meer en meer als een spectrum, zoals bij autisme *spectrum* stoornis. Deze nieuwe kijk op ontwikkelingsstoornissen leidt tot nieuwe mogelijkheden binnen de kinderpsychiatrie: we kunnen onze benadering van diagnostiek en behandelingen aanpassen en verbeteren op het individu en niet langer het 'label' behandelen. Maar de vertaalslag van onderzoek gericht op dit soort verfijning naar de klinische praktijk brengt ook uitdagingen met zich mee.

De volgende alinea's beschrijven eerst twee belangrijke uitdagingen (de one-size-fits-all aanpak en symptoombestrijding) en vervolgens de hypotheses waarop de onderzoeken in dit proefschrift gebaseerd zijn en een mogelijk passende rationele behandeling (bumetanide). Aansluitend worden twee bruikbare benaderingen besproken (stratificatie en passende uitkomstmaten), die kunnen bijdragen aan het uiteindelijke doel van dit proefschrift: het verbeteren en verfijnen van effectieve behandelingen voor ontwikkelingsstoornissen richting meer gepersonaliseerde zorg.

De huidige visie op behandeling: one-size-fits-all en symptoombestrijding

De behandeling van ontwikkelingsstoornissen met medicatie is tot nu toe gericht op het verminderen van symptomen: het meest problematische zichtbare gedrag. Zoals driftbuien bij ASS of impulsiviteit en verminderde concentratie bij ADHD. Deze aanpak wordt echter steeds vaker bekritiseerd omdat het de diversiteit in gedrag en diversiteit in onderliggende etiologie (een oorzaak of de ontstaansgeschiedenis) negeert. Ondanks dat we veel verschil zien tussen kinderen geclassificeerd met *dezelfde* stoornis en juist veel overlap zien tussen kinderen met *verschillende* stoornissen, wordt er in de psychiatrie nog steeds vaak gezocht naar een wondermiddel dat effectief is voor alle kinderen met één specifieke aandoening: de '**one-size-fits-all'-aanpak**. Dit staat in schril contrast met de enorme genetische diversiteit en etiologische complexiteit van ontwikkelingsstoornissen, waardoor het noodzakelijk lijkt dat we juist meer persoonlijke en rationele behandelingen ontwikkelen.

Door vooruitgang in de neurowetenschappen hebben we meer kennis gekregen over mogelijke onderliggende, etiologische processen die leiden tot verschillende vormen van ontwikkelingsstoornissen, welke ook wel *shared mechanistic pathways* worden genoemd. Desondanks is de ontwikkeling van nieuwe behandelingen nog steeds vaak gericht op **symptomatische, in plaats van rationele, mechanisme-gerichte behandeling**. Er worden bijvoorbeeld vaak antidepressiva voorgeschreven voor angstklachten of stimulantia ('ritalin') voor concentratieproblemen die vaak aanwezig zijn bij ASS, maar hoe deze samenhangen met het onderliggende mechanisme van ASS is onduidelijk. Een mogelijk uitgangspunt voor het ontwikkelen van gerichte 'rationele' behandelingen zijn mono-genetische aandoeningen (waarbij één mutatie in één gen verantwoordelijk is voor [een deel van] het ziektebeeld) die sterk geassocieerd zijn met ontwikkelingsstoornissen. Zoals Downsyndroom of Fragiele X (niet onderzocht in dit proefschrift) en tubereuze sclerose complex (TSC; onderzocht in dit proefschrift). Onderzoek naar mono-genetische aandoeningen blijkt heel nuttig als uitgangspunt om meer begrip te krijgen van onderliggende biologische mechanismen van verschillende aandoeningen. Onderzoek doen naar kinderen met een mono-genetische aandoening, in plaats van kinderen met een bepaalde klinische diagnose, lijkt een veelbelovende manier om nieuwe toepasbare, medicatie strategieën te ontdekken.

Het uitgangspunt voor dit proefschrift was dat experimenteel en klinisch onderzoek rationele behandelingen hebben geopperd voor ontwikkelingsstoornissen. De gemene deler is de hypothese dat ontwikkelingsstoornissen gebaseerd zijn op

afwijkende neuronale netwerkorganisatie en -activiteit die ontstaat in de vroege ontwikkeling (in de volgende paragrafen ga ik hier dieper op in). Hierop voortbouwend zijn in het afgelopen decennium innovatieve, rationele behandelingen ontstaan, waarvan sommigen gebaseerd zijn op "hergebruik" van bestaande medicijnen. Dat is gunstig gezien de onderliggende werkingsmechanismen en bijwerkingen van deze medicijnen al bekend zijn waardoor ze sneller gebruikt kunnen worden - als ze effectief blijken. Een van deze rationele behandelingen is gebaseerd op de excitatie/inhibitie disbalans hypothese, waarmee afwijkende neuronale netwerkactiviteit bij ontwikkelingsstoornissen uitgelegd kan worden.

