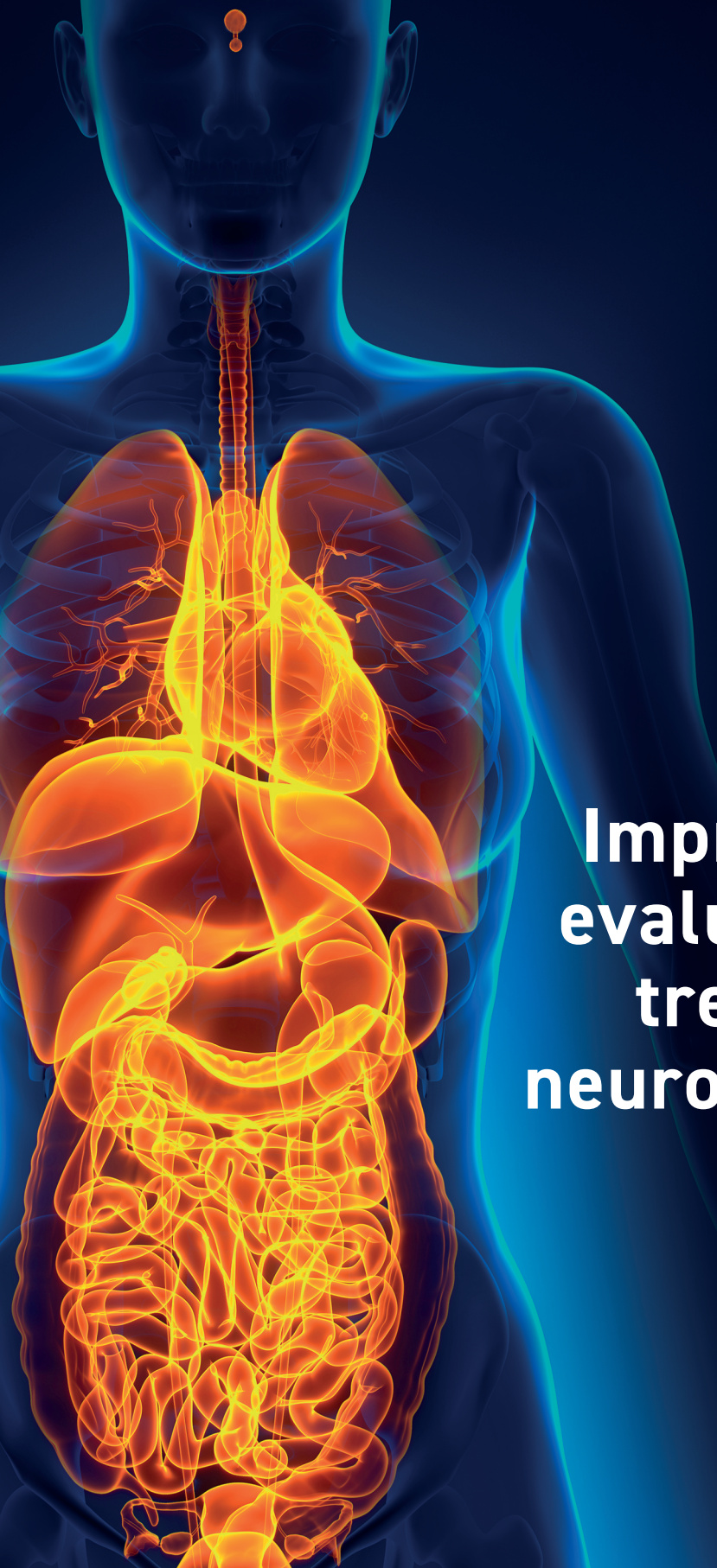

Improving the evaluation and treatment of neuroendocrine disorders

Julie Refardt

IMPROVING THE EVALUATION AND TREATMENT OF NEUROENDOCRINE DISORDERS

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IMPROVING THE EVALUATION AND TREATMENT OF NEUROENDOCRINE DISORDERS

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Molecular Imaging of Neuroendocrine Neoplasms.
Refardt J, Hofland J, Wild D, Christ E.
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Theranostics in neuroendocrine tumors: an overview of current approaches and future challenges.
Refardt J, Hofland J, Kwadwo A, Nicolas GP, Rottenburger C, Fani M, Wild D, Christ E.
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GENERAL INTRODUCTION INTO NEUROENDOCRINOLOGY

Neuroendocrine cells are spread throughout the body and combine the nervous and the endocrine system (1). They are characterized by their ability to synthesize and secrete hormones and peptides upon neuronal stimulation as well as their dependence on peptide hormone receptor expression on the cell surface (2). Neuroendocrine cells are essential for multiple body functions and responses. They form various organs such as the pituitary, the parafollicular (c-cells) of the thyroid or the adrenal medulla, but are also scattered throughout the gastrointestinal tract and the lungs, forming the diffuse neuroendocrine system (1). In addition to the general regulation of hormonal pathways, they are closely involved in feedback mechanisms. Accordingly, any disruption of these tightly regulated structures can lead to various symptoms and disorders (2).

Neuroendocrinology includes, among others, the two important areas of hypothalamic-pituitary axis disorders and neuroendocrine neoplasms (NENs).

The hypothalamus-pituitary axis plays an important role in the regulation of reproduction, growth, energy metabolism, stress response and fluid balance (3, 4). For the latter, the communication between the hypothalamus and the posterior pituitary regarding the production and secretion of arginine vasopressin (AVP) is essential. AVP is the most important hormone in the regulation of fluid homeostasis and is physiologically secreted from the posterior pituitary by axons of hypothalamic neurons upon elevated serum osmolality or decreased circulating blood volume (5). AVP then promotes water reabsorption via the V2 receptors in the kidneys. Once osmolality falls below the individual osmotic threshold, AVP levels become undetectable, leading to renal excretion of solute-free water. Inadequate or insufficient secretion of AVP can lead to disturbances of the body's osmotic balance and sodium homeostasis (4).

NENs are tumors that originate mainly from neuroendocrine cells of the pituitary, thyroid, lung, gastroenteropancreatic (GEP) system, and adrenal glands (1, 6). The improved diagnostic measures and awareness are associated with an increase in incidence rate over the last years (7).

A minority of NENs has the ability to produce amines or peptides, leading to paraneoplastic hormonal syndromes. The carcinoid syndrome is the most prevalent NEN-associated syndrome and includes flushing and diarrhea (8). Patients with carcinoid syndrome are at risk to develop valvular heart disease and mesenterial fibrosis but also electrolyte disturbances (9), leading to decreased quality of life and prognosis (10).

Generally, a better understanding of the heterogeneity of presenting symptoms and focusing management and treatment on patient-relevant outcomes are needed in neuroendocrine diseases. In the following, the two areas of neuroendocrine disorders are introduced in more detail and open issues are addressed.

Neuroendocrine disorders affecting the water sodium homeostasis

The most common outcome of a deranged water sodium homeostasis is hyponatremia. Hyponatremia is defined as a serum sodium level of <135 mmol/l and is characterized by a relative excess of body water compared to total body sodium (11). Being present in up to 30% of hospitalized patients (12), hyponatremia is an important condition and linked to increased morbidity and mortality (13, 14). The pathophysiological mechanism underlying most hyponatremic states is inappropriately elevated AVP levels, the so-called syndrome of inappropriate antidiuresis (SIAD, formerly referred to as SIADH) (15, 16). In SIAD, AVP release is not fully suppressed despite hypoosmolality, leading to water retention and subsequent hyponatremia (*Figure 1*). There are numerous causes leading to SIAD, including ectopic production of AVP by some tumors including NENs (8, 17). Hyponatremia may however also be triggered by other endocrine conditions such as adrenal insufficiency or hypothyroidism (5).

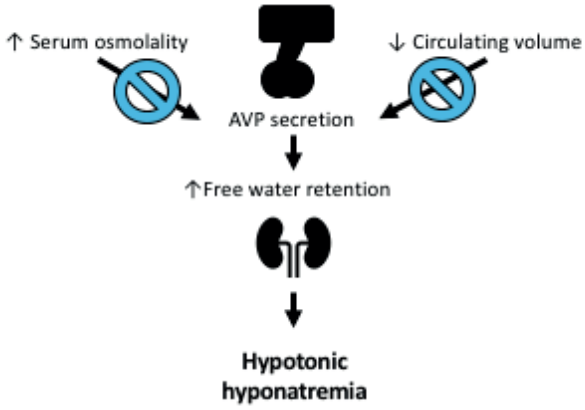

Figure 1 Schematic illustration of the pathophysiology behind the syndrome of inappropriate antidiuresis (SIAD). Despite normal serum osmolality and circulating volume, AVP is secreted from the posterior pituitary, leading to free water retention in the kidneys and following hypotonic hyponatremia.

Hypernatremia is much less common than hyponatremia and results from a decrease in total body water in relation to the sodium content (18, 19). It has been primarily described in

inpatients (20, 21). Its main endocrine cause includes disorders affecting AVP release from the posterior pituitary, thereby causing uncontrolled polyuria and consecutive polydipsia (22).

Both hypo- and hypernatremia are associated with clinical complications and inferior outcome in several disorders (23-25). However, data on clinical symptoms are still sparse and the impact of dysnatremia on outcome in NENs is currently unknown. **The first aim of this thesis is therefore to evaluate the impact of hyponatremia on patient's general well-being as well as neurocognitive and neuromuscular function. The second aim is to assess if dysnatremia impacts treatment outcome in NEN patients.**

Diagnosis and treatment of AVP-dependent fluid disorders

The diagnosis and treatment of hypernatremia in the absence of polyuria is straightforward, as in most cases it is due to limited access to water or excessive water loss and is therefore treated by fluid replacement (18). If polyuria is present, the most important endocrine cause is AVP-deficiency (formerly referred to as central diabetes insipidus (26)), with its treatment focusing on the underlying disorder and desmopressin substitution (4, 27).

Unfortunately, the diagnostic approach to hyponatremia is more complex and time-consuming, due to the broad spectrum of possible causes (11). In addition, while the indication for treating acute, severe symptomatic hyponatremia to treat cerebral edema is unquestioned, the rationale for treating the more common subtype of chronic hyponatremia is less clear (23).

The recommended first-line treatment for SIAD-induced hyponatremia is fluid restriction, which has often only limited efficacy (28). Medical treatment options include vasopressin receptor antagonists (vaptans), which are very costly and bear the risk of plasma sodium overcorrection (11, 29, 30), and urea, known for its poor palatability and therefore low compliance (31). Difficult diagnosis and limited therapeutic options may explain why treatment of hyponatremia is inadequate in more than 70% of hospitalized patients (32). Accordingly, improved diagnostic measures and better treatment options are desperately needed. Due to the complexity of the disease, a clinical model would be helpful for its better evaluation.

Sodium glucose co-transporter 2 (SGLT2) inhibitors are used as drugs for diabetes mellitus due to their induction of pronounced glucosuria (33, 34). Glucosuria leads to osmotic diuresis with consequent increased excretion of free water (35), which might also be of interest for SIAD-induced hyponatremia characterized by retention of free water. Because the SGLT2 inhibitor empagliflozin has a favorable safety profile with positive effects on cardiovascular and renal outcomes (36, 37), SIAD patients could benefit from such treatment.

Therefore, the third and fourth aims of this work are to develop an SIAD model to better investigate this most common cause of hyponatremia and to evaluate the use of SGLT2 inhibitors as a new treatment option for SIAD.

Neuroendocrine neoplasms

NENs are a diverse group of tumors arising from the neuroendocrine cells located throughout the body (38). Identical to their origins, they are able to synthesize and secrete hormones and are characterized by the overexpression of specific peptide hormone receptors on the cell surface (39). Among the various locations, neuroendocrine cells of the gastrointestinal tract, pancreas and lungs represent the main origin of NENs (*Figure 2*).

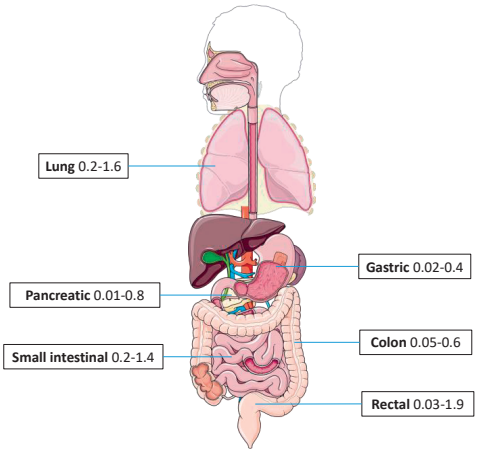

Figure 2: Neuroendocrine neoplasm locations and incidence rates
Illustration of the different neuroendocrine neoplasm origins and their incidence rates (cases / 100'000 / year). Adapted according to *Hofland J. et al, 2019 Endocrine reviews*

NENs are classified according to their origin, extension and histological differentiation (40). Based on mitotic count and Ki-67 index – a nuclear protein marker associated with cellular proliferation rate (41, 42) – GEP NENs are classified as well-differentiated grade 1 – 3 neuroendocrine tumors (NETs) or poorly differentiated neuroendocrine carcinomas (NEC) (41-43). Compared to other tumors, NENs are mostly slow-growing tumors and are therefore diagnosed with a latency of several years and at metastatic stage in up to 50% (44). Although NENs with paraneoplastic syndrome are clinically more apparent, their diagnosis still remains difficult and is often delayed because the symptoms can be confused with other, more common causes (8).

NENs are prone to develop liver metastases as the predominant distant metastatic site because of the portal venous drainage (44, 45). However, liver failure in NEN patients is rare, even in

cases with extensive metastatic liver disease (46). Nevertheless, there have been case reports of hyperammonemia in NENs (47-53). Hyperammonemia occurs when there is a defect in the detoxification or overproduction of ammonia, which can lead to encephalopathy (46, 54). To date, there is no cohort of NEN patients who develop hyperammonemia to further describe this disorder.

Accordingly, the fifth objective of this work is to describe the clinical characteristics and outcome of NEN patients who develop hyperammonemia and to determine possible predictive factors.

Somatostatin receptors

Somatostatin receptors (SSTs) are targetable G-protein coupled peptide hormone receptors on the surface of neuroendocrine cells (55, 56). There are five subtypes of SSTs, with the subtype 2 (SST2) being the most commonly expressed receptor. It consists of two isoform variants (SST2A and SST2B) and somatostatin binding leads to activation of different signal transduction pathways (57). The activation of SSTs leads to inhibition of the adenylyl cyclase and reduction of intracellular calcium levels, thereby inhibiting cell proliferation and peptide hormone secretion. SSTs are expressed in most neuroendocrine tissues including the pituitary, the gastrointestinal system, pancreas and thyroid, but are also found in the kidneys, lungs and immune cells. Importantly, SSTs are overexpressed and homogenously distributed on the tumor surface of most well-differentiated NENs (56, 58). This revolutionary discovery has played a key role in the development of targeted diagnostic and therapeutic interventions, also known as theranostics, as discussed in more detail below.

Diagnosis and treatment of neuroendocrine neoplasms

The cornerstone in the diagnosis and staging of NENs nowadays is the use of molecular imaging (59). The key for molecular imaging is the use of a radiotracer with a vector and a radioactive component. While the vector targets a specific pathological feature of the tumor, the radioactive component makes the target visible. The most common radioisotopes used in clinical practice include the positron emitters Gallium-68 (68-Ga) and Fluorine-18 (18F) or the gamma emitter Iodine-123 (123I). Visualization is obtained through planar scintigraphy, single photon emission computed tomography (SPECT) or positron emission tomography (PET), which are usually combined with CT or MRI to allow exact anatomical localization (59). A comparison of different molecular imaging techniques is shown in *Figure 3*.

Due to the homogeneous overexpression of SSTs on the NEN tumor surface, SST-based imaging is the main functional imaging modality currently in use (60, 61).