De excitatie/inhibitie (E/I) disbalans hypothese en rationaal voor behandeling

Twintig jaar geleden werd de *E/I-disbalans hypothese* geïntroduceerd. Een E/I-disbalans staat voor een verstoorde balans tussen stimulerende (exciterende) en remmende (inhiberende) hersenactiviteit. De onderzoekers die de hypothese introduceerden waren geïnspireerd door hun observaties dat kinderen met ASS vaak óók epilepsie of epileptiforme afwijkingen hadden. Ze stelden dat ASS en aanverwante stoornissen werden gekenmerkt door een *verhoogde* E/I-balans in neurale circuits, wat leidt tot hyperprikkelbaarheid van deze circuits. Deze aanname is belangrijk gebleken en een waardevol concept om ons begrip van ontwikkelingsstoornissen verder uit te diepen. Echter was de initiële aanname dat overprikkeling in het brein leidt tot overprikkeling in gedrag te simpel, aangezien excitatie en inhibitie zeer complexe processen zijn.

Er zijn nadien veel studies geweest die een E/I-disbalans in diermodellen van ASS hebben gevonden. Een correctie van deze disbalans met medicatie leidde tot een normalisatie van autisme-achtige gedragingen. Ook in andere ontwikkelingsstoornissen is bewijs van een verstoorde E/I-balans gevonden. Epilepsie wordt bijvoorbeeld traditioneel beschouwd als het resultaat van een verhoogde E/I-balans en ook mono-genetische ontwikkelingsstoornissen zoals TSC, Fragile-X syndroom en Rett syndroom laten een verstoorde E/I-balans zien. In deze stoornissen wordt de E/I-disbalans vaak gelinkt aan verstoorde *GABAerge inhibitie*. Aangezien GABA een belangrijke neurotransmitter is met een inhiberende functie in het centraal zenuwstelsel, kan een verstoring in dit systeem verstrekkende gevolgen hebben.

De E/I-balans beïnvloeden via chloride

Een specifiek GABAerg -inhibitoir- mechanisme dat kan leiden tot E/I-disbalans in ontwikkelingsstoornissen is een verandering in de *chlorideconcentratie* in neuronen. In het tekstvak hieronder wordt dit verder uitgelegd. Bij de normale ontwikkeling vindt

er een opeenvolging van processen plaats rond de geboorte die gekenmerkt wordt door een drastische afname van de chlorideconcentratie in neuronale cellen. Deze afname veroorzaakt een verschuiving in de werking van GABAerge transmissie: van stimulerend (depolariserend) naar remmend (hyperpolariserend): ook wel de *GABA shift* genoemd. Oftewel, ook al kennen wij GABA vooral als de belangrijkste *remmende* neurotransmitter in de hersenen, tijdens de ontwikkeling *stimuleert* het in eerste instantie neurotransmissie in onrijpe neuronen. De GABA shift wordt veroorzaakt door een verandering in de activiteit van twee chloride co-transporters: NKCC1 en KCC2. NKCC1 zorgt voor meer chloride in de neuronen en KCC2 voor minder. Nog ontwikkelende en onrijpe neuronen hebben hogere chlorideconcentraties vergeleken met volwassen neuronen. Er is gespeculeerd dat deze GABA shift in sommige ontwikkelingsstoornissen niet (goed) optreedt, waardoor neuronen een onrijpe status behouden. Dit systeem biedt een mogelijke manier om symptomen van ontwikkelingsstoornissen te verbeteren door GABAerge-signalering te beïnvloeden via een behandeling die gericht is op het herstellen van de chlorideregulatie. En dit is zelfs al mogelijk met bestaande, goed bestudeerde medicijnen.

De chlorideconcentratie van een neuron, wat is dat precies?

De fundamentele cellen van het zenuwstelsel worden ook wel zenuwcellen of **neuronen** genoemd. Deze neuronen zorgen voor het ontvangen, verwerken en doorgeven van signalen. Een neuron bestaat uit een cellichaam, dendrieten (signaal ontvangen) en een axon (signaal doorgeven). Alle signalen die binnenkomen op de dendrieten worden opgeteld en bepalen de elektrische lading van het cellichaam. In rust, als er geen signaal binnenkomt of weggaat, bestaat er een potentiaalverschil: de binnenzijde van de cel is negatiever geladen dan de buitenzijde (-70mV). Als er wel signalen binnenkomen en de **elektrische lading** een drempelwaarde (-50mV) bereikt heeft, dan kan het neuron vuren. Er ontstaat dan een actiepotentiaal die via het axon een signaal doorgeeft.