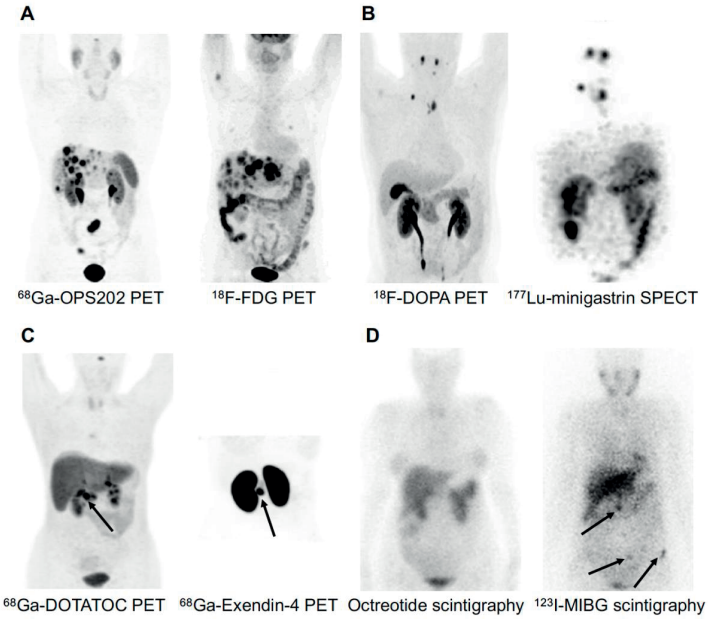

Figure 3 Overview of different molecular imaging techniques

Maximal intensity projections (A-C) and planar images (D) of the most important scans, shown exemplarily for different neuroendocrine neoplasms:

A) Neuroendocrine tumor: Gallium-68 labelled somatostatin receptor antagonist (68Ga-OPS202) PET and Fluorine-18 fluorodeoxyglucose (18F-FDG) PET

B) Medullary thyroid carcinoma: Fluorine-18 dihydroxyphenylalanine (18F-DOPA) PET and Lutetium-177 minigastrin SPECT

C) Insulinoma: Gallium-68 DOTATOC PET and Gallium-68 exendin-4 PET

D) Pheochromocytoma: octreotide scintigraphy and Indium-123 metaiodobenzygluanidine (123I-MIBG) scintigraphy

Arrows show the localization of the insulinoma (C) and pheochromocytoma metastases (D).

PET=positron emission tomography; SPECT=single photon emission computed tomography

Adapted according to *Refardt J, et al. J Clin Endocrinol Metab. 2022*

Planar scintigraphy using the gamma emitter 111-Indium-pentetreotide (Octreoscan™) was the first commercially approved SST imaging technique and for many years the reference standard (62). However, it has many downsides, as scintigraphy requires a lengthy, patient-unfriendly scanning process (63) and precise anatomical localization is difficult. With the increasing use of PET, new tracers using 68Ga-labelled DOTA-peptide somatostatin analogs (DOTATATE, DOTATOC, DOTANOC) were developed (64). The main difference between the analogs is their affinity to the different SST subtypes: while the octreotide used for DOTATOC and DOTANOC targets the SST subtypes 2, 3 and 5, octreotate used for DOTATATE has an enhanced affinity for the SST subtype 2 (64). However, this difference is not clinically relevant and their use subject to availability. The main advantages of 68Ga-SST PET over SST scintigraphy are better spatial resolution, resulting in higher diagnostic

accuracy, as well as approximately 75% lower radiation dose and higher patient comfort, as the scan can be performed after only one hour (60, 65). Importantly, sufficient uptake of radiolabeled SSA on 68Ga-SST PET/CT is essential for both unlabeled and radiolabeled SSA treatment (66).

Unlabeled and radiolabeled somatostatin analogues (SSA) are used therapeutically in NENs. Two randomized double blind placebo controlled trials (PROMID-trial (67) / CLARINET-trial (68)) showed a prolongation of time to progression and progression-free survival (PFS) through treatment with the unlabeled SSAs octreotide LAR and lanreotide in intestinal and pancreatic NETs, respectively. SSA inhibit tumor growth but are also used for symptom control in functioning tumors (9).

In addition to unlabeled SSA, the use of radiolabeled SSAs – known as peptide receptor radionuclide therapy (PRRT) - has become a highly efficient treatment option for NENs (69). PRRT systemically delivers beta-emitting radionuclides, specifically targeting cells expressing high levels of SSTs. In the phase III NETTER-1 trial, PRRT using 177Lu-DOTATATE in midgut neuroendocrine tumors, led to improved response rates with prolonged PFS, as well as improved symptom control and quality of life, compared to a high dose of octreotide LAR (70, 71). In addition to this study, there is increasing evidence for the efficacy of PRRT in all SST-positive NETs (66, 72, 73). Furthermore, its good tolerability with side effects mainly involving renal and bone marrow toxicity (66, 74) makes PRRT a promising and evolving therapy option.

However, as sufficient SST expression is a prerequisite for treatment with PRRT, patients with low SST expression are at a disadvantage as other treatment options are currently limited. Systemic therapy using chemotherapy or targeted therapy has partial efficacy in NENs with often significant toxicity (75). This likely contributes to the poorer survival rates of patients with SST-negative NET compared to SST-positive tumors (76). This difference in outcome could also be resultant to distinct biological behavior, as SST-negative tumors tend to have a higher proliferative rate (77, 78). However, studies comparing outcomes and covariates between matched SST-negative and -positive NET patients are missing. In addition, finding a way to upregulate SST in those patients would be of great importance and enable new treatment options.

Accordingly, the sixth aim of this thesis is to compare outcomes of metastatic SST-negative and -positive NET patients using a propensity score method and covariant adjustment. Further aims are to study the mechanisms behind SST expression and to find a way to upregulate SST in SST-negative NET patients.

The role of epigenetics in neuroendocrine neoplasms

Recent evaluation of whole-exome/genome sequencing showed a very low mutation rate for well-differentiated NENs of all origins (79, 80). Accordingly, epigenetic changes seem to be more important in the development of NENs, especially in small intestinal NENs (SI-NENs) (81, 82). Epigenetic changes affect gene expression without changing the DNA sequence and consist of DNA methylation and histone modification (81). The role of DNA methylation in tumorigenesis of NENs has been demonstrated in several studies showing an association between promoter methylation of the RASSF1A and CTNNB1 genes with extensive disease and poor overall survival in SI-NENs (83-85).

Histone modifications also contribute to tumorigenesis, with a small study suggesting a potential role of the histone H3K4 locus in SI-NENs (86). Other well-known histone marks are H3K27me3, which is associated with transcriptional repression, and H3K9Ac, which is associated with active gene transcription of SST2 in NETs (87).

In accordance with the importance of epigenetic changes in tumorigenesis of NENs, research focus has also been set on epigenetic drugs to modify SST2 expression (*Figure 4*).

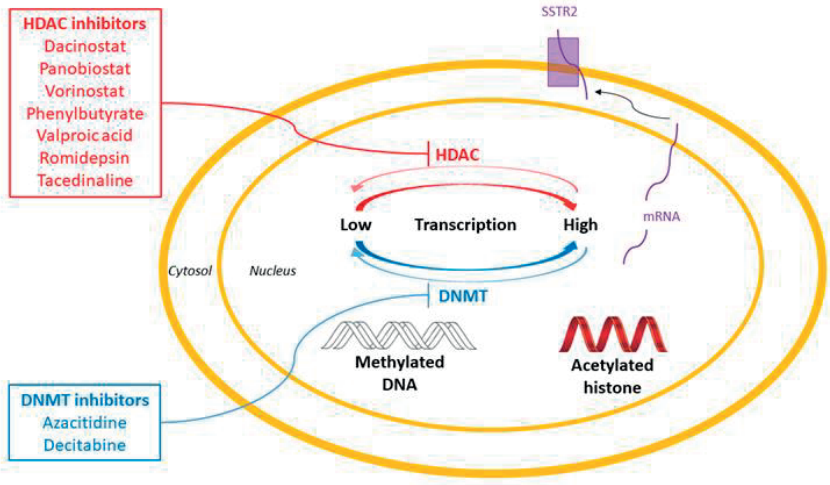

Figure 4: Schematic overview of drugs inducing upregulation of SST2
DNMT inhibitors reduce DNA methylation while HDAC inhibitors stimulate histone acetylation. This induces upregulation of somatostatin receptor subtype 2. Adapted from *Taelman et al J Nucl Med, 2016.*
DNMT = DNA methyltransferase; HDAC = Histone deacetylase; SST2 = somatostatin receptor subtype 2

The use of epigenetic drugs to modify cancer treatment response has been widely described (88, 89). Several in vitro studies have demonstrated an increase in SST2 expression levels by reducing DNA methylation and increasing histone acetylation levels of the SST2 gene pro-

moter region in NET cell lines (87, 90, 91). The use of the anti-epileptic drug valproic acid, a histone deacetylase (HDAC) inhibitor, upregulated SST2 mRNA and protein expression in NET cells and stimulated the binding and uptake of radiolabeled SSAs in vitro (87, 91). Similar results were shown when different HDAC inhibitors were evaluated in vitro. In addition, the different NET cell lines showed a higher radio sensitivity after treatment with VPA.

The DNA methyltransferase (DNMT) inhibitor 5-aza-2'-deoxycytidine also stimulated SST2 expression and SSA binding and showed a synergistic effect to valproic acid (87). A single animal study showed that epigenetic treatment with 5-aza-2'-deoxycytidine on mice carrying NET cells (90) led to increased tumoral uptake of radiolabeled SSAs. Treatment of mice with this DNMT inhibitor also increased tumor-to-background and tumor-to-kidney ratios, indicating its potential for improving tumor detectability and treatment. However, the DNMT inhibitor used in these studies has a high toxicity profile, possibly limiting its use in NEN patients.

Despite the promising results from the in vitro and in vivo studies, there is only a single study on SST2 upregulation by HDAC inhibitor vorinostat in five NET patients already expressing SST2 at baseline (92). Further studies in SST-negative patients are needed to assess the use of epigenetic treatment in NENs.

The seventh and eighth objectives of this work are, first, to further investigate the regulation of SST expression in SI-NET and, second, to conduct a proof-of-concept study to investigate the effect of epigenetic drug treatment on NET patients with low SST expression.

AIMS AND OUTLINE OF THIS THESIS

Taken together, the general aims of the studies presented in this thesis are:

1. To evaluate the impact of hyponatremia on patient relevant outcomes
2. To assess the impact of dysnatremia on treatment outcome in NEN patients
3. To develop an SIAD model for diagnostic and therapeutic evaluations
4. To assess the use of SGLT2 inhibitors as a new treatment option for SIAD
5. To investigate the impact of developing hyperammonemia in NEN patients
6. To evaluate the influence of low SST expression on outcome in advanced stage NEN patients
7. To investigate the regulation of SST expression in SI-NET
8. To assess the effect of epigenetic drug treatment on NET patients with low SST expression

Part 1 focuses on the impact of neuroendocrine disorders on patient relevant outcomes.

Chapter 2 describes the impact of chronic hyponatremia on neurocognitive and neuromuscular function, while **chapter 3** evaluates the prognostic importance of dysnatremia before or during treatment with PRRT.

Chapter 4 describes the outcome of neuroendocrine tumor patients who develop hyperammonemia while **chapter 5** focusses on the impact of having a neuroendocrine tumor with low SST expression.

Part 2 then takes the focus towards diagnosis and treatment of neuroendocrine disorders.

Chapter 6 discusses the possibility of evaluating new diagnostic and therapeutic strategies in healthy volunteers with artificially induced syndrome of inappropriate antidiuresis (SIAD), while **Chapter 7** shows first promising results of this approach. **Chapter 8** then describes the effect of a new treatment approach in patients with chronic SIAD.

Chapter 9 focusses on the regulation of somatostatin receptor expression in small intestinal neuroendocrine tumors, with **Chapter 10** then reporting the effects of epigenetic drug treatment on somatostatin receptor expression in neuroendocrine tumor cell lines and patients.

Lastly, **chapter 11** and **12** provide a general discussion and summary of the presented data.

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PART 1

Outcome of neuroendocrine disorders

2 Impact of chronic hyponatremia on neurocognitive and neuromuscular function

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ABSTRACT

Background: Chronic hyponatremia is common and associated with increased morbidity and mortality. However, whether treatment improves outcome in patients without significant symptoms is unclear. We here assessed the therapeutic outcome on clinical symptoms, neurocognitive and neuromuscular function in patients with chronic non profound hyponatremia.

Material and methods: Prospective case-control study in 19 patients from the University Hospital Würzburg with chronic non profound hyponatremia without clinically apparent symptoms. At baseline and after a 14-day treatment period of hyponatremia, patients were assessed by specific clinical symptoms questionnaire, neurocognitive and neuromuscular function was analysed by five attention tests and a gait test consisting of 3 steps "in tandem." The results were compared to a control group of healthy volunteers.

Results: Compared to healthy volunteers, patients with mild (n = 10, mean serum sodium 132 ± 1.2 mmol/L) and moderate hyponatremia (n = 9, mean 126 ± 3.3 mmol/L) performed significantly worse in the neurocognitive subtests alertness (P = 0.018), divided attention (P = 0.017) and go/no-go (P = 0.026). Performance in the neuromuscular subtests was also lower in the patient group without reaching significance. The extent of hyponatremia had no impact on the specific test and questionnaire results. Fourteen-day treatment of hyponatremia improved clinical symptoms in all patients (P = 0.003) and neurocognitive function in sodium-normalised patients (go/no-go test, P = 0.029).

Conclusion: Chronic hyponatremia is symptomatic and impairs neurocognitive and neuromuscular function. Short-time therapeutic intervention led to improved clinical symptoms and neurocognitive function, but had no effect on neuromuscular function. Larger trials with long-term treatment are needed to specify the therapeutic need in chronic hyponatremia.

1. INTRODUCTION

Hyponatremia is the most frequent electrolyte disorder in clinical practice (1,2) with incidences between 15% and 30% of acutely or chronically hospitalised patients (3,4). Clinical symptoms of hyponatremia essentially depend on its severity and rapidity of onset (5). Acute hyponatremia manifests promptly (<48 hours) and can cause dramatic neurological complications including death due to intracranial hypertension. In contrast, the more common chronic forms of hyponatremia manifest over time and remain clinically rather inconspicuous as the brain adapts to the extracellular hypo-osmolality (5). As a consequence, chronic manifestation of hyponatremia remains often unnoticed by both patients and physicians alike (7) and current treatment recommendations are restricted to patients with acute and/or severely symptomatic hyponatremia (8-12).

Although accumulating clinical evidence suggests that even non profound chronic hyponatremia associates with attention deficits and gait disturbances (13), increased risk of osteoporosis, bone fractures (14-16) as well as cognitive impairment (17) and death (2,18-20), it remains unclear whether correction of decreased sodium levels is able to improve patient's clinical outcome. While Renneboog et al (13) reported an improvement in the gait analysis of moderate chronic hyponatremia after serum sodium normalisation, the results in the attention tests were less clear. Two further clinical studies showed an improvement of mental cognition and quality of life after normalisation of chronic hyponatremia (21,22), but did not assess the neurocognitive and neuromuscular function. Another trial failed to show

an improvement in the overall neurocognitive composite scores after inducing normonatremia except for the domain of rapid motor movements (23) while a study using neuromuscular test showed no effect of hyponatremia correction on muscle strength but improvement in the nerve conduction tests (24).

The aim of this prospective case-control study was the investigation of the subclinical manifestation of chronic non profound hyponatremia on subjective well-being as well as neurocognitive and neuromuscular function in clinically unimpaired subjects by assessment before and after therapeutic short-term intervention as well as by direct comparison to healthy normonatremic volunteers.

2. MATERIAL AND METHODS

2.1 Subjects and study design

In this prospective case-control study, adult patients from the outpatient clinic of the University Hospital of Würzburg with documented chronic mild (serum sodium level 130-134 mmol/L) or moderate (serum sodium level 121- 129 mmol/L) hypotonic hyponatremia without available exclusion criteria (like clinically apparent adverse effects or physical limitations, ongoing treatment for electrolyte correction, adverse clinical symptoms, clinical signs of tumour disease, history of myocardial or cerebrovascular ischaemia within the last 3 months, pregnancy or breastfeeding, hypo- (<3.5 mmol/L) or hyperkalemia (>5 mmol/L), red-green visual impairment, severe renal insufficiency (GFR <30 mL/min), impaired liver function (equivalent CHILD Pugh stage B-C), acute or chronic heart failure (equivalent NYHA III-IV), or uncontrolled diabetes mellitus (random plasma glucose level >200 mg/dL)), were eligible for study enrolment.