Het **chloride-ion** is negatief geladen (Cl-). Als er relatief veel chloride-ionen in de cel zijn, is er een hoge **chlorideconcentratie**. De cel is dan erg negatief geladen en kan niet vuren. **Co-transporters** kunnen reguleren hoeveel ionen de cel ingaan (importer) of uitgaan (exporter). NKCC1 is een chloride importer en kan hierdoor zorgen voor meer chloride in de cellen. Wanneer je de activiteit van NKCC1 dempt of blokkeert, komt er dus minder (negatief geladen) chloride de cellen in en kan de neuron makkelijker de drempelwaarde bereiken om een signaal door te geven.

Bumetanide als potentiële behandeling om E/I te herstellen

Bumetanide is een van de meest bestudeerde medicijnen die invloed heeft op chloride concentraties. Bumetanide is een NKCC1-*antagonist*, wat wil zeggen dat het de werking van NKCC1 *dempt* of zelfs verhindert. Oftewel, de import van chloride wordt geblokkeerd waardoor chloride concentraties verlaagd worden en de GABAerge transmissie mogelijk hersteld kan worden. Bumetanide is van origine gebruikt als plaspil en wordt al bijna 50 jaar ingezet bij volwassenen en kinderen met hartfalen of oedeem (de aanwezigheid van vocht op plaatsen in het lichaam waar vocht normaal niet of nauwelijks aanwezig is) en heeft alleen milde, aan de werking gerelateerde bijwerkingen.

Ben-Ari en Lemonnier pionierden met het potentieel van bumetanide om gedrag van kinderen met een ontwikkelingsstoornis te verbeteren. Zij baseerden dit op verschillend preklinisch en klinisch onderzoek waaruit zij concludeerden dat kinderen met ASS vaak onrijpe neuronale netwerken hebben en dat onrijpe neuronen vaak stimulerend in plaats van remmend GABA hebben door verhoogde chloride concentraties. Een medicijn dat chloride concentraties verlaagt, zou volgens hen kunnen leiden tot verbeteringen in netwerkfunctie en daaropvolgend in gedrag en leren.

Verschillende onderzoeken hebben laten zien dat bumetanide inderdaad inhibitoire GABAerge transmissie kan herstellen in muismodellen van ASS. Vervolgens hebben studies met kinderen en adolescenten laten zien dat ASS-symptomen verminderden na bumetanide en spontaan oogcontact juist toenam, wat gepaard ging met een normalisatie van hersenactiviteit. Naast effecten op gedrag hebben studies in verschillende stoornissen ook het potentieel van bumetanide laten zien op *cognitieve functies* (functies die te maken hebben met het verwerken van informatie, zoals aandacht en geheugen). Alle studies bij elkaar genomen lijkt bumetanide een potentieel effectieve behandeling voor verschillende ontwikkelingsstoornissen.

Stratificatie en passende uitkomstmaten om nieuwe klinische studies te verbeteren

Een uitdaging in de kinderpsychiatrie is dat er geen testen beschikbaar zijn die kunnen bepalen welke behandeling het meest geschikt is voor een patiënt en hoe we de effectiviteit van een behandeling precies kunnen bepalen en definiëren. Het gebruik van biologische testen als diagnostische hulp wordt door sommigen toegejuicht en door anderen betwist (er zijn bijvoorbeeld al veel biologische testen voor onder andere ADHD voorgesteld, maar de resultaten kunnen vaak niet gerepliceerd worden in grote groepen). Toch lijkt het intuïtief onlogisch om negatief te staan tegenover

het gebruik van testen die potentieel gunstige, of juist mogelijke bijwerkingen op bepaalde medicatie kunnen voorspellen. Dit wordt ook wel **stratificatie** genoemd. Men zou in theorie bijvoorbeeld kunnen stratificeren op basis van genetica, neurofysiologie, cognitie en gedrag. En juist omdat ontwikkelingsstoornissen zo verschillend zijn, zouden we met stratificatie meer behandeleffect kunnen aantonen in vergelijking met de normale diagnostische categorieën, zeker wanneer er gerichte behandelingen getest worden in groepen die gestratificeerd zijn op basis van een bekend onderliggend mechanisme.

Naast stratificatie, hangt het succes van de inzet van nieuwe behandelingen af van de beschikbaarheid van **relevante uitkomstmaten**, waarmee ook verandering door behandeling gemeten kan worden. Idealiter is zo'n uitkomstmaat gericht op het perspectief van de patiënt of ouder/verzorger, wat hij of zij echt belangrijk vindt. Helaas is dit vaak nog niet het geval. De categorieën en symptomen zoals beschreven in het handboek van de psychiatrie (DSM-5) komen niet altijd overeen met de gedragingen die patiënten en ouders het meeste in de weg zitten. Daarnaast zijn veel uitkomstmaten, zoals vragenlijsten, initieel ontwikkeld om patiënten te diagnosticeren en zijn ze daardoor vaak erg lang, onbetrouwbaar als je ze vaak herhaalt en minder geschikt om verandering te vangen. Bovendien zijn de vragenlijsten vaak geen directe afspiegeling van uitdagingen in het dagelijks leven. Er is dus een alternatief nodig, waarbij individueel gekozen symptomen beoordeeld kunnen worden en een systeem dat gebruikt kan worden in klinische trials en waarbij de uitkomsten vergeleken kunnen worden.

Om deze uitdagingen samen te vatten: de ontwikkeling van behandelingen voor ontwikkelingsstoornissen is op dit moment gericht op symptomen in plaats van onderliggende mechanismes en maakt gebruik van een one-size-fits-all-benadering, waarbij factoren die de uitkomst voor een individu kunnen voorspellen, grotendeels onbekend zijn of genegeerd worden.

In dit proefschrift hebben we daarom drie klinische onderzoeken en een kwalitatief onderzoek uitgevoerd door middel van (i) verschillende stratificatiestrategieën en (ii) het evalueren van bumetanide, een op mechanismen gebaseerde behandeling en tot slot (iii) het identificeren van patiëntrelevante behandelresultaten. Met verschillende stratificatie methoden hebben we geprobeerd recht te doen aan de variatie in onderliggende oorzaken en de verscheidenheid aan symptomen binnen en tussen verschillende ontwikkelingsstoornissen. We verwachtten dat wanneer we meer gelijke groepen creëerden door bijvoorbeeld te kijken naar overlap in oorzaken of overlap in bepaalde gedragskenmerken, we minder variatie zouden zien in de reactie op de behandeling.

De onderzoeken en bevindingen in het proefschrift

In de BATSCH-studie (**hoofdstuk 2**) stratificeerden we behandeling op basis van een mono-genetische stoornis - TSC - en hebben we derhalve patiënten met deze aandoening onderzocht. De reden om specifiek TSC te onderzoeken is dat er wordt aangenomen dat de hersenafwijkingen bij TSC, met name de tubers, een bron van overmatige en ongewenste hersenactiviteit vormen. In de tubers is een verstoorde balans tussen stimulerende en remmende hersenactiviteit (E/I balans) aangetoond en deze is gerelateerd aan verstoringen in chloride regulatie (zie bovenstaande textbox). Bumetanide zou de chloride concentratie in de hersencellen kunnen verlagen en zo overmatige hersenactiviteit verminderen. We bestudeerden het effect van bumetanide in BATSCH niet alleen op gedragssymptomen maar ook op hersenfuncties door gebruik van auditieve electroencephalography (EEG) taken (bij EEG worden de veranderingen in elektrische activiteit van de hersenen gemeten), cognitieve taken en gedrag door middel van vragenlijsten. We ontdekten dat bumetanide inderdaad het prikkelbare (*irritability*), sociale en hyperactieve gedrag kon verbeteren. Daarnaast vonden de patiënten hun eigen kwaliteit van leven hoger na bumetanide behandeling. Op het hersen functioneren leek bumetanide een normaliserend effect te hebben bij één van de taken, maar op de cognitieve taken zagen we geen verschil na de behandeling.

De BAMBI-studie (**hoofdstuk 3 en 4**) was gericht op de ontwikkeling van kenmerken (*markers*) die op basis van cognitie en/of EEG maten een positief effect op bumetanide konden stratificeren. Daarnaast werd onderzocht of eerder aangetoonde positieve effecten van bumetanide op sociaal gedrag van kinderen met ASS konden worden gerepliceerd (**hoofdstuk 3**). Het effect van bumetanide bij kinderen met een ASS-diagnose werd middels een dubbel-blind gerandomiseerd en placebo-gecontroleerde trial onderzocht. Anders dan voorgaande studies lieten zien, vonden wij geen superieur effect van bumetanide op sociaal (en prikkelbaar [*irritability*] en sensorisch) gedrag. Wel zagen we een effect op repetitief gedrag, wat een ander kernsymptoom is van ASS. Dit effect leek meer aanwezig bij vrouwelijke deelnemers. We vonden ook dat leeftijd mogelijk invloed had op het effect van bumetanide: we zagen een grotere verbetering op sociaal gedrag bij jongere kinderen.