In addition, 13 healthy normonatremic volunteers were included, of whom 6 were later excluded as their average age (25-26 years) was significantly below the average patient age. Therefore, 7 control subjects (n = 5 male) were enrolled in this study.

2.2 Assessments and intervention

2.2.1 Symptoms questionnaire

To investigate the influence of chronic non profound hyponatremia on patient's subjective well-being, a specific questionnaire testing for subclinical symptoms of hyponatremia (headache, confusion, mental slowness, irritability, orientation to place/time/person, tiredness, vertigo, nausea, emesis, gait instability, falls and muscle cramps) and its severity (measured as the sum of score values: 0 = no, 1 = mild, 2 = moderate, 3 = severe) was designed.

2.2.2 Neurocognitive functional tests

Intactness of the functions of attention as prerequisite for effective behaviour in everyday life was assessed using the standardised computer test system "Test of Attentional performance (TAP) 2.1" (25). The TAP has been developed in 1994 (26) by Zimmermann & Fimm as an assessment of attention deficits in adults, and has since been validated and used as a reliable tool for attentional impairment in different trials (27-31). Within this procedure, simple reaction paradigms were chosen, in which participants had to react selectively to well discriminable, nonverbal visual and acoustic stimuli by a simple motoric reaction (a simple keypress). During the tests, participants were seated in a quiet room in front of a black computer screen. After a short pre-test phase, the tests evaluated the following tasks:

- *Intrinsic alertness* refers to the condition of general wakefulness that enables to quickly and appropriately respond to any given demand. In this task in which a cross appears on the monitor at randomly varying intervals, reaction time (by pressing a key) is measured in milliseconds (ms) in response to the optical stimulus.
- *Phasic alertness*: In this second condition, reaction time is measured in response to a critical stimulus preceded by a cue stimulus presented as a waning tone. "Phasic arousal" or "temporal orientation of attentional focus" is measured as median reaction time (MDRT).
- *Divided attention*: The capacity to pay attention to several things at once is of great importance in everyday life and requires the ability for divided attention to simultaneously ongoing processes. In this test, a visual and an auditory task must be processed in parallel and answered by pressing a key. Deficits of divided attention are measured in errors.
- *Sustained attention:* Continued maintenance of attention is required in tasks with very different cognitive demands, ranging from simple stimulus detection tasks to tasks with a high cognitive load. Concentrating on a task is a typical requirement in working life that involves focussing attention on a mentally demanding activity for a sustained period of time. In this test, a sequence of stimuli is presented on the monitor with stimuli varying in a range of feature dimensions: colour, shape, size and filling. A target stimulus, requiring the participant to press a key, occurs whenever it corresponds in one or the other of two predetermined stimulus dimensions with the preceding stimulus (i.e. same shape, but different colours, size, filling). Performance of sustained attention is measured in errors.
- *Go/no-go:* refers to the ability to perform an appropriate reaction under time pressure and to simultaneously inhibit an inappropriate behavioural response. The go/no-go paradigm tests this form of behavioural control, in which it is important to suppress a reaction triggered by an external stimulus to the benefit of an internally controlled behaviourally response. The focus of attention in this paradigm is directed to predictably occurring stimuli that require a selection reaction, that is, to react or not react. The participant is asked to remember "2 of 5" target stimuli, presented in an alternating sequence on the screen and to press the key in response to their appearance. Participants with a deficit in this form of behavioural control show either a much higher number of incorrect reactions (measures of error), or much higher reactions time compared with the simple reaction tasks (measured in milliseconds (ms)).

Each sub-test was evaluated based on the reaction times or errors. An error was defined as an omission, non-stimulated reaction or latency. All tests were evaluated separately once per study participant and visit, thus two times per patient and once per control subject.

2.2.3 Neuromuscular tests

The neuromuscular function was assessed by performing a three standing-exercise test using the ground force reaction platform Balance-X-Sensor (Soehnle Professional product) (32). The Balance-X-Sensor digitally measures and logs the forces that the test person exerts on the platform over time. The purpose is to measure the quantitative control capability of motorial and sensorial systems, required to maintain balance, and displays the results on an integrated computer, resulting in the following readout parameters:

- *Force vector area (cm^2),* that is the balance area of the body's center of gravidity, representing steadiness when standing and walking. A small area indicates a high degree of steadiness.
- *External muscle activity (Hz),* that is the speed of postural muscles, representing training condition. A high power indicates excellent muscle strength and endurance.
- *Orthostatic power (watts),* measures the muscle power exerted when standing during the test, representing the interaction of vestibular organ, nerve conduction and muscle function condition. Here, low power indicates an efficient interaction.

All exercise tests were first evaluated with eyes open and afterwards with closed eyes. Patients could choose their supporting leg freely but had to stick to their choice for all the tests. The tests were rated successfully if participants were able to stand by themselves and were not touching the handrails. Each test was performed twice and the average measurement was recorded. The following exercises were assessed with increasing level of difficulty:

i. Romberg's standing test: holding a position with both feet together and hands by the sides for 10 seconds.
ii. Semi-tandem stand: holding a position with the heel of one foot placed to the side of the big toe of the opposite foot for 10 seconds.
iii. Tandem stand: holding a position for 10 seconds with the feet placed at a precise distance apart (heel to toe) without taking a supporting step.

Symptoms and tests were assessed in the patient group under hyponatremic conditions as well as after a 14-day treatment intervention including salt tablets (3-9 g/day) in 84%, fluid restriction in 63%, stop of triggering medication in 11% of patients according to treatment guidelines (8,9).

The study was approved by the local Ethics Committee of the University of Würzburg (no. 34/08), written informed consent was obtained from all subjects before participation.

2.3 Statistical analysis

Data analysis involved five comparisons:

- Patients with untreated chronic hyponatremia versus healthy subjects

- Patients with untreated chronic mild hyponatremia versus untreated chronic moderate hyponatremia
- Patients with chronic hyponatremia before versus after treatment
- Patients with improved versus patients with corrected chronic hyponatremia after treatment
- Patients with corrected chronic hyponatremia before vs after treatment

Analysis endpoints included:

- Subjective symptoms of hyponatremia (sum of score values over all single symptoms: 0 = no, 1 = mild, 2 = moderate, 3 = severe)
- Outcomes of neurocognitive functional tests (alertness, phasic alertness, sustained attention, divided attention, go/no-go)
- Outcomes of neuromuscular balance tests (force vector area [cm^2], orthostatic power [watts] and external muscle activity [Hz]).

The analyses with regard to symptoms of hyponatremia and neurocognitive functional tests were conducted using standard linear models (LM), with the factor of main interest (patient vs. healthy subject, severity of hyponatremia, that is mild vs. moderate, before vs. after treatment, corrected vs. improved hyponatremia) as explanatory factor.

The assumptions of LMs were assessed by graphical inspection of the residuals. Neuromuscular balance tests and neurocognitive tests were analysed using linear mixed-effects models (LMM) with a random intercept per study participant to account for the non-independence of multiple measurements on the same participant. Apart from the explanatory factor of main interest (see above), the test mode (Romberg, semi-tandem, tandem) and the test condition (eyes closed vs open) were used as explanatory factors. The assumptions of LMMs were also assessed by graphical inspection of the residuals. Because the normality assumption was violated, balance area and standing performance were log-transformed.

To evaluate a possible correspondence between the severity of hyponatremia before and after treatment, a correspondence analysis using multidimensional scaling was performed (33) and Fisher's exact test was applied to test the independence of severity before and after treatment. For this analysis, we used a finer categorisation of the severity of hyponatremia, splitting the moderate category (121-129 mmol/L) into moderate (125-129 mmol/L) and severe (121-124 mmol/L).

All analyses were conducted using the statistical software package R (R Core Team, 2015, Version 3.2.2 (34)), using two-sided statistical tests and a significance level, α, of 5%. Effect size estimates are reported together with 95% confidence intervals.

Reporting of the study conforms to STROBE statement along with references to STROBE statement and the broader EQUATOR guidelines (35).

3. RESULTS

Nineteen patients (11 female) aged 45 to 79 (60.8 ± 8.85) years were included in the study. The main aetiologies of hyponatremia were chronic renal insufficiency (47%, 9 patients) and chronic SIADH (36%, 7 patients), two patients had diuretic-induced hyponatremia and one chronic cardiac failure. Baseline serum sodium levels ranged from 121- 133 mmol/L (128.8 mmol/L ± 3.9), including 10 patients with mild and nine patients with moderate hyponatremia (*Table 1*). The control group consisted of nine healthy volunteers (5 male), mean age 57 years.

Fourteen-day treatment of hyponatremia resulted in an average increase of 4.7 mmol/L (±3.0) to a serum sodium level of 133.5 mmol/L (±3.5), with nine patients reaching normonatremia (=corrected hyponatremia, 136 mmol/L ± 1.8) and 10 patients with improved, but persistent hyponatremia (= improved HN, 130.8 mmol/L ± 2.1) (*Table 1*). The correspondence analysis suggested an association between mild HN at baseline and corrected HN after treatment as well as between moderate HN at baseline and mild HN after treatment and severe HN at baseline and moderate HN after treatment, but the association was not statistically significant (Data not shown).

Table 1 Serum sodium levels during study intervention

Serum sodium *before* 14-day treatment (mmol/L)	128.8 (3.9)
n mild hyponatremia	10
Mean (SD) / Range	131.7 (1.2) / 130-133
n moderate hyponatremia	9
Mean (SD) / Range	125.6 (3.3) / 121-129
Serum sodium *after* 14-day treatment (mmol/L)	133.5 (3.5)
n corrected hyponatremia	9
Mean (SD) / Range	136.4 (1.8) / 135-140
n improved hyponatremia	10
Mean (SD) / Range	130.8 (2.1) / 127-134

Table 1 Serum sodium levels before and after 14-day treatment of hyponatremia
n=number, SD=standard deviation

All patients answered the clinical symptoms questionnaire. Two patients were clinically not able to accomplish the neurocognitive functional tests; one patient was only able to complete

the first part. Two patients failed to conduct the neuromuscular balance tests according to study protocol.

3.1 Clinical symptoms

The most intensely reported symptoms before treatment of chronic non-profound hyponatremia were headache, tiredness and gait instability (*Figure 1*). However, there was no difference in the severity of the symptoms between patients with mild and patients with moderate hyponatremia (estimate mild vs moderate hyponatremia 0.6 (−6.2, 7.34), p = 0.86).

Symptoms ameliorated significantly through the therapeutic intervention (estimate after vs before therapy −4.9 (−6.6, −2.1), p = 0.003), whilst no difference was seen in patients reaching normonatremia compared to patients with persistent hyponatremia (estimate improved vs corrected hyponatremia 0.1 (−3.0, 3.2), p = 0.94).

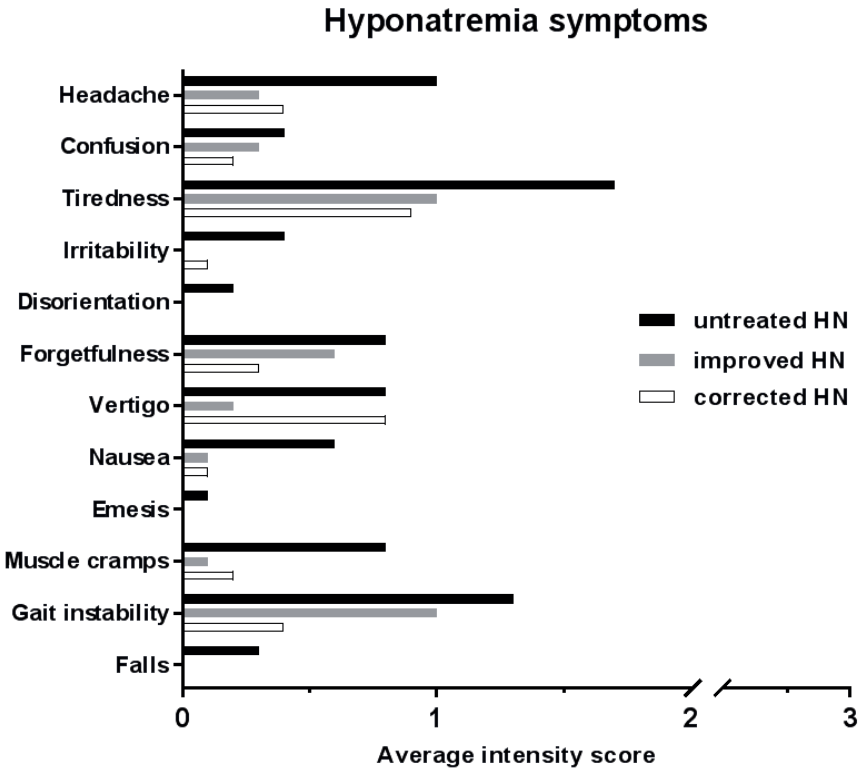

Figure 1 Average Intensity score (0 = none, 1 = mild, 2 = moderate, 3 = severe) of patients reporting symptoms of hyponatremia (HN)
improved HN=improved but not normalised hyponatremia after treatment;
corrected HN=corrected hyponatremia after treatment

3.2 Neurocognitive functional tests

The descriptive comparison of the tests of all study participants is shown in *Table 2*. Compared to the healthy control subjects, patients with untreated chronic hyponatremia performed significantly worse in the neurocognitive subtests "intrinsic alertness," "divided attention" and "go/no-go" (estimate untreated HN vs healthy volunteers: 51.3 (9.8, 92.9), p = 0.018; 5.0 (1.0, 9.0), p = 0.017; 111.6 (14.5, 208.7), p = 0.026). Severity of hyponatremia (mild vs moderate) in untreated patients had no effect on test performance (results not shown). Short-term therapeutic intervention with normalisation of serum sodium levels led to an improvement in the "go/ no-go" evaluation (estimate improved vs corrected hyponatremia

121.8 (CI 14.0, 229.5), p = 0.029) but showed no clear effect on other neurocognitive tasks.

Table 2 Neurocognitive functional test performance

	Controls	Patients before treatment		Patients after treatment	
		Mild HN	Moderate HN	Improved HN	Corrected HN
Intrinsic alertness (ms)	213.4 (59.9)	271.6 (41.9)	254.9 (37.5)	308.6 (95.9)	262.6 (62.1)
Phasic alertness (MDRT)	0.0 (0.1)	0.1 (0.1)	0.0 (0.1)	0.0 (0.0)	0.1 (0.1)
Sustained attention (errors)	10.0 (11.9)	12.7 (13.3)	16.1 (11.1)	16.1 (12.8)	8.2 (9.6)
Divided attention (errors)	1.8 (1.6)	7.0 (5.3)	6.4 (5.7)	6.6 (5.7)	4.7 (4.4)
Go/no-go (ms)	490.4 (115.3)	640.9 (100.4)	546.3 (95.7)	680.8 (96.3)	559.0 (110.4)

Table 2 Descriptive comparison as mean (standard deviation) according to different neurocognitive functional tests

HN=hyponatremia; ms=milliseconds; MDRT, median reaction time;

3.3 Neuromuscular balance tests (Balance X-sensor)

The descriptive comparison of the tests of all study participants is shown in *Table 3*.

The balance tests revealed a trend for an increased force vector area in untreated patients with chronic non profound hyponatremia compared to healthy control subjects (estimate 0.5 (−0.1, 1.1; p = 0.088), indicating a lower degree of steadiness. This finding was consistent to the higher gait instability (*Figure 1*). However, severity of hyponatremia (mild vs moderate) had no influence on neuromuscular test performance (results not shown).