Om het nut van een cognitieve stratificatie marker in klinische trials te onderzoeken, werd ook het effect van bumetanide op cognitieve testen en de neurocognitieve netwerkorganisatie onderzocht tijdens de BAMBI-studie in **hoofdstuk 4**. Gezien een goed afgestemde balans tussen E/I cruciaal is voor adequate informatieverwerking, werd verwacht dat medicatie die E/I kan beïnvloeden, een positief effect heeft

op problemen in de informatieverwerking. Een groot aantal tests werd gebruikt om verschillende belangrijke *cognitieve domeinen* te meten, zoals aandacht en impulscontrole. Voordat deelnemers gestart waren met de medicatie zagen we grote verschillen in het uitvoeren van de taken, veel kinderen hadden met name moeite met het remmen van impulsen. Na de bumetanide behandeling vonden we geen effecten op de belangrijkste cognitieve componenten en we vonden ook geen cognitieve verslechtering (wat je bij andere medicatie soorten soms wel ziet). Met innovatieve analyses werd de samenhang tussen verschillende cognitieve domeinen voor en na bumetanide behandeling gemeten. Hiermee vonden we wel een aantal interessante verschillen na bumetanide, die mogelijk wijzen op een meer subtiele verschuiving en alleen detecteerbaar zijn wanneer we de organisatie van componenten als geheel beschouwen en niet louter kijken naar individuele cognitieve taken.

Vervolgens hebben we in **hoofdstuk 5** onderzocht of er alternatieve manieren zijn om vooraf op bepaalde eigenschappen te stratificeren, om zodoende de variatie in behandeleffect te verminderen. In een dubbel-blind gerandomiseerd en placebo-gecontroleerd onderzoek hebben we kinderen met een ontwikkelingsstoornis, ASS, ADHD en/of epilepsie, gestratificeerd op basis van problemen in de prikkelverwerking. De verwachting was dat het hebben van prikkelverwerkingsproblemen een betere voorspeller van bumetanide effect zou zijn dan het hebben van een specifieke diagnose. Bijna driekwart van de patiënten werd geclassificeerd met ASS; 20% met ADHD, en 2 kinderen hadden epilepsie. Helaas deden er niet genoeg kinderen mee om conclusies te trekken. We beschouwen de studie daarom als een *proof-of-concept* studie, met aanbevelingen voor vergelijkbaar en toekomstig onderzoek. Opvallend genoeg vonden we wel een superieur bumetanide effect op prikkelbaar gedrag (*irritability*) in deze kleine steekproef. Dit positieve effect van de bumetanide behandeling leek alleen niet af te hangen van de mate van prikkelverwerkingsproblemen die het kind bij aanvang van de studie had. De uitdagingen die we tijdens de werving van dit onderzoek tegenkwamen en de aanbevelingen die hieruit voortvloeiden zijn met name gericht op het samenwerken met expertisecentra voor een evenwichtige balans tussen de groepen en een focus op relevante uitkomstmaten die cross-diagnostische kernmerken kunnen vangen, in plaats van diagnose-specifieke symptomen.

Maar hoe zit het dan met die relevante uitkomstmaten? Volgend op onze interesse in de verstoorde prikkelverwerking als een kenmerk van verschillende stoornissen (cross-diagnostisch), evenals een mogelijk aanknopingspunt van een mechanisme-gerichte behandeling, is het van belang om uitkomstmaten voorhanden te hebben die de verstoorde prikkelverwerking kunnen meten. Ondanks dat deze cruciaal zijn om te gebruiken in klinische trials gericht op prikkelverwerkingsproblemen, ontbreken

deze relevante en gepersonaliseerde uitkomstmaten momenteel. Om hieraan bij te dragen hebben we naast de klinische studies, ook een kwalitatief onderzoek uitgevoerd: de prikkel-PROM-studie, beschreven in **hoofdstuk 6**. We hebben in dit onderzoek ouders uitgebreid geïnterviewd waaruit relevante thema's geïdentificeerd konden worden van prikkelverwerkingsproblemen in het dagelijks leven van kinderen met ASS, die als uitkomsten in klinische studies gebruikt kunnen worden. De thema's konden vertegenwoordigd worden in 11 uitkomstmaten. Vervolgens hebben we deze uitkomstmaten vergeleken met een bestaand generiek portaal, de PROMIS-itembanken. In deze itembanken zitten uitsluitend vragen die vanuit de patiënt en ouders ontwikkeld zijn en dit vragenlijst portaal is heel bruikbaar voor klinisch onderzoek: het is erg gebruiksvriendelijk, minder tijdrovend dan normale vragenlijsten en de scores kunnen tussen verschillende stoornissen en onderzoeken vergeleken worden. Zeven van de 11 uitkomsten konden worden gemeten met de PROMIS-item banken en vier met bestaande PROM's. Opmerkelijk genoeg waren de meest gerapporteerde thema's *indirecte* gevolgen van een verstoorde prikkelverwerking, zoals vermoeidheid of slaapproblemen, een onderscheid dat in het veld nog niet vaak is gemaakt, maar mogelijk wel belangrijk is voor het ontwikkelen van nieuwe behandelingen.

Wat betekenen deze resultaten?

De resultaten van de studies geven ons een idee van het effect van bumetanide bij kinderen met een ontwikkelingsstoornis: met name prikkelbaar en repetitief gedrag lijkt te verbeteren. Aangezien we met name een duidelijk effect zien bij patiënten met TSC, zou men kunnen concluderen dat stratificatie op basis van een genetische stoornis zinvol is. Daarentegen lijkt bumetanide geen effect te hebben op het aantal epileptische aanvallen, al hebben er te weinig kinderen met epilepsie deelgenomen aan de studies om hier uitsluitsel over te geven. Ook is het belangrijk om bij het testen van nieuwe medicatie, of bestaande medicatie voor een nieuwe doelgroep, de bijwerkingen te bestuderen. We kunnen uit onze studies concluderen dat bumetanide een relatief veilig medicijn is in vergelijking met medicatie die vaak voor kinderen met een ontwikkelingsstoornis voorgeschreven wordt, zoals stimulantia of antipsychotica. De meest voorkomende en tevens voorspelde bijwerking was te weinig kalium in het bloed (hypokaliaemie), wat eenvoudig voorkomen kon worden met kalium tabletten.

Naast de verschillende uitkomstmaten (bijvoorbeeld vragenlijsten of checklists van bijwerkingen) waarmee we de effecten van bumetanide konden rapporteren, hebben we ook veel geleerd van de gesprekken met kinderen en ouders over hun perspectief. We konden een aantal terugkerende thema's identificeren: 1) meer cognitieve

capaciteit (voorbeeld: een leraar vertelde de ouders van een deelnemer dat zij meer informatie tot zich kon nemen en beter nadacht voordat ze een antwoord gaf, wat resulteerde in beter geformuleerde antwoorden), 2) minder boosheid en frustratie en 3) meer 'aanwezig' zijn in de sociale- en leeromgeving. Veel ouders noemde dat deze subtiele veranderingen resulteerden in voortuitgang in de ontwikkeling ofwel op school of in de thuisomgeving. Een verbetering in de thuisomgeving en minder stress in het gezin werd ook opgemerkt met een checklist die als 'extra meting' werd gebruikt in de BATSCH-studie. Hieruit kwam ook naar voren dat het aantal driftbuien en agressieve uitbarstingen verminderde en ze daarnaast ook van kortere duur waren. Hoewel de checklist meer als extra en *exploratieve* meting is meegenomen, bleek het heel waardevol te zijn in het beschrijven van verbeteringen in het dagelijks leven en dagelijks functioneren.

Wat kunnen vervolgstudies leren van deze onderzoeken?

Er zijn een aantal zaken die we in vervolgstudies anders zouden aanpakken of die we juist zouden toevoegen. Het is bijvoorbeeld lastig voor kinderen met een laag cognitief niveau om neuropsychologische- of EEG-taken met instructies te volbrengen. Vervolgstudies zouden hun taken moeten aanpassen aan het vermogen van de kinderen, zodat bij elk kind veranderingen in het neurocognitieve profiel of de mate van hersenactiviteit gemeten kan worden. Daarnaast is het belangrijk om bewust te zijn van de vocht-afdrijvende eigenschappen van bumetanide, waardoor het lastig is om deelnemers van een *dubbel*-blind gerandomiseerd placebo gecontroleerd onderzoek helemaal "blind" te houden of zij het medicijn of de neppil hebben gekregen. Vervolgstudies kunnen dit omzeilen door een neppil te gebruiken met vocht-afdrijvende eigenschappen, of door nieuwe moleculen te ontwikkelen die *wel* NKCC1 in de hersenen inhiberen maar *niet* in de nieren.

Een ander belangrijke aanbeveling die voortvloeit uit de beschreven onderzoeken in dit proefschrift is de nadruk op patiënt-gerapporteerde uitkomstmaten (PROMs). Tijdens de onderzoeken merkten we dat er een kloof was tussen de uitkomstmaten (vragenlijsten) zoals beschreven in de richtlijnen voor klinische onderzoek en wat ouders graag aan verbeteringen zouden zien. Daarnaast vonden veel ouders de conventionele vragenlijsten gebruiksonvriendelijk: ze waren lang, herhaaldelijk en veel vragen waren niet relevant of de antwoordopties niet passend. Het gebruiken van het PROMIS-systeem in vervolgonderzoek, waarin verschillende PROMs gebruikt worden om relevante en op de persoon toegespitste situaties uit te vragen, lijkt een stap in de goede richting. Dit systeem kan ook gebruikt worden voor verschillende ontwikkelingsstoornissen, waardoor je makkelijker een cross-diagnostische studie kan opzetten.

Een laatste aanbeveling is het verder bestuderen van de E/I-hypothese, waarop onze studies gebaseerd zijn. Omdat er veel verschillende processen betrokken zijn bij een gebalanceerde E/I, is het belangrijk om deze goed in kaart te brengen met behulp van beeldende technieken die hersenactiviteit kunnen meten na inname van bumetanide. Op deze manier kan ook bekeken worden in welke mate bumetanide door de barrière rondom de hersenen heen komt en hoe het mogelijk leidt tot een verandering in gedrag.