Table 3 Neuromuscular balance test performance

	Controls	Patients before treatment		Patients after treatment	
		Mild HN	Moderate HN	Improved HN	Corrected HN
Force vector area (cm^2)					
Romberg	1.41 (1.16)	2.98 (3.52)	2.69 (3.03)	2.16 (1.18)	4.21 (4.99)
Semitandem	2.91 (2.33)	3.78 (3.97)	3.57 (2.17)	3.74 (3.28)	6.79 (5.57)
Tandem	11.87 (12.71)	8.24 (6.88)	8.80 (8.85)	5.85 (10.80)	13.59 (12.55)
Orthostatic power (watts)					
Romberg	5.95 (0.37)	5.37 (0.72)	5.56 (1.10)	4.96 (1.83)	5.75 (0.93)
Semitandem	5.21 (0.46)	4.96 (0.61)	5.12 (1.12)	4.50 (1.55)	4.78 (1.18)
Tandem	4.92 (0.54)	4.51 (0.61)	5.06 (0.94)	4.36 (1.40)	4.72 (1.14)
External muscle activity (Hz)					
Romberg	0.000 (0.000)	0.000 (0.000)	0.002 (0.007)	0.005 (0.014)	0.001 (0.003)
Semitandem	0.003 (0.004)	0.001 (0.001)	0.004 (0.005)	0.006 (0.010)	0.005 (0.004)
Tandem	0.016 (0.011)	0.009 (0.010)	0.013 (0.014)	0.013 (0.023)	0.040 (0.073)

Table 3 Descriptive comparison of different neuromuscular balance tests as mean (standard deviation) according to different neuromuscular balance tests with eyes closed.
HN=hyponatremia; improved HN=improved but not normalised hyponatremia after treatment; corrected HN=corrected hyponatremia after treatment;

Short-term treatment intervention resulted in an overall decline of the functional tests for external muscle activity and orthostatic power (estimate patients after vs before therapy 0.4, (0.2, 0.6), $p = 0.001$ rsp -0.2 (-0.4, -0.1), $p < 0.001$), while the treatment effect on serum sodium increase had no beneficial effect on neuromuscular test performance (results not shown).

4. DISCUSSION

The discrimination of subtle clinical deterioration of chronic hyponatremia in patients without otherwise serious clinical compromise is intriguing and of great clinical relevance for treatment consideration of all hyponatremic patients. The main finding of our study is that patients with chronic non profound hyponatremia indicate reduced clinical well-being and suffer from specific functional neurocognitive and neuromuscular deficits. Our data confirm the results from Renneboog et al (13) concerning the neurocognitive impairment of patients with hyponatremia. In addition, we also show that the sense of balance is impaired. Notably, patients with mild hyponatremia were similarly affected as patients with moderate hyponatremia. This underlines the clinical relevance of mild hyponatremia which is often wrongly classified as asymptomatic.

Therapeutic intervention ameliorated symptoms of mild to moderate hyponatremia. Treatment also led to the improvement of neurocognitive function in sodium-normalised patients. However, unexpectedly it reduced neuromuscular function independently of reaching normonatremia. This may be due to the short treatment period of only two weeks when tests were repeated. Adaptation to higher serum sodium levels might need time which may vary individually. Accordingly, different rates of re-adaptation of brain solutes after normalisation from hypotonic hyponatremia in mice and rats were shown over a period of many days, in contrast to electrolytes which normalised within 24 hours (5).

A second possible explanation is certainly the small patient number with only nine patients reaching normonatremia after intervention, mirroring the difficulties in outpatient treatment and recruitment of "isolated" chronic hyponatremia in otherwise largely uncompromised patients. Possibly, as the analysis suggested a correspondence between degree of hyponatremia and chances of normalisation after treatment, a trial with longer duration and consequent correction of hyponatremia would show a beneficial effect of serum sodium normalisation on neurocognitive and neuromuscular function. Accordingly, in the two comparable studies which showed a benefit after serum sodium correction, the time point for the second neuromuscular testing was defined as achievement of normonatremia (13,24).

The major strength of our study is our study design with standardised and computerised measurement methods and reproducible processes, allowing a detailed assessment of hyponatremia associated symptoms as well as neurocognitive and neuromuscular functional impairment. However, our study also has limitations. First, the relative small sample size with multiple comparison as the strict inclusion criteria made it difficult to recruit patients suffering from isolated chronic hyponatremia. At the same time, these criteria are a key advantage of the study and ensured that severe comorbidities and their treatment did not confound the treatment effect of hyponatremia. In contrast, the studies of Schrier and Renneboog et al (13,21) also included patients with severe chronic diseases, where improvement of the assessed symptoms may not only be attributed to a normalised serum sodium level. Second, there is a possible selection bias since we only included patients fit enough to perform the different tests. Ideally, in a further study, a bed side test to evaluate the different neurocognitive and -muscular functions should be used with the possibility to include all available patients. Lastly, our control group showed an unexpected variability of the assessed parameters, possibly affecting the statistical significance of the comparison. With the majority of the control group being males compared to the 58% female patients, a possible gender bias cannot be excluded. Also without a second evaluation, any potential over time variability of the measured variables cannot be excluded. Subsequent studies should consider this aspect in order to specify any possible treatment effect from the factor time.

5. CONCLUSION

Chronic mild to moderate hyponatremia is functionally relevant and interferes with subjective well-being as well as attenuation in specific neuromuscular and neurocognitive functional performance. Short-time therapeutic intervention ameliorated symptoms of hyponatremia and neurocognitive function, but failed to improve neuromuscular function. Similar trials with long-term treatment are needed to specify the therapeutic need of patients with chronic mild to moderate hyponatremia.

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3 Prognostic value of dysnatremia for survival in neuroendocrine neoplasm patients

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ABSTRACT

Objective: Hyponatremia and hypernatremia are common electrolyte abnormalities in patients with malignancy and have been independently associated with worse survival outcomes. To date, there are no data on the impact of dysnatremia on survival outcomes in patients with neuroendocrine neoplasms (NENs).

Design: This study involves retrospective cohort analysis from a tertiary care center of NEN patients treated with peptide receptor radionuclide therapy (PRRT) with a cumulative activity of at least 3.7 GBq 177Lu-DOTATATE between the years 2000 and 2015.

Methods: Comparison of overall survival of patients with the occurrence of hyponatremia (serum sodium < 135 mmol/L) or hypernatremia (serum sodium > 145 mmol/L) before starting or during PRRT was performed.

Results: A total of 649 patients were included. Hyponatremia occurred in 57 patients during the observation period and was associated with a shorter median overall survival (95% CI) of 25 months (14-36) compared to 55 months (48-61) of the 512 normonatremic patients ($P < 0.001$), adjusted hazard ratio (HR): 1.48 (95% CI: 1.04-2.12). Overall survival time was reduced regardless of whether hyponatremia was present at baseline or during PRRT. In contrast, hypernatremia occurred in 80 patients and was associated with a longer median overall survival (95% CI) of 94 months (47-140) compared with the 512 normonatremic patients ($P = 0.018$), adjusted HR: 0.61 (95% CI: 0.40-0.92). This association was driven by the patients with hypernatremia during PRRT. No association between dysnatremia and progression-free survival after PRRT was observed.

Conclusions: The occurrence of hypo- or hypernatremia in PRRT-treated NET patients is associated with opposing outcomes with regard to overall survival. Sodium levels might have a prognostic role in these patients.

1. INTRODUCTION

Hyponatremia and hypernatremia are among the most common electrolyte disorders (1, 2). Hyponatremia has a high prevalence of up to 40% in patients with malignancies (3, 4) and reflects an excess of total body water relative to total body sodium content. It can be divided into hypovolemic (e.g. due to diarrhea), euvolemic (e.g. due to the syndrome of inappropriate antidiuresis (SIAD)) or hypervolemic (e.g. due to cardiac failure) causes (5). Hypernatremia, on the other hand, is much less common and results from a decrease in total body water in relation to the sodium content (6, 7). Hypo- and hypernatremia have been associated with worse outcomes and increased mortality in a variety of diseases including cancer patients (2-4, 8, 9). Whether dysnatremia is directly responsible for the increased mortality or is only a marker for the severity of the underlying disease remains controversial however (10).

Neuroendocrine neoplasms (NENs) are a diverse group of rare tumors originating from neuroendocrine cells located mainly in the intestine, pancreas and lung (11). They are classified according to their origin, extension and histological differentiation (12). A minority of NENs have the ability to produce amines or peptides, leading to paraneoplastic hormonal syndromes (13) which can cause dysnatremia due to hypovolemia or SIAD (14, 15).

NENs are usually diagnosed in metastatic setting (16) which is associated with impaired patient survival and requires systemic palliative therapy. The presence of somatostatin receptors (SSTs) on the majority of NENs enables the possibility for treatment with unlabelled somatostatin analogues (SSAs) or radio-labelled SSAs as in peptide receptor radionuclide therapy (PRRT) (17-19).

Despite the prevalence of dysnatremia and its association with worse outcome, there are to the best of our knowledge no data on the effect of dysnatremia in NEN patients beyond case reports. The aim of the current study was therefore to investigate the association of hypo- and hypernatremia with overall survival of NEN patients. For this, we used a well-characterized prospectively collected cohort of NEN patients undergoing PRRT.

2. MATERIALS AND METHODS

2.1 Patients

The data of this retrospective analysis was obtained from a prospective phase II study of patients with a SST-positive tumor undergoing PRRT with 177Lu-DOTATATE at the ENETS Centre of Excellence for Neuroendocrine Tumors at Erasmus MC Rotterdam, the

Netherlands (20). The study was approved by the local Institutional Review Board of the Erasmus MC and all patients provided written informed consent.

Patients were selected if they received PRRT with at least 100 mCi (3.7 GBq) 177Lu-DOTATATE between the years 2000 to 2015 and had their treatment follow-up in the Netherlands. Patients were eligible for PRRT if tumor uptake on 111In-DTPA-octreotide scintigraphy (OctreoScan) was equal to or higher than the physiological uptake of the liver. Additional criteria included serum haemoglobin ≥6.0 mmol/L, total white blood cell (WBC) count ≥2x109/L, platelet count ≥75x109/L, serum creatinine ≤150 μmol/L or creatinine clearance ≥40ml/minute and Karnofsky performance status ≥50.

Tumors were graded according to the 2019 WHO guidelines (21). Treatments were recorded from time of NEN diagnosis until first PRRT cycle. The highest serum chromogranin A levels (reference <94 ug/L) during the follow-up period were reported.

2.2 Treatment and follow up

177Lu-DOTATATE was prepared locally as previously described (22). To reduce renal toxicity, an intravenous infusion of amino acids (2.5% arginine and 2.5% lysine in 1L 0.9% NaCl) was co-administered with the radiopharmaceutical. The intervals between PRRT were 6 to 10 weeks, with a maximum of 16 weeks in case of longer lasting toxicity. The cumulative intended activity was 800 mCi (29.6 GBq) 177Lu-DOTATATE.

Treatment response was assessed according to the Response Evaluation Criteria in Solid Tumors 1.1 (RECIST 1.1) criteria (23). Uptake on scintigraphy was scored on planar images using the Krenning Scale Scoring system (24).

Routine laboratory values were collected at baseline and 4-6 weeks after each PRRT. This entire period was defined as one treatment cycle. Follow-up visits occurred at 6 weeks, 3 months and 6 months after the last treatment cycle and thereafter every 6 months.

2.3 Laboratory assessments and diagnosis of dysnatremia

Serum sodium levels were analyzed by indirect ion selective electrode method (cobas® 8000 modular analyzer, Roche diagnostics).

Hyponatremia was defined as serum sodium level <135 mmol/L in absence of hyperglycemia; hypernatremia was defined as serum sodium level >145 mmol/L. Renal impairment was defined as having a creatinine clearance <90ml/minute.

Possible causes of dysnatremia were evaluated using the available clinical, laboratory, and radiological data.

2.4 Primary and secondary outcomes

The primary endpoint was to compare survival outcomes between NEN patients who developed hypo- or hypernatremia at baseline or within the first 4 cycles of PRRT to patients who remained normonatremic. Secondary endpoints included pre-defined subgroup analyses of patients being dysnatremic at baseline or developing dysnatremia during PRRT.

2.5 Statistics

Descriptive statistics were used to characterize demographic and clinical data, expressed as median with interquartile range (IQR) or frequencies with percentage. The association of categorical variables was assessed by Pearson chi-square test, continuous variables by Mann–Whitney U test.

Outcome analysis was divided into two groups. First, patients who developed hyponatremia were compared to normonatremic patients. Second, the same analyses were repeated with hypernatremic compared to normonatremic patients.

Survival rates, defined as survival from date of first PRRT to last date of follow-up or death, were calculated with the Kaplan-Meier method. The log-rank test was used to compare survival differences between groups. A Cox proportional hazard model was used to calculate mortality hazard ratios (HR) and 95% confidence intervals (CI). The Cox proportional hazards model was used to calculate crude (significance level $p<0.05$) and adjusted hazard ratios (HRs), using a forward approach (Wald) to avoid overfitting. The occurrence of dysnatremia, sex, tumor origin, tumor grade and tumor treatment were used as categorical variables, the remaining factors as continuous variables. To correct for potential immortal time bias, time dependent Cox proportional hazards models were performed whenever dysnatremia occurred after the baseline visit (25).

Secondary analyses involved evaluation of the timing of the occurrence of dysnatremia and the treatment response including progression-free survival (PFS), defined as time from baseline until progression (according to the RECIST 1.1 criteria (23)) or death / loss of follow up.

Statistical analyses were performed using SPSS version 25.0 (IBM Corp., Armonk, NY) and GraphPad Prism7. P values <0.05 were considered statistically significant.

3. RESULTS

3.1 Patient characteristics

Six-hundred-fifty-one patients fulfilled the selection criteria. Two patients with hyperglycemic-induced hyponatremia were excluded, leaving a total of 649 patients. During the observation period, 57 (9%) NEN patients had an occurrence of hyponatremia, 80 (12%) an occurrence of hypernatremia and 512 (79%) stayed normonatremic, *Table 1*. Dysnatremia was mild in both groups, with median (IQR) serum sodium levels of 133 mmol/L (132-134) in the hyponatremic and 147 mmol/L (146-148) in the hypernatremic group. The patients developing hyponatremia were older, had a higher rate of impaired kidney function, a lower performance status, higher chromogranin A levels and received more often chemotherapy prior to PRRT compared to normonatremic patients.

Table 1 Patient characteristics

Patient characteristics	**Any hyponatremia**	**Always normonatremic**	**Any hypernatremia**
	n=57	n=512	n=80
Age in years, median (IQR)	66 (57-74) ##	60 (52-68)	62 (56-69)
Sex, female, n (%)	31 (54)	237 (46)	37 (46)
Karnofsky index, median (IQR)	80 (70-90) ##	90 (80-100)	90 (80-90)
Site of origin, n (%)			
- *Gastroduodenal*	2 (3.5)	13 (2.5)	1 (1)
- *Midgut*	19 (33)	217 (42)	28 (35)
- *Hindgut*	1 (2)	18 (3.5)	6 (7.5)
- *Pancreas*	22 (39)	154 (30)	20 (25)
- *Lung*	3 (5)	26 (5)	4 (5)
- *Unknown*	10 (18)	84 (16)	21 (26) ##
Grade, n (%)			
- *1*	6 (10.5)	114 (22)	35 (44)
- *2*	11 (19)	147 (29)	22 (27.5)
- *3*	1 (2)	11 (2)	2 (2.5)
- *NEC*	0	4 (1)	1 (1.5)
- *unknown*	39 (68.5)	236 (46)	20 (25)
Hormone secreting, n (%)	16 (28)	192 (38)	31 (39)
Sodium, mmol/L, median (IQR)	133 (132-134) ##	141 (139-142)	147 (146-148) ##
Causes of dysnatremia, n (%)			
- *Hypervolemia*	14 (25)	na	0
- *Hypovolemia*	10 (18)	na	42 (53)
- *Medication induced*	8 (14)	na	4 (5)
- *Malnutrition*	7 (12)	na	0

Table 1 Patient characteristics (*continued*)

Patient characteristics	Any hyponatremia	Always normonatremic	Any hypernatremia
- *SIAD*	2 (4)	na	0
- *Other*	1 (2)	na	2 (3)
- *Unknown*	15 (26)	na	33 (41)
Renal impairment, n (%)	21 (37) ##	99 (19)	8 (10) #
Chromogranin A, µg/L, median (IQR)	2410 (527-12002) ##	738 (284-2810)	650 (306-4082)
Uptake on SST-based imaging, median (IQR)	3 (3-4)	3 (3-4)	3 (3-4)
- *2, n (%)*	5 (9)	43 (8.5)	12 (15)
- *3, n (%)*	32 (56)	320(62.5)	50 (62)
- *4, n (%)*	20 (35)	149 (29)	19 (24)
PRRT cycles, median (IQR)	4 (3-4)	4 (3-4)	4 (4-4)
PRRT activity in mCi, median (IQR)	800 (600-800)	800 (600-800)	800 (700-800)
Previous chemotherapy, n (%)	9 (16) #	38 (7.5)	3 (4)
Previous external beam radiotherapy, n (%)	3 (5)	34 (6.5)	3 (4)
Previous surgery, n (%)	18 (32)	207 (40.5)	31 (39)
Died, n (%)	41 (72) ##	249 (49)	26 (33) ##

Values are shown as frequencies (%) or median (IQR). n = number; IQR = interquartile range. SIAD = syndrome of inappropriate antidiuresis, SST = somatostatin receptor
/ ## = p-value <0.05 / <0.01 versus normonatremic patients

However, no direct correlation between these factors and serum sodium levels was found (data not shown). Patients developing hypernatremia, on the other hand, were similar to the normonatremic patients, but less likely to have renal impairment. Distribution of tumor origin was similar between all three groups, with the exception of a higher rate of NEN of unknown origin in the hypernatremic group.