Wat heeft de toekomst voor ons in petto?

Wat heeft een patiënt aan een "gemiddelde" van de hele groep die onderzocht is in een studie? Dat is toch niet relevant voor die persoon zelf? Op basis van de onderzoeken in dit proefschrift kunnen we concluderen dat bumetanide voor *sommige* kinderen met een ontwikkelingsstoornis inderdaad een geschikte behandeling lijkt – maar niet voor *alle* kinderen. Het lastige van de huidige gouden standaard in klinisch onderzoek – placebo-gecontroleerd gerandomiseerd – is dat het individuele verschillen moeilijk mee kan nemen. Doordat het onderzoek gestandaardiseerd is, kunnen onder andere op het individu toegespitste taken en vragenlijsten niet worden meegenomen, maar krijgt iedereen dezelfde (type) versie. Ook zouden we op basis van bijvoorbeeld eerder medicatiegebruik of verwachte bijwerkingen bij een specifiek persoon, een gepersonaliseerd doseerschema willen gebruiken – ook dit is vaak lastig in een conventionele onderzoeksopzet. Een alternatief hiervoor, waarin het draait om de vraag "werkt deze specifieke behandeling voor dit specifieke persoon?" lijkt gelukkig binnen bereik. Het begrip *N-of-1 trials* staat voor een gepersonaliseerde onderzoeksopzet: van het kiezen van geschikte uitkomstmaten tot het aanbieden van een bepaalde behandeling en individuele terugkoppeling van het effect hiervan. Op de lange termijn zouden we hiermee het diagnostische systeem in de kinderpsychiatrie kunnen uitbreiden naar een systeem waarin de labels niet domineren, maar waarin de individuele problemen van het kind centraal staan en welke het startpunt vormen voor de meest optimale behandeling en begeleiding.

CONCLUSIE

Het doel van dit proefschrift was bijdragen aan het verbeteren van rationele en mechanisme-gerichte behandelmogelijkheden voor ontwikkelingsstoornissen. We hebben onderzocht of het groeperen of *stratificeren* op basis van onder andere zichtbare kenmerken (zoals prikkelverwerkingsproblemen) of oorzakelijke verbanden (zoals een genetische stoornis) resulteert in een duidelijker behandeleffect. De resultaten van de onderzoeken in dit proefschrift laten het volgende zien:

- Bumetanide kan een positief effect hebben op gedrag bij verschillende ontwikkelingsstoornissen, met name prikkelbaar en repetitief gedrag verbeteren. De neuronale mechanismen die ten grondslag liggen aan deze gedragseffecten, moeten nader worden verhelderd;

- We benadrukken de noodzaak van gestratificeerde psychiatrie gericht op het bieden van meer persoonlijke behandelmogelijkheden alleen voor *die* kinderen die er waarschijnlijk baat bij hebben en de noodzaak van betere uitkomstmaten. Stratificatie op basis van genetische aandoeningen lijkt een veelbelovende aanpak, net zoals stratificatie op basis van een potentieel 'shared developmental pathway', zoals het bestaan van prikkelverwerkingsproblemen;

- De onderzoeksopzet van N-of-1 studies kunnen een krachtig alternatief zijn om belangrijke nadelen van gerandomiseerd placebo-gecontroleerde studies te overwinnen en met name bruikbaar binnen de heterogene populatie van ontwikkelingsstoornissen;

- Het gebruik van PROMIS-itembanken in klinisch onderzoek kan een oplossing zijn om zowel directe als indirecte gevolgen van prikkelverwerkingsproblemen te evalueren en kan bijdragen aan de ontwikkeling van nieuwe behandelingen gericht op de prikkelverwerking.

Concluderend benadrukken deze bevindingen het belang van een verschuiving van een traditioneel diagnose-gedreven benadering waarbij neurobiologische kennis buiten beschouwing wordt gelaten, naar een meer gestratificeerde en subtype-gedreven benadering. Dit dient altijd in het grotere plaatje van het dagelijks leven van de patiënt en zijn of haar sociale omgeving te gebeuren, waarop de behandelmogelijkheden afgestemd moeten worden. Hopelijk zal dit de weg vrijmaken om patiënt-gedreven behandelmogelijkheden te kunnen bieden, die een verscheidenheid aan klinische en biologische informatie integreert om aan te geven wat de meest effectieve en minst schadelijke behandeling kan zijn voor elke individuele patiënt met een ontwikkelingsstoornis.

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DANKWOORD

DANKWOORD

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Kroelkoning **Thomas**, jij bent 'pas' in jaar 4 ingestapt in de PhD-trein en hebt vooral de thuiswerk-tijden en laatste loodjes van dit proefschrift meegemaakt. Die uiteraard

gepaard gingen met de nodige perikelen en melancholie... En dan is dit proefschrift ook nog eens abracadabra voor je (saahaai). Hang in there! Ik heb het gevoel dat we nog maar net zijn begonnen aan onze reis en ik kan niet wachten om meer avonturen te beleven met jou.

De bro to my do, **Jelmer** onze academische interesses kunnen niet veel verder uit elkaar liggen maar stiekem lijken we toch best veel op elkaar. Wij kennen de pieken en dalen van een broer-zus relatie, maar zelfs in de lows weet ik dat ik altijd bij jou terecht kan. Jij bent een bijzonder mooi en oprecht persoon en ik gun je de wereld broer. Helaas zijn **Marleen** en jij niet aanwezig bij de verdediging, maar ik weet dat je apetrots op me bent en vanaf de Kilimanjaro een klein vreugdedansje zal doen (als er nog wat zuurstof in die tank zit). Lieve **papa** en **mama**, een stukje tekst in dit dankwoord kan onmogelijk recht doen aan mijn dankbaarheid voor jullie. Ik probeer het toch. Bedankt voor jullie onvoorwaardelijke liefde en eindeloze betrokkenheid. Jullie enthousiasme, altijd open armen, goede raad en mentale support gedurende de afgelopen jaren gaven mij het gevoel dat ik bergen kon verzetten. Daarnaast geniet ik volop van onze avonturen: met pa fietsen naar Parijs, op de bonnefooi door Spanje met kerst, met ma van berghut naar berghut hiken in de Dolomieten of met een felucca over de Egyptische Nijl varen. Maar de weekenden voor de houtkachel op de boerderij zijn ook onvergetelijk. Lieve folks, jullie zijn ronduit fantastisch.

Een onconventionele start vraagt om een idem einde. Ik sluit graag af met de wijsheid van neuroloog **Oliver Sacks**, wiens inzichten ik tijdens mijn eerste college Psychologie leerde kennen en welke nog altijd relevant blijken: *"In examining disease, we gain wisdom about anatomy and physiology and biology. In examining the person with disease, we gain wisdom about life."*

CURRICULUM VITAE

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Dorinde Marije van Andel was born on January 27th, 1991 on an organic farm in Zeewolde, the Netherlands. After completing her A-levels in 2009 at RSG Slingerbos in Harderwijk, she started her studies in Psychology at the University of Groningen. During this program she studied cognitive psychology for a semester at the State University of New York in Geneseo (USA). She completed her Bachelor's degree cum laude in 2013. Subsequently, she spent a year traveling through Oceania and Asia. In 2014 she started the research master Cognitive and Clinical Neuroscience (track Neuropsychology) at Maastricht University, from which she graduated cum laude in 2016. Her final research internship and master thesis concerned neurophysiological functioning in a large international autism cohort, carried out within the NICHE lab at the department of Psychiatry at UMC Utrecht. In 2016 she started her PhD project in the multidisciplinary team of the Sensory Processing Program (department of Psychiatry, UMC Utrecht) which focused on the combination of providing care and conducting research in children with a neurodevelopmental disorder. Here she could develop as a researcher, and also discovered her strong interest in diagnostics and clinical care. Dorinde is looking forward to start her training as a clinical psychologist and she has a strong desire to contribute to improved access to mental health care.

Dorinde Marije van Andel is geboren op 27 januari 1991 op een biologische boerderij in Zeewolde. Na het behalen van haar VWO-diploma in 2009 aan het RSG Slingerbos in Harderwijk is ze gestart met de bachelor Psychologie aan de Rijksuniversiteit van Groningen. Binnen dit programma studeerde ze een semester cognitieve psychologie aan de State University of New York in Geneseo (Amerika). In 2013 haalde ze cum laude haar diploma. Daaropvolgend reisde ze een jaar door Oceanië en Azië. In 2014 begon ze aan de onderzoeksmaster Cognitive and Clinical Neuroscience (specialisatie Neuropsychologie) aan Maastricht Universiteit, waarvan ze cum laude afstudeerde in 2016. Haar afstudeerstage en masterscriptie betroffen het neurofysiologisch functioneren van een groot internationaal autisme cohort bij het NICHE-lab op de afdeling Psychiatrie in het UMC Utrecht. In 2016 startte ze op dezelfde afdeling haar PhD-project, maar nu bij het multidisciplinaire team van het Zorgprogramma Prikkelverwerking waarin zorg en onderzoek van kinderen met een ontwikkelingsstoornis centraal stonden. Hier kon ze zich ontwikkelen als onderzoeker en ontdekte tevens haar passie voor diagnostiek en klinische zorg. Dorinde kijkt uit naar een carrière als behandelend psycholoog en wil zich graag inzetten voor toegankelijke(re) zorg.

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