The presumed main causes of hyponatremia were hypervolemia due to impaired kidney, liver or heart function (25%), followed by hypovolemia (18%). Hypernatremia, on the other hand, was mainly due to hypovolemia (53%), with diarrhea as the leading cause (41%). Etiology of hypo- and hypernatremia remained unclear in 26% and 41%, respectively.

3.2 Survival outcome and hyponatremia

During the observation period, 72% (41/57) of the patients with hyponatremia died, compared to 49% (249/512) in the normonatremic group (p=0.001). Hyponatremic patients had a shorter median (95% CI) survival time of 27 months (12-42) compared to 55 months (49-61) for normonatremic patients (p=0.001), *Figure 1.*

To further investigate this observation, subgroup analyses for patients being hyponatremic at baseline (n=24) or patients being hyponatremic during treatment with PRRT (n= 33) was carried out. Compared to normonatremic patients, hyponatremia remained associated with a decreased median (95% CI) survival time, independently whether it occurred at baseline (27 months (15-38), p=0.003) or during PRRT (25 months (7-43), p=0.004). There was also no difference between the two dominant primary sites of midgut and pancreatic origin. In both groups, hyponatremia compared to normonatremia was associated with a decreased median (95% CI) survival time (midgut NENs: 23 months (18-29), p=0.001; pancreatic NENs: 37 months (8-77), p=0.074).

No difference was seen within the hyponatremia group, in particular no difference in survival was seen between patients with repeated hyponatremia compared to patients with a single episode.

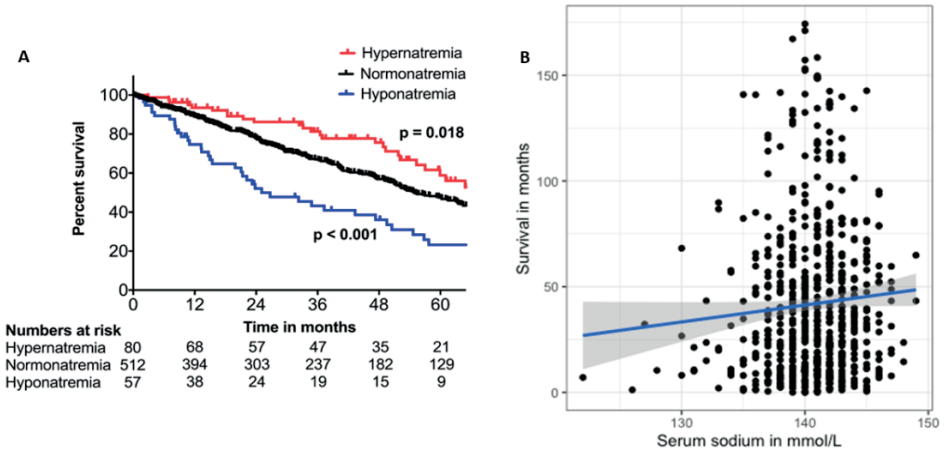

Figure 1 Association of serum sodium levels with survival in NEN patients:

A) Kaplan-Meier analysis showing overall survival of NEN patients who developed hyponatremia or hypernatremia compared to normonatremic patients. P-values indicate difference in survival according to log-rank test.

B) Baseline serum sodium levels in relation to survival.

Blue line indicates smooth local regression including 95% confidence interval.

3.3 Survival outcome and hypernatremia

Of the 80 NEN patients with hypernatremia at baseline or during PRRT, 26 (33%) died. Contrary to the findings seen with hyponatremia, hypernatremic patients had a longer median overall survival (95% CI) of 94 months (47-141) compared to 55 months (49-61) in the 512 normonatremic patients, p=0.018, *Figure 1*.

Subgroup analyses of patients being hypernatremic at baseline (n=30) and patients being hypernatremic during PRRT (n=50), showed that the observation was mainly driven by the occurrence of hypernatremia during PRRT: compared to normonatremic patients, median (95% CI) survival was 55 months (38-73) for patients being hypernatremic at baseline (p=0.65), but was not reached for PRRT-associated hypernatremia (p=0.011). Meanwhile, hypernatremia was only associated with longer median (95% CI) survival in pancreatic NENs (94 months (42-145), p=0.01), which was again driven by hypernatremia occurring during PRRT (hypernatremia at baseline: p=0.12; hypernatremia during PRRT: p=0.019).

Similar to the hyponatremic group, no difference was seen between patients with repeated hypernatremia and patients with a single occurrence of hypernatremia.

3.4 Regression analysis of dysnatremia and survival

In univariable analyses, hyponatremia, hypernatremia, age, Karnofsky index, having a grade 1 tumor, PRRT dosage and receiving prior surgery or chemotherapy were identified to correlate with overall survival (*Figure 2*). In a multivariable analysis of these factors, hyponatremia remained an independent predictor of shorter overall survival with a HR (95% CI) of 1.48 (1.04-2.12), p=0.031, while hypernatremia remained associated with a reduced risk with a HR (95% CI) of 0.61 (0.40-0.92), p=0.019, *Table 2*. A higher Karnofsky index score and a higher activity of PRRT were additional independent factors for a better outcome, while treatment with chemotherapy was associated with an increased risk of mortality.

Although renal function was not a predictor of mortality according to the univariable analysis, the multivariable analysis was repeated with inclusion of this variable because of the higher prevalence in hyponatremic patients. In the selected model with the parameters defined above including the variable renal function, hyponatremia remained an independent predictor of worse outcome (p=0.031).

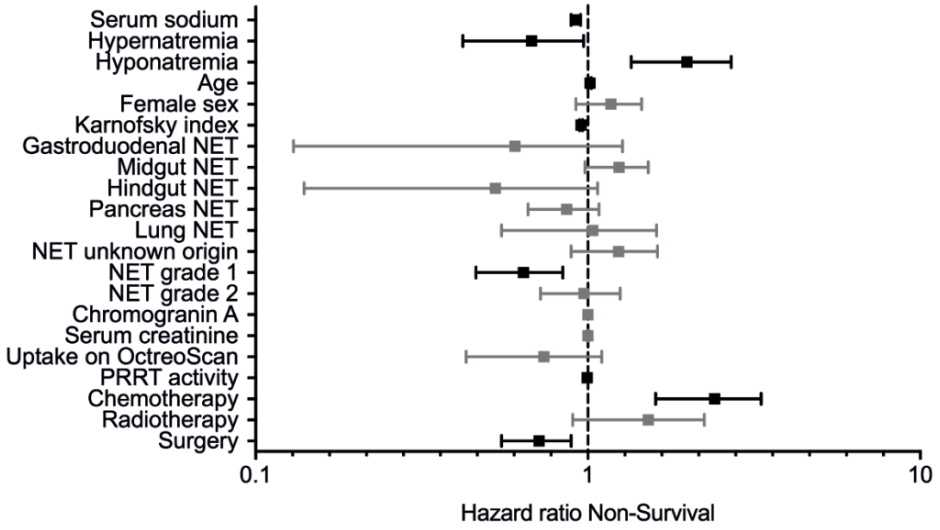

Figure 2 Forest plot showing the effect of patient characteristics on mortality risk: Univariable regression analysis of patient characteristics.

Serum sodium (in mmol/L), Age (in years), Karnofsky index, Chromogranin A (in μg/L), serum creatinine (in μmol/L) and PRRT activity (in mCi) were evaluated as continuous variables, the remaining as categorical variables.

The squares reflect the hazard ratio, while the whiskers indicate the lower and upper limits of the 95% confidence interval. Black colour indicates significant variables.

NEN = neuroendocrine neoplasm, PRRT = peptide receptor radionuclide therapy

Table 2:

	Hazard Ratio	95% CI	p value
Hyponatremia	1.48	1.04-2.12	**0.031**
Hypernatremia	0.61	0.40-0.92	**0.019**
Age	1.01	0.99-1.02	0.10
Grade 1	0.82	0.57-1.20	0.08
Karnofsky index	0.96	0.95-0.97	**<0.001**
Chemotherapy	2.59	1.81-3.71	**<0.001**
Surgery	0.83	0.65-1.06	0.10
PRRT total activity	0.996	0.995-0.997	**<0.001**

Table 2: Multivariable regression analysis using a forward selection model (Wald) showing the hazard ratio for mortality with 95% confidence interval (CI) for the impact of dysnatremia during the observation period.
PRRT = peptide receptor radionuclide therapy, CI = Confidence interval

When stratified according to the timing of hyponatremia, the HR (95% CI) was 2.20 (1.35-3.58) for hyponatremia at baseline and 1.53 (0.80-1.83) for hyponatremia during PRRT, however the adjusted HRs did not reach significance.

Baseline hypernatremia was not related to survival outcome. However, PRRT-associated hypernatremia was associated with a lower mortality risk with a HR (95% CI) of 0.51 (0.30-0.88) and remained an independent predictor of a better outcome in the multivariable regression analysis (p = 0.015).

3.5 Treatment outcome and dysnatremia

PFS (95% CI) was similar between the three groups, but there was a trend towards worse outcome for hyponatremic patients with 20 months (11-28), compared to 24 months (22-26) and 33 months (26-39) for normo- and hypernatremic patients, respectively (Hypo- vs normonatremic: p=0.07; Hyper- vs normonatremic: p=0.08) *Figure 3*.

There was no difference in the radiological response to PRRT between patients with hyponatremia, normonatremia, or hypernatremia.

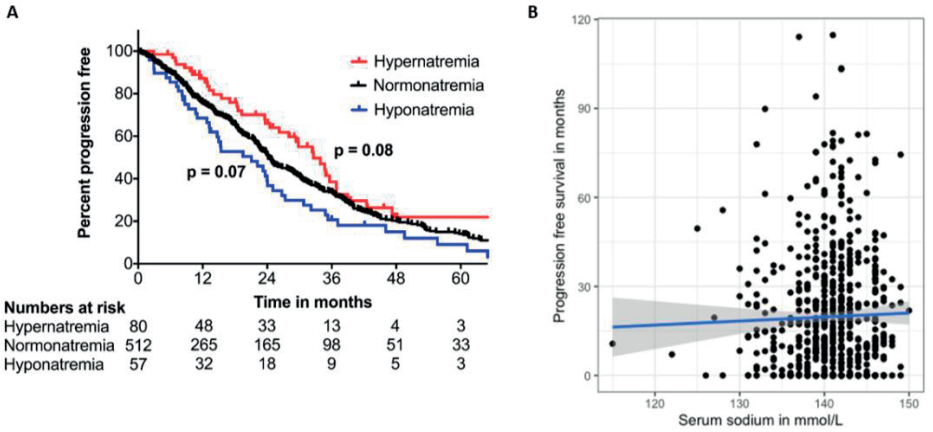

Figure 3 Association serum sodium levels with treatment response

A) Kaplan-Meier analysis showing progression free survival of NEN patients who developed hyponatremia or hypernatremia compared to normonatremic patients.

P-values indicate difference in progression free survival according to log-rank test.

B) Baseline serum sodium levels in relation to progression free survival.

Blue line indicates smooth local regression including 95% confidence interval.

4. DISCUSSION

Our study showed that the occurrence of hypo- or hypernatremia in PRRT-treated NEN patients is associated with opposing outcomes with regard to overall survival. While hyponatremia was prognostic for a higher mortality risk regardless of its occurrence before or during PRRT in our cohort, hypernatremia was associated with a better outcome. The latter association was driven by the patients developing hypernatremia during PRRT.

Hyponatremia is a known prognostic factor for worse outcome both in cancer and non-cancer patients (3, 4, 26). However, to our knowledge, despite some case reports, this is the first study to describe its impact on NEN patients. The exact relationship between hyponatremia and mortality is complex, as hyponatremic patients are more likely to have severe and complicated underlying diseases (27). A similar observation was made for our cohort, where hyponatremic patients were older, had a lower performance status, higher rate of renal impairment and more aggressive tumors – as suggested by the higher number of patients receiving prior chemotherapy. Given the higher chromogranin A levels in patients with hyponatremia, one might also suspect a higher tumor burden; however, these levels may also be influenced by renal dysfunction (28). Despite these factors, hyponatremia remained an independent predictor of worse outcome in multivariable analysis. This supports the hypothesis that hyponatremia is not only a marker for the severity of the underlying disease but might have its own negative effect. For example, hyponatremia has been suggested to have a direct pleiotropic effect on cancer promotion and progression in patients with advanced solid tumors (29). Hypotonic stress due to hyponatremia is thought to stimulate transcription of SGK1, a protein involved in the cellular stress response that has been shown to play an important role in cell proliferation and metastatic seeding (30). This might be one of the reasons why malignant SIAD has been associated with an increased mortality risk compared to non-malignant SIAD (31, 32). Other studies found a possible adverse effect of hyponatremia on cardiomyocytes (33) as well as acceleration of cellular senescence (34), which might contribute to the neurocognitive and -muscular dysfunction described in observational studies (35, 36). This may also have played a role in the findings of Cuesta et al. (37), who showed that patients with SIAD had poorer survival rates whilst having less morbidity. Interestingly, the increased mortality risk also seems to be independent of the severity of hyponatremia (8, 26). This was also seen in our cohort, where the majority had only mild hyponatremia (serum sodium 130-134 mmol/l). Although hyponatremia remained an independent prognostic factor for mortality in the adjusted analysis, this observation was not confirmed on the subgroups of patients with hyponatremia at baseline or during PRRT. This could be a power issue in view of the lower number of patients, however possibly hyponatremia is both: a surrogate marker and a reason for worse outcome as previously discussed by Hoorn and Zietse (10).

Although hypernatremia has also been associated with worse outcome in cancer patients (2, 9, 38), there is limited data compared to hyponatremia. While hyponatremia is common in outpatients and hospitalized patients, hypernatremia is mainly described in inpatients (39, 40). The most common cause of hypernatremia is lack of free water due to inadequate intake or excessive loss (6). In our outpatient cohort, hypovolemia was also the main cause of hypernatremia. Remarkably, the association of better outcome with hypernatremia was driven by the patients who developed it during PRRT. This points toward a dynamic, possibly treatment-associated effect, which was most prominent in the pancreatic NENs in our

cohort. PRRT-associated flare of carcinoid symptoms (41) and subsequent fluid loss could constitute a possible explanation. However, the lack of association in midgut NENs, the group with the highest frequency of carcinoid syndrome, argues against this theory. Another explanation could be PRRT-induced tumor lysis (42), which could lead to urea-induced diuresis, as observed in a study of catabolic ICU patients who developed hypernatremia (43). Since no difference between the groups with regard to treatment response was found in our cohort, these effects were probably only minor, which is reflected in the generally mild hypernatremia. However, since our data cannot shed light on these pathophysiological mechanisms, these evaluations remain purely speculative.

A limitation of our analysis is its retrospective design. While data were derived from a prospective phase 2 study (20), some factors like tumor grade are incomplete because either the histopathologic report was missing or incomplete for mitotic count or Ki67% index. Furthermore, the registry data had not been collected for purpose of investigating dysnatremia. In order to better assess cause and pathophysiology of the observed electrolyte disorders, additional parameters such as serum and urinary osmolality or serum cortisol levels would be required. Also, since the majority of our patients had mild hyponatremia, no statement can be made concerning the influence of hyponatremia severity on outcome in NEN patients.

Another limitation is the possible selection bias due to the inclusion of only patients eligible for PRRT. The prevalence of hyponatremia in particular might have been higher if patients with more severe renal dysfunction or lower performance status had been included.

Nevertheless, through the detailed and careful analysis of the available data, we were able to point out the prognostic role of dysnatremia in NEN for the first time. Our data should be confirmed in a larger, more diverse NEN cohort, in order to validate that sodium levels are a prognostic factor for these patients.

In conclusion, we show that the occurrence of hypo- or hypernatremia in PRRT-treated NEN patients is associated with opposing outcomes with regard to overall survival. While hyponatremia was confirmed as a predictor of worse outcome, the role of PRRT-induced hypernatremia and its association with increased overall survival has to be further investigated.

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4 Prognostic significance of hyperammonemia in neuroendocrine neoplasm patients with livermetastases

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Endocrine related cancer 2022

ABSTRACT

Neuroendocrine neoplasms (NENs) are rare usually slow-growing tumors, often presenting with extensive liver metastases. Hyperammonemia due to insufficient hepatic clearance has been described in NEN cases, however no systematic evaluation of risk factors and outcomes of NEN-associated hyperammonemia exists so far. This case report and retrospective review of NEN patients developing hyperammonemia from the years 2000-2020 at the Erasmus Medical Center in Rotterdam, the Netherlands, aimed to describe these patients and determine prognostic factors to improve evaluation and treatment. Forty-four NEN patients with documented hyperammonemia were identified. All patients had liver metastases with 30% (n=13) showing signs of portal hypertension. Patients who developed encephalopathy had higher median ammonia levels, but there was no association between the severity of hyperammonemia and liver tumor burden or presence of liver insufficiency. Eighty-four percent (n=37) of patients died during follow-up. The median (IQR) time from diagnosis of hyperammonemia to death was 1.7 months (0.1-22.7). Hyperbilirubinemia, hypoalbuminemia, elevated INR, presence of liver insufficiency, encephalopathy and ascites were associated with worse outcome. Their role as independent risk factors for mortality was confirmed using the Child-Pugh score as a summary factor ($p<0.001$). No difference was seen concerning overall survival between our hyperammonemia patients and a propensity score matched control stage IV NEN cohort. In conclusion, hyperammonemia comprises a relevant and potentially underdiagnosed complication of NEN liver metastases and is associated with worse outcome. Assessment of signs of encephalopathy, risk factors and the Child-Pugh score could be helpful in selecting patients in whom ammonia levels should be measured.

1. BACKGROUND

Hyperammonemia occurs when there is a defect in the detoxification or overproduction of ammonia. It can lead to encephalopathy, ranging from mild cognitive impairment to coma (1, 2). While the metabolism of ammonia to urea and excretion via the kidneys is primarily impaired in inborn errors of metabolism, there are also several secondary causes that can be grouped into four main categories (1). The first are factors inhibiting the conversion of ammonia to urea such as severe malnutrition with low carnitine or arginine levels, drug-induced mitochondrial dysfunction, or urea cycle disorders. Second are factors that reduce the urea cycle capacity, as observed in liver insufficiency. A third cause of secondary hyperammonemia is the presence of shunting of the liver, such as portosystemic shunting as in portal hypertension or intrahepatic shunting due to high liver tumor load. Fourth, hyperammonemia can occur due to overproduction or due to infections with urease-producing bacteria, e.g. urinary tract or skin infections (3).

Ten cases of hyperammonemia in neuroendocrine neoplasm (NEN) patients have been described in the literature to date (4-10). NENs are a diverse group of rare malignant tumors arising from neuroendocrine cells and are mainly localized in the intestine, pancreas and lung (11). NENs are graded based on histological differentiation into well-differentiated neuroendocrine tumors or poorly differentiated neuroendocrine carcinomas (12). Around one fourth of NENs produce amine or peptide hormones and are called functional tumors (13). Due to their usually slow growth, NENs are often diagnosed at a metastatic stage and are prone to develop liver metastases (14, 15). However, liver failure in NEN patients is rare, even in cases with extensive metastatic liver disease (1). Treatment of metastatic NENs depends on their presentation and includes surgery, liver-directed therapy, (radiolabelled) somatostatin analogues, targeted treatment, and chemotherapy (16). The majority of the previously reported cases of hyperammonemia in NEN patients suffered from high liver tumor burden, with different triggering factors leading to the elevated ammonia levels.

Based on an index case of a NEN patient with hyperammonemia presenting in our clinic, awareness of this NEN-related complication was increased. Subsequently all NEN cases treated at our center of excellence during the last 20 years were reviewed. As no cohort of NEN patients developing hyperammonemia has been described to date, our aim was to describe clinical characteristics and outcome as well as to define prognostic factors in order to ameliorate evaluation and management of these patients.

2. METHODS

2.1 Collection of clinical data

Clinical data of this case report and retrospective case review was obtained from the years 2000-2020 from the database of the ENETS Centre of Excellence for Neuroendocrine Tumors Erasmus MC Rotterdam, the Netherlands. The Local Institutional Review Board of the Erasmus MC approved the database and a waiver of patient informed consent for the retrospective analysis was issued.

While an extensive work-up was performed in the index patient, data was assessed retrospectively for the other patients. Patients were selected if they had hyperammonemia defined as an ammonia level above the upper reference limit of 45 μmol/L. Patient, tumor, laboratory and radiological characteristics were recorded at the time point of hyperammonemia occurrence according to careful review of patient files as well as evaluation of the liver images by a radiologist. Tumor grade and stage were documented according to the 2010 WHO grading and ENETS guidelines (17, 18). Treatments were recorded over the entire follow-up period.

Liver insufficiency was defined as the simultaneous presence of bilirubin and INR levels above the normal range and albumin levels below the normal range. Signs of portal hypertension were defined as having thrombopenia and/or splenomegaly, presence of collaterals or thrombotic occlusions of the portal or liver veins or ascites without peritoneal carcinomatosis (19). The Child-Pugh and Model for End-stage Liver-Disease sodium (MELD-Na) prognostic scores were calculated according to the published scoring systems (20, 21). The Child-Pugh score includes the factors ascites, bilirubin, albumin, International Normalized Ratio (INR) of prothrombin time and encephalopathy, while the MELD-Na score includes the factors creatinine, bilirubin, INR and sodium. Due to missing values, the Child-Pugh and MELD-Na scores could not be calculated in n=12 and n=11 patients respectively.

For survival comparison, our previously presented cohort of metastatic (= stage IV) grade 1 or 2 NEN patients (22) was used. The data of this cohort were obtained from 2000-2019 and are derived from two prospective observational studies of NEN patients from the ENETS Centre of Excellence for Neuroendocrine Tumors Erasmus MC Rotterdam, the Netherlands and the Swiss national register of neuroendocrine tumors SwissNET. Of the total 325 patients, seven patients were also in the hyperammonemia cohort and hence excluded. To make the data more comparable, only patients with reported liver metastases were included, leaving a total number of 247 patients.

2.2 Statistics

Descriptive statistics were used to characterize demographic and clinical data, expressed as median with interquartile range (IQR) or frequencies with percentage (%). Binary variables were analyzed using logistic regression models, continuous variables were analyzed with linear regression models. A Cox proportional hazard model was used to calculate mortality hazard ratios (HR) and 95% confidence intervals (CI), with time to event counting from first onset of hyperammonemia until death or loss of follow-up. The proportionality of hazards was evaluated using the Cox regression analysis with time-dependent covariates. The assumption of proportionality of hazards was tested and was not broken in any of the Cox regression models.

For the control cohort, propensity score (PS) matching was used. PS matching is used to optimize the post-weighting balance of covariates between groups (23, 24). Using the PS methodology, all hyperammonemia patients and all control patients with liver metastases were assigned a weight between 0 and 1 according to the covariates age, sex, hormonal syndrome, site of origin and additional metastases. Grading was not used as a covariate, since this variable is not known in 23% of the hyperammonemia cohort. PSs were then used to match hyperammonemia patients to control patients with liver metastases in a 1:1 ratio using the nearest neighbor matching method. Successful matching was indicated by absence of statistical significance between the variables.

Overall survival rates, i.e. survival from date of diagnosis to last date of follow-up or death, were calculated with the Kaplan-Meier method. The log-rank test was used to compare overall survival differences between groups.

Statistical analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY) and R 3.3.3 open-source. P values <0.05 were considered statistically significant.

3. RESULTS

3.1 Index case

A 65-year-old patient with a metastatic pancreatic NEN presented with progressive liver metastases two years after diagnosis in our center. Peptide receptor radionuclide therapy (PRRT) using ^{177}Lu-DOTATATE was initiated. In the meantime, however, the patient became disoriented in time and place, nauseous and developed a fine hand tremor. Interestingly, symptoms transiently improved within one day of the administration of PRRT. Diagnostic work-up revealed hyperammonemia of 138 µmol/L without signs of liver dysfunction. Magnetic resonance imaging showed liver tumor burden mainly concentrated in the left hepatic

lobe without signs of intra- or extrahepatic portosystemic shunts. Diagnostic angiography showed signs of portal hypertension with a recanalized umbilical vein, high portal venous pressure and retrograde flow in the left portal vein, but no evidence for shunting. After initial improvement under treatment with lactulose and rifaximin, the patient again developed progressive encephalopathic symptoms. Despite negative imaging, intrahepatic shunting was suspected and selective intra-arterial radiotherapy (SIRT) injecting 90Yttrium-labelled microspheres to the left hepatic artery was performed to reduce the liver tumor burden. Two weeks after the SIRT, the patient already reported a significant improvement in quality of life. Ammonia levels taken 3 weeks after the procedure decreased to 70 μmol/L. The procedure was repeated 2.5 years after the first intervention due to a relapse in symptoms and recurrent hyperammonemia of 144 μmol/L, in the absence of radiological signs of progressive disease. Again, SIRT was successful and hyperammonemia remained controlled with lactulose and rifaximin. One year after the second intervention, the patient's symptoms recurred and plasma ammonia levels rose to 139 μmol/L. Arginine treatment was started after which the ammonia levels decreased to 80 μmol/L.

3.2 Retrospective analysis of hyperammonemia cases

Patient characteristics

Our analysis revealed 75 NEN patients with available ammonia levels, of which 44 NEN patients had documented hyperammonemia during their disease course (*Table 1*). The median (IQR) ammonia level in this NEN cohort was 83 μmol/L (59-141). Patients were predominantly male (64%) and presented with well-differentiated (93%) NENs of the pancreas (36%), small intestines (31%) and other origins (33%). All patients had stage IV metastatic disease with liver metastases. Additional metastatic sites were identified in 68% of patients. The main treatment modalities before onset of hyperammonemia involved somatostatin analogues (71%) or PRRT (59%) and surgical interventions (41%). Eighty-four percent of the patients died, with a median (IQR) follow up time of 55 months (31-105) since NEN diagnosis and a median (IQR) follow-up time of 1.7 months (0.1-22.7) since the diagnosis of hyperammonemia.

Table 1

A) Characteristics of the 44 NEN patients with hyperammonemia		
Sex, male	n (%)	28 (64)
Age, years	median (IQR)	65 (54-70)
BMI, kg/m^2	median (IQR)	21.8 (18.5-25.6)
NEN origin		
Gastro-duodenal	n (%)	3 (6.7)
Midgut	n (%)	14 (31)
Hindgut	n (%)	0
Pancreas	n (%)	16 (35,6)
Lung	n (%)	3 (6.7)
Unknown	n (%)	8 (17.8)
Grade		
NET grade 1	n (%)	11 (25)
NET grade 2	n (%)	20 (45.5)
NET grade 3	n (%)	1 (2)
NEC	n (%)	2 (4.5)
Unknown	n (%)	10 (23)
Metastases		
Liver	n (%)	44 (100)
Non-mesenteric lymph nodes	n (%)	17 (39)
Mesenteric lymph nodes	n (%)	15 (34)
Lung	n (%)	5 (11)
Bone	n (%)	6 (14)
Hormonal syndrome	n (%)	19 (43)
Laboratory		
Ammonia, µmol/L	median (IQR)	83 (59-141)
Chromogranin A, ng/L	median (IQR)	3724 (293-56468)

Treatment during follow-up period		
Somatostatin analogues	n (%)	**32 (73)**
Before HA		*18 (56)*
After HA		*1 (3)*
Before and after		*13 (41)*
PRRT	n (%)	**30 (68)**
Before HA		22 (73)
After HA		4 (13.5)
Before and after		4 (13.5)
Chemotherapy	n (%)	**9 (21)**
Before HA		*4 (44.5)*
After HA		*4 (44.5)*
Before and after		*1 (11)*

Table 1 (*continued*)

A) Characteristics of the 44 NEN patients with hyperammonemia			
Targeted treatment	n (%)		**8 (18)**
Before HA			*3 (38)*
After HA			*3 (38)*
Before and after			*2 (25)*
Surgery	n (%)		**19 (43)**
Before HA			*17 (90)*
After HA			*1 (5)*
Before and after			*1 (5)*
Liver embolization	n (%)		**11 (25)**
Before HA			*8 (73)*
After HA			*3 (27)*
Before and after			*0*
Follow up time, months	median (IQR)		55 (31-105)
Time diagnosis HA to death or loss of follow up, months	median (IQR)		1.7 (0.1-22.7)
Patients died	n (%)	37	84)

Table 1 Characteristics of the patients with hyperammonemia. Values are shown as frequencies (%) or median (IQR).
BMI = body mass index; HA = Hyperammonemia; IQR = interquartile range; n = number; NEN roendocrine neoplasm; NEC = neuroendocrine carcinoma; PRRT = peptide receptor radionuclide therapy;

The main reasons for ammonia measurement were encephalopathy in 31%, general deterioration in 30% and post-interventional evaluation or evaluation due to acute triggers (e.g. sepsis) in 16%, respectively. The suspected underlying cause for hyperammonemia was tumor progression in the majority of patients (57%), followed by post-interventional complications in 18%, for details see *Table 1*.

Risk factors associated with hyperammonemia

In our cohort of hepatic-metastasized NEN patients, abnormal liver transaminases were present in 71%, with 39% fulfilling the criteria of liver insufficiency at the time of hyperammonemia diagnosis, *Table 2*. Eighty-two percent of patients had an impaired kidney function, cardiac failure was present in 7% of patients.

Table 2 Risk factors associated with hyperammonemia

Laboratory parameters at time point hyperammonemia		
Liver function		
Liver transaminase levels normal	n (%)	13 (29)
>normal, <10x elevated	*n (%)*	*20 (46)*
>10x / <100x elevated	*n (%)*	*7 (16)*
>100x elevated	*n (%)*	*4 (9)*
Bilirubin, (Norm 0-16 µmol/L)	median (IQR)	20 (10-33)
Hyperbilirubinemia	n (%)	*23 (52)*
Albumin, (Norm 35-50g/L)	median (IQR)	27 (23-36)
Hypalbuminemia	n (%)	*31 (71)*
Kidney function		
Creatinine, (Norm 55-90 µmol/L)	median (IQR)	116 (78-154)
GFR	median (IQR)	53 (37-81)
GFR 60-90ml/min	*n (%)*	*7 (16)*
GFR 30-59ml/min	*n (%)*	*22 (50)*
GFR <30ml/min	*n (%)*	*7 (16)*
Coagulation factors		
Thrombocytes, (Norm 150-370 10^9/L)	median (IQR)	184 (95-288)
Thrombopenia	*n (%)*	*16 (36)*
Prothrombin time, (Norm 10.9-13.3)	median (IQR)	16.5 (14.6-23.4)
INR, (Norm <1.1)	median (IQR)	1.4 (1.3-1.8)
Assessment liver function		
Liver insufficiency (high bilirubin, low albumin, elevated INR)	n (%)	17 (39)
Signs of encephalopathy	n (%)	14 (31)
Child-Pugh Score	median (IQR)	8 (7-10)
MELD-Na-score	median (IQR)	17 (11-23)
Radiology		
Tumor burden liver		
0-10 %	*n (%)*	*10 (22,7)*
11-25 %	*n (%)*	*2 (4,5)*
26-50 %	*n (%)*	*7 (15,9)*
>50 %	*n (%)*	*20 (45,5)*
Missing	*n (%)*	*5 (11,4)*
Bilobar metastatic involvement liver	n (%)	32 (73)

Table 2 Risk factors associated with hyperammonemia (*continued*)

Radiology		
Hepatomegaly	n (%)	24 (55)
Portal / superior mesenteric vein thrombosis	n (%)	7 (16)
Splenomegaly	n (%)	5 (12)
Collateral vessels	n (%)	6 (14)
Ascites (malignant & non-malignant)	n (%)	30 (68)
Ascites due to portal hypertension	*n (%)*	*9 (30)*
Signs of portal hypertension (thrombopenia, splenomegaly, large portal vein / collaterals, thrombotic occlusions)	n (%)	13 (29)

Table 2 Risk factors associated with hyperammonemia in neuroendocrine neoplasm patients. Values are shown as frequencies (%) or median (IQR).
GFR = glomerular filtration rate; INR = international normalized ratio of prothrombin time; IQR = interquartile range; n = number; MELD-Na = Model for End-stage Liver-Disease sodium

In the majority (68%) of the patients both liver lobes were affected by metastases with a liver tumor burden greater than 50% in 46% of all patients. On imaging, ascites (both malignant and non-malignant) was present in 68% of the patients. Other signs of portal hypertension were seen in 29% of the patients.

The maximum levels of plasma ammonia did not correlate with liver transaminases (R2<0.001) There was also no difference in hyperammonemia severity according to liver tumor load (p=0.15) or between patients with and without liver insufficiency or signs of portal hypertension (p=0.29 and p=0.16 respectively), *Figure 1.*

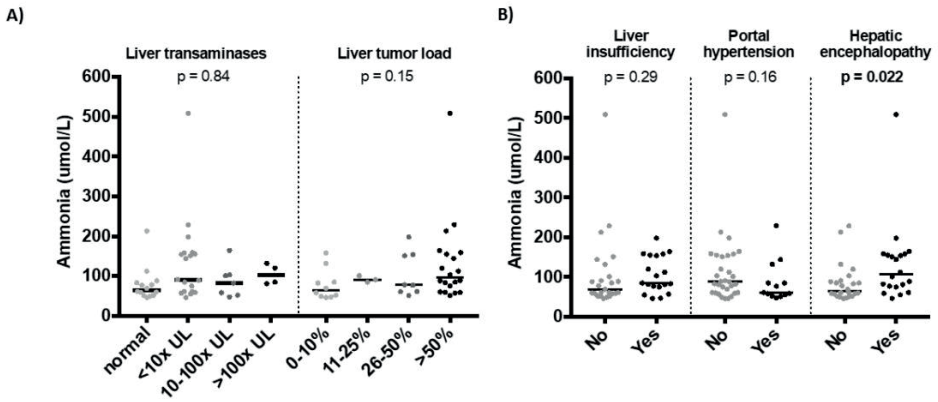

Figure 1 Plasma ammonia levels in neuroendocrine neoplasm patients stratified according to different risk factors:
A) Liver transaminases and liver tumor load
B) Liver insufficiency, signs of portal hypertension and hepatic encephalopathy
Data is shown as median with individual data points. The p-values depict the difference within the groups, bold writing indicating significance. UL = upper limit of normal

In patients with extensive liver disease, tumor progression was identified as the main cause for hyperammonemia, while in patients with a low tumor liver burden acute triggers such as interventions or surgery were the main cause. There was no difference in survival time between the two groups from the moment of hyperammonemia.

The median (IQR) plasma ammonia levels in patients developing encephalopathy was higher with 107 μmol/L (76-157) compared to 65 μmol/L (53-90) in patients without encephalopathy, *Figure 1*.

Impaired kidney function was observed in the majority of the hyperammonemia patients (*Table 1*), however no correlation was observed between maximum levels of plasma ammonia and creatinine levels (R2=0.025, p=0.31).

Risk factors associated with mortality

To identify variables associated with mortality in NEN patients with hyperammonemia, a univariable regression analysis was performed on known risk factors associated with liver failure or hyperammonemia, *Figure 2*. The maximum level of plasma ammonia was not associated with increased mortality in our cohort. In contrast, hyperbilirubinemia (bilirubin levels HR (95% CI) 1.013 (1.003-1.024), p=0.01), hypoalbuminemia (albumin levels HR (95% CI) 0.95 (0.91-0.99), p=0.007) and elevated INR (INR levels HR (95% CI) 1.92 (1.26-2.94), p=0.003) were markers for worse outcome. The presence of liver insufficiency (HR 3.53, 95% CI 1.69-7.38), p=0.01), encephalopathy (HR 2.4, 95% CI 1.2-4.5, p=0014) and ascites (HR 2.66, 95% CI 1.21-5.83, p=0.015) were also associated with increased mortality risk.

Since our cohort size and event rate did not allow us to combine all identified factors in a multivariable regression analysis, we decided to test classic prognostic scores for end-stage liver diseases: Child-Pugh and MELD-Na. While the Child-Pugh score includes all defined factors, the MELD-Na score includes only bilirubin and INR. Both scores were associated with an increased mortality risk in NEN patients with hyperammonemia with a HR (95% CI) of 1.50 (1.21-1.87, p<0.001) for the Child-Pugh score and 1.14 (1.06-1.22, p<0.001) for the MELD-Na score. Median (IQR) survival time from onset of hyperammonemia was 51 months (31-105) for Child-Pugh class A (=5-6 points), compared to 0.8 month (0.3-19) for class B (=7-9 points) and 0.1 month (0.1-1.7) for class C (=10-15 points), p=0.004, *Figure 2*.

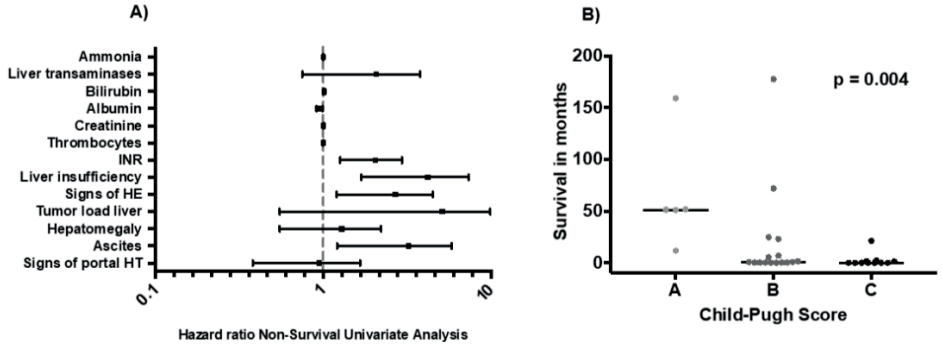

Figure 2 Risk factors for the development of hyperammonemia

A) Forest plot showing the effect of the pre-defined risk factors on mortality risk according to univariable regression analysis.

The black squares reflect the hazard ratio, while the whiskers indicate the lower and upper limits of the 95% confidence interval.

HE = encephalopathy; HT = Hypertension; INR = international normalized ratio of prothrombin time;

B) Survival time from onset of hyperammonemia to death in relation to Child-Pugh scores.

Data is shown as median with individual data points. The p-value depicts the difference within the groups, bold writing indicating significance.

Control = NEN control population, HA = hyperammonemia population; NEN = neuroendocrine neoplasm;

To evaluate the impact of hyperammonemia on overall survival, we compared our patients with 44 propensity score (PS) matched stage IV NEN patients. PS matching led to two well-balanced groups, which showed no difference in overall survival, p=0.65. Accordingly, no evaluation concerning the possible identification of risk factors could be performed.

Treatment of hyperammonemia in our cohort

The majority (59%) of our patients did not receive specific treatment for hyperammonemia. Of the treated 18 patients, 83% received lactulose orally, in seven cases in combination with rifaximin. Additional supportive medication with L-arginine and dexamethasone was given in three cases. Two patients, among them our index case, received SIRT with 90Yttrium-labelled microspheres. One patient was treated with PRRT with 177Lu-DOTATATE to lower the liver burden, and in one patient portal hypertension was successfully treated with portal vein stenting.

Of the patients who received only lactulose and/or rifaximin, the majority (61%) died within 3 months of hyperammonemia onset (median (IQR) plasma ammonia level 151 (86-162) μmol/L). All patients with interventional treatment had a survival of at least 11 months from hyperammonemia onset (median (IQR) plasma ammonia level 69 (60-138) μmol/L).

4. DISCUSSION

Hyperammonemia is an underrecognized complication of liver metastases in NEN patients. The presence of hyperammonemia is accompanied by a high short-term mortality rate and several risk factors for worse outcome could be defined. The commonly used Child-Pugh score proved to be a useful tool for assessing mortality risk in these patients.

Hyperammonemia in NEN patients can occur due to several causes. Because NEN are usually slow-growing tumors, progressive hepatic tumor infiltration can lead to intrahepatic shunting. Decreased liver capacity due to tumor infiltration contributes to the reduced clearance of ammonia. This was likely the main cause in 46% of our patients presenting with a liver tumor burden above 50% and has also been described in previous case reports (8). Hepatic failure due to tumor infiltration however is rare in NEN patients (1). This was also reflected by our cohort where the majority of patients had intact liver function and only four patients showed signs of liver failure. The second main cause for hyperammonemia in our cohort was portosystemic shunting, caused by obstructions of the portal venous system or microvascular portosystemic shunting within the liver metastases. Portosystemic shunting as cause of encephalopathy in NEN patients has been described in other case reports (5, 7) and was most likely also one of the main causes in 13% of our patients. Additional causes include decreased ammonia excretion due to impaired kidney function or acute stressors such as surgical or other interventions, infections or medication associated toxicities (1, 4, 25). In 27% of our patients, the occurrence of hyperammonemia was indeed associated with an acute trigger factor. Interestingly, this was the main cause in our patients with a tumor liver load below 10%.

Lastly, an alternative mechanism involving the production of hormones or neurotransmitters have been proposed (9). The fact that no difference was seen in hyperammonemia severity according to liver tumor load, liver insufficiency or signs of portal hypertension indicates different mechanisms. In addition, the number of patients with functional active NENs (43%) was higher in this cohort than the control cohort (32%) (22). That functional active NENs could interfere with the complex detoxification process of ammonia seems plausible and should be further investigated.

Given the likely underreporting of hyperammonemia in NEN patients, the main challenge will be to identify the patients in whom ammonia levels should be measured. An important clinical indicator is the development of encephalopathy, which was documented in 31% of our patients. Encephalopathy can be missed in patients presenting with mild symptoms (26) like mild behavior changes but still alert. This is likely underreported in the presented cohort. Awareness of the treating physician prompting ammonia measurement in metastatic NEN

patients presenting with behavior changes, disorientation, memory problems or hand tremor is critical. These symptoms are debilitating and severely reduce quality of life of the patient and/or patient's family. Furthermore, although patients who developed encephalopathy had higher levels of ammonia, lower levels were not exclusive of the development of symptoms. Similar findings have been described in other studies (2, 27). Interestingly, the severity of hyperammonemia failed to identify patients at risk for a reduced outcome, which underlines the need for additional prognostic factors.

In our cohort, the parameters hyperbilirubinemia, hypoalbuminemia, elevated INR as well as the presence of ascites and hepatic encephalopathy were associated with increased risk of mortality. Since these factors might be challenging to combine for the individual patient, we chose to evaluate the use of the known risk scores for liver insufficiency Child-Pugh and MELD-Na. While a higher rating was associated with an increased mortality risk in both scores, the Child-Pugh score included all defined risk factors and showed a higher association with worse outcome. The Child-Pugh score was developed as a predictive tool for mortality in cirrhotic patients and categorizes patients into good liver function (A), moderately impaired liver function (B) and advanced liver dysfunction (C) (20). The clear association between Child-Pugh score and survival in our NEN patients with liver metastases shows that this score can be employed for prognostication.

Although overall survival rate of hyperammonemia patients did not differ from PS-matched stage IV control NEN patients, the median survival time after diagnosis of hyperammonemia was only 1.7 months. This underlines that these patients require prompt recognition and initiation of specialized treatment for hyperammonemia and underlying causes. Hyperammonemia is also accompanied by reduced survival rates in patients with acute and chronic liver failure and irrespective of complete liver failure (28, 29).

A careful evaluation of possible causes and specific treatment options is crucial following the demonstration of hyperammonemia. Treatment for hyperammonemia given to the patients in the presented cohort was limited, with the majority receiving no treatment. This is most likely due to the low awareness of the impact of hyperammonemia. Evaluation should include nutritional status assessment as well as carnitine and arginine measurement, since both are important intermediates in ammonia detoxification via the urea cycle (30). Late-onset or acquired urea cycle disorders may also become apparent in patients with chronic malnutrition, rapid weight loss or other factors causing metabolic stress (31). For patients in whom the cause of hyperammonemia is not easily reversible, treatment focuses on lowering ammonia levels. This can be achieved in most cases by reducing ammonia production and absorption from the intestines, as with the antibiotic rifaximin. Lactulose can also reduce absorption by converting ammonia to ammonium, which is then excreted in the stool (32, 33). Other

treatment options are L-arginine, a non-essential amino acid that reduces ammonia levels by increasing urea production (34, 35). In the presented case, the administration of L-arginine resulted in a transient amelioration of symptoms, since L-arginine is given together with PRRT for nephroprotection.

In case of hyperammonemia due to shunting, reduction of liver tumor mass could be the preferred strategy, for example with SIRT as in our case, or with interventions such as trans-arterial embolization (TAE) or transarterial chemo-embolization (TACE) (5-7, 10). According to the available limited data, TAE might be preferred in severely symptomatic patients aiming at rapid reduction of ammonia levels (5). While these procedures might prolong survival in patients with otherwise high mortality rates, they could also trigger hyperammonemia due to acute deterioration of liver function. Therefore, their use should be carefully evaluated in a multidisciplinary setting.

The main limitation of our study are its retrospective design and likely selection bias. All but three patients were diagnosed with hyperammonemia due to patient deterioration or the appearance of an acute trigger factor. Patients with mild symptoms could have been missed, especially in view of the low awareness of hyperammonemia in NEN patients. This is also reflected in the low number of NEN patients with available ammonia levels. In addition, due to the limited number of actively treated patients, no statement can be made about the effect of ammonia-lowering treatments on the outcome. Despite these limitations, this study is the first to systematically evaluate risk and possible prognostic factors in NEN patients with hyperammonemia.

In conclusion, our study revealed that NEN patients developing hyperammonemia have a worse outcome independent of the severity of hyperammonemia. Ammonia levels should be measured when neurocognitive symptoms or risk factors are present. Child-Pugh scoring could be helpful for risk stratification in these patients.

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5 Inferior outcome of neuroendocrine tumor patients negative on somatostatin-receptor imaging

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ABSTRACT

Sufficient expression of somatostatin receptor (SST) in well-differentiated neuroendocrine tumors (NETs) is crucial for treatment with somatostatin analogs (SSAs) and peptide receptor radionuclide therapy (PRRT) using radiolabeled SSAs. Impaired prognosis has been described for SST-negative NET patients; however, studies comparing matched SST-positive and -negative subjects who have not received PRRT are missing. This retrospective analysis of two prospectively maintained NET databases aimed to compare matched metastatic grade 1 or 2 SST-positive and -negative NET patients. SST-negativity was defined as having insufficient tumor uptake on diagnostic SST imaging. Patients that underwent PRRT were excluded. Seventy-seven SST-negative and 248 SST-positive grade 1-2 NET patients were included. Median overall survival rates were significantly lower for SST-negative compared to SST-positive NET patients (53 months vs 131 months; $P < 0.001$). To adjust for possible confounding by age, gender, grade and site of origin, 69 SST-negative NET patients were propensity score matched to 69 SST-positive NET patients. Group characteristics were similar, with the exception of SST-negative patients receiving more often chemotherapy and targeted treatment. The inferior survival outcome of SST-negative compared to SST-positive NET patients persisted with a median overall survival of 38 months vs 131 months ($P = 0.012$). This relationship upheld when correcting for the main influencing factors of having a higher grade tumor or receiving surgery in a multivariate Cox regression analysis. In conclusion, we showed that propensity score-matched SST-negative NET patients continue to have a worse prognosis compared to SST-positive NET patients despite receiving more aggressive treatment. Differences in tumor biology likely underlie this survival deficit.

1. INTRODUCTION

Neuroendocrine neoplasms (NENs) consist of a diverse group of tumours arising from neuroendocrine cells and are mainly localized in the intestine, pancreas and lung (1). Classification of NENs is based on their origin, extension and histological differentiation (2). Based on their histology, mitotic count, the expression of the nuclear protein marker Ki-67 and additional markers of differentiation, NENs are classified as well-differentiated grade 1, 2 or 3 neuroendocrine tumours (NETs) or poorly differentiated grade 3 neuroendocrine carcinomas (3). Around 25% of NENs produce hormones or peptides and are so called functioning tumours (4). However, the majority of NENs are slow growing non-secreting tumours, leading to a late diagnosis at a metastatic stage (5).

Characteristic features of well-differentiated NETs are the expression of hormone receptors on its tumour surface, with the somatostatin receptor subtype 2 (SST2) being expressed in up to 90% of gastroenteropancreatic NETs (6, 7). According to this feature, SSTR-based imaging is the current standard for diagnosing and staging NETs (8). Furthermore, treatment with unlabelled somatostatin analogues (SSAs) or radio-labelled SSAs as in peptide receptor radionuclide therapy (PRRT) have shown prolongation of progression free survival (PFS) in metastatic NET patients (9-11). However, since sufficient uptake of radiolabelled SSAs on SSTR imaging is a prerequisite for those therapies (12), SSA treatment is questionable and PRRT not feasible in SSTR-negative NET patients. To overcome this limitation, systemic treatment options such as chemotherapy and targeted therapy are used in these patients, but chemotherapy is not an option in metastatic midgut NETs (13). This could partly explain the poorer survival rates in SSTR-negative NET patients (14). Other data have suggested that a more aggressive tumour biology was responsible for the worse outcome, but these results were obtained in uncontrolled studies (15, 16). Studies comparing outcomes and covariates between matched SSTR-negative and -positive NET patients are missing.

Accordingly, the aim of this retrospective analysis was to compare outcomes of metastatic grade 1-2 SSTR-negative and -positive NET patients from the databases of two large NEN cohorts. We hypothesized that the poorer outcome of SSTR-negative NET patients would persist after patient matching using the propensity score method and covariant adjustment.

2. METHODS

2.1 Patient characteristics

The data of this retrospective analysis was obtained from the years 2000-2019 from two prospective observational studies of NEN patients from the ENETS Centre of Excellence for

Neuroendocrine Tumours Erasmus MC Rotterdam, the Netherlands and the Swiss national register of neuroendocrine tumours SwissNET. Local Institutional Review Boards (IRBs; Rotterdam: IRB of the Erasmus MC; Switzerland: Federal Office of Public Health) approved both databases. Informed consent was obtained of all Swiss patients, the IRB of the Erasmus MC approved a waiver of patient informed consent for the retrospective analysis.

Patients were selected if they had advanced stage well-differentiated grade 1 or 2 NETs. Patients that received treatment with PRRT were excluded from this study because of its beneficial effect on survival in favor of SSTR-positive tumours. Tumour characteristics, grade and stage were documented at the time of diagnosis according to the 2010 WHO grading and ENETS guidelines (17). Treatments were recorded over the entire follow-up period. Highest serum chromogranin A levels (reference <94ug/L) of the follow up period were used.

SSTR negativity was defined as having an uptake lower than the liver on SSTR-scintigraphy (18) and equal to or lower than the liver on SSTR-PET (19) at the time of diagnosis.

2.2 Primary and secondary outcomes

The primary endpoint was to compare survival outcomes between patients with a SST-negative NET and those with a SST-positive NET. Secondary endpoints were to define prognostic factors for worse outcome.

2.3 Statistics

Descriptive statistics were used to characterize demographic and clinical data, expressed as median with interquartile range (IQR) or frequencies with percentage (%). The association of categorical variables was assessed by Pearson chi-square test, continuous variables by Mann–Whitney U test.

Outcome analysis was divided into three parts. First, data of all eligible patients were evaluated according to their SST status. Survival rates (= survival from date of diagnosis to last date of follow-up or death) were calculated with the Kaplan-Meier method, the log-rank test was used to compare survival differences between groups. A Cox proportional hazard model was used to calculate mortality hazard ratios (HR) and 95% confidence intervals (CI). Only variables that significantly affected survival in univariate analysis were included in the Cox model. The proportionality of hazards was evaluated using the Cox regression analysis with time-dependent covariates. The assumption of proportionality of hazards was tested and was not broken in any of the Cox regression models.

Second, all analyses were repeated in a propensity score (PS)-matched cohort. PS analysis optimizes the post-weighting balance of covariates between groups (20, 21). Using the PS methodology, all patients were assigned a weight between 0 and 1 according to the covariates age, gender, tumour grade and site of origin. Using caliper matching without replacement we used PSs to match SSTR-negative to SSTR-positive NET patients in a 1:1 ratio. A caliper distance of an absolute difference in PS of 0.1 was employed, leading to 69 matched patients in each group, with 8 SSTR-negative NET patients failing to match within the defined scope. Successful matching was indicated by absence of statistical significance between the variables.

Third, subgroup analysis involving all patients treated with SSA were performed for overall survival and PFS as described above. PFS was defined as survival without progression from the start of SSA treatment to the last date of follow-up or progression. PS-matching as described above was again used to better compare the SSTR-negative and -positive patients. Also, effect of SSA treatment among the SSTR-negative patients was investigated.

Statistical analyses were performed using SPSS version 25.0 (IBM Corp., Armonk, NY) and R 3.3.3 open-source. P values <0.05 were considered statistically significant.

3. RESULTS

3.1 Complete cohort

Patient characteristics

After excluding all patients receiving PRRT, a total of 77 SST-negative NET patients and 248 SST-positive NET patients met the inclusion criteria and were included into the analysis. SST-status was defined according to SST-scintigraphy in 255 (79%) patients, while in 28 (9%) and 42 (13%) Ga-DOTATOC or Ga-DOTATATE PET/CT was used respectively. The two patient groups differed with regard to the distribution of the site of origin, grade, metastatic locations and received treatment (see *Table 1* for details). Median (IQR) follow-up time for SST-negative and -positive NET patients was 34 (15-67) and 51 (16-83) months, respectively ($p=0.093$).

Table 1 Characteristics complete cohort

Complete cohort	SST status negative (n=77)	SST status positive (n=248)	p value
Sex (female), n (%)	41 (53)	119 (48)	0.4
Age at diagnosis, years (IQR)	63 (55-68)	63 (54-71)	0.3
Primary tumor site, n (%)			**<0.001**
• Gastro-duodenal	0	4 (2)	
• Midgut	24 (31)	172 (69)	
• Hindgut	1 (1.3)	4 (2)	
• Pancreas	13 (17)	35 (14)	
• Lung	24 (31.2)	11 (4)	
• Unknown	15 (19.5)	22 (9)	
Grade, n (%)			**0.009**
• 1 / 2	29 (38) / 48 (62)	136 (55) / 112 (45)	
o *Gastro-duodenal*	*0 (0)*	*2 (50) / 2 (50)*	
o *Midgut*	*13 (54) / 11 (46)*	*104 (60) / 68 (40)*	
o *Hindgut*	*0 (0) / 1 (100)*	*2 (50) / 2 (50)*	
o *Pancreas*	*4 (31) / 9 (69)*	*9 (26) / 26 (74)*	
o *Lung*	*9 (38) / 15 (62)*	*7 (64) / 4 (36)*	
o *unknown*	*3 (20) / 12 (80)*	*12 (54) / 10 (46)*	
Hormone secreting, n (%)	23 (30)	78 (32)	0.6
Chromogranin A, ng/l (IQR)	255 (91-952)	261 (101-907)	0.9
Site of metastasis, n (%)			
• Lymphnodes	60 (78)	217 (89)	0.2
• Liver	61 (79)	193 (78)	0.4
• Lung	11 (14)	13 (5)	**0.001**
• Bone	21 (27)	28 (11)	**<0.001**
Treatment, n (%)			
• Somatostatin analogues	35 (46)	152 (61)	**0.014**
• Chemotherapy	23 (30)	12 (5)	**<0.001**
• Targeted therapy	22 (29)	4 (2)	**<0.001**
• Liver directed therapy	6 (8)	23 (9)	0.7
• Surgery	45 (58)	177 (71)	**0.033**
o *curative*	*19 (42)*	*113 (64)*	
o *palliative*	*26 (58)*	*64 (36)*	
Died, n(%)	43 (56)	72 (29)	**<0.001**

Table 1 Patient characteristics of the complete cohort according to SST status.Values are shown as frequencies (%) or median (IQR). p-Values refer to differences between the groups.
SST = somatostatin receptor ; n = number; IQR = interquartile range

Outcomes

At last follow-up, 56% (n=43/77) of the SST-negative NET patients had died, compared to 29% (n=72/248) of the SST-positive NET patients (p<0.001). Median (95% CI) overall survival time was significantly lower for SST-negative NET patients with 53 months (26-80) compared to 131 months (89-173) for SST-positive NET patients, (p<0.001, *Figure 1*).

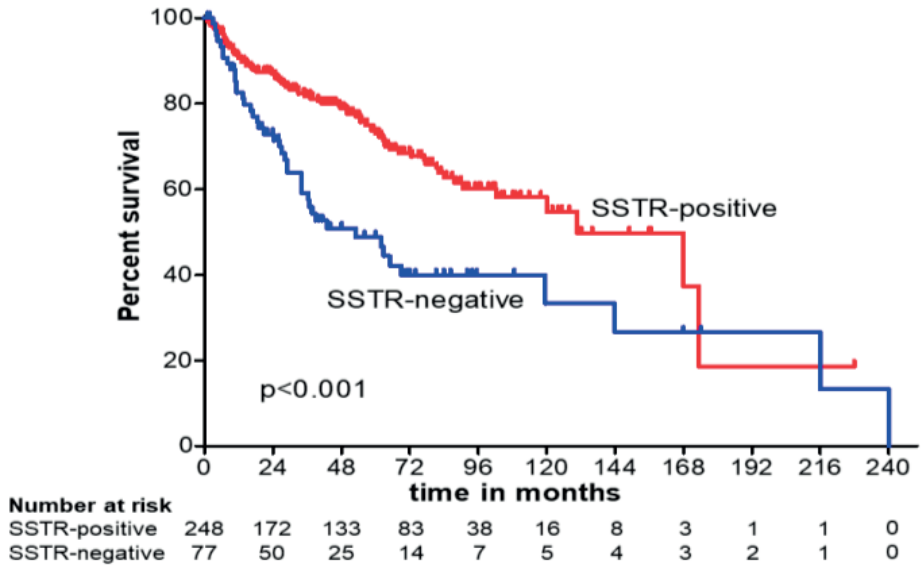

Figure 1 Overall Survival Complete Cohort

Kaplan Meier analysis showing overall survival of the complete cohort, divided into SST-positive (n=248) and SST-negative (n=77) patients.

p-Value indicates difference in survival (Log Rank Test); SSTR = somatostatin receptor

Multivariate Cox regression analysis confirmed SST-negativity as an independent risk factor for mortality with a hazard ratio (95% CI) of 1.85 (1.21-2.83), p=0.005. Additional determinants for worse outcome were age above 65 years (HR 2.11 (1.42-3.12), p=0.001),

having a grade 2 tumour (HR 1.75 (1.17-2.61), p=0.007) and the presence of bone metastases (HR 1.80 (1.14-2.82), p=0.011) (*Table 2*).

Table 2: Cox Regression analysis of the complete cohort.

Complete Cohort	Univariate Cox Regression Analysis	Multivariate Cox Regression Analysis	Hazard Ratio (95% CI)
SST negativity	**<0.001**	**0.005**	**1.85** (1.21-2.83)
Sex (female)	0.80		
Age > 65 years	**<0.001**	**0.001**	**2.11** (1.42-3.12)
Primary tumour site	**0.001**	0.97	
Grade 2 vs 1	**<0.001**	**0.007**	**1.75** (1.17-2.61)
Site of metastasis			
• Lymph nodes	0.07		
• Liver	**0.011**	0.10	
• Lung	0.60		
• Bone	**0.001**	**0.011**	**1.80** (1.14-2.82)
Treatment			
• Somatostatin analogues	0.20		
• Chemotherapy	**0.001**	0.28	
• Targeted therapy	**0.008**	0.06	
• Liver-directed therapy	**0.005**	0.95	
• Surgery	**<0.001**	**<0.001**	**0.39** (0.26-0.57)
- curative vs palliative	*0.42*		
- primary tumour site	*0.69*		

Table 2 Only variables reaching significance in the univariate analysis were included into the multivariate analysis.
SST = somatostatin receptor; 95% CI = 95% confidence interval; vs = versus

Having received any surgical intervention was associated with a significantly reduced overall mortality risk (HR 0.39 (0.26-0.57), p<0.001). However, neither the intent of surgery (curative versus palliative: p=0.42) nor the primary tumour site (p=0.69) had an effect on survival outcome. *Figure 2* shows survival outcomes of SST-negative and -positive patients according to their tumour grade and surgical status.

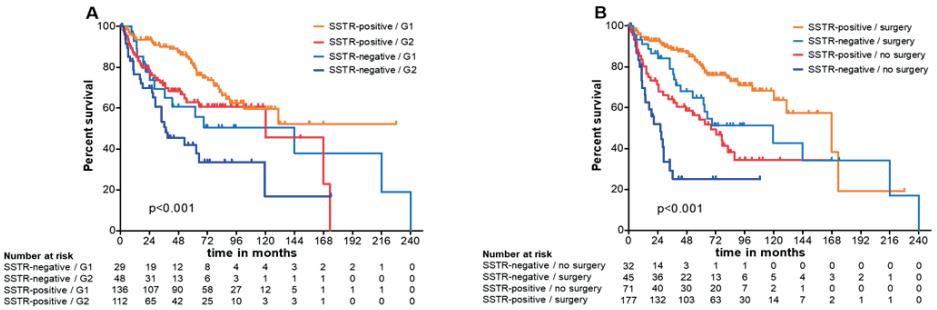

Figure 2 Overall survival complete cohort according to covariates

Kaplan Meier analysis showing overall survival of the complete cohort, according to
A) SST-status and tumour grade 1 (n=165) or 2 (n=160) and
B) SST-status and having received a surgical intervention (n=222) or not (n=103).
p-Value indicates difference in survival between the groups (Log Rank Test).
SSTR = somatostatin receptor; G1 = grade 1; G2 = grade 2;

3.2 Propensity score-matched cohort

Patient characteristics

Propensity score (PS) matching led to two well-balanced groups of 69 patients each with a median (IQR) follow-up time for SST-negative NET patients of 34 (14-64) months and SST-positive NET patients of 44 (9-76) months, p=0.53. With the exceptions of SST-negative NET patients having a higher prevalence of lung metastases and more often receiving chemotherapy and targeted treatment than patients with SST-positive NETs, there were no significant differences between the two groups (*Supplementary Table S1*).

Outcomes

During the observation period, more SST-negative NET patients (59%, n=41/69) than matched SST-positive (32%, n=22/69, p=0.001) NET patients died. Despite the PS matching, SST-negative NET patients still had a worse median (95% CI) overall survival of 38 (19-56) months compared to SST-positive NET patients with 131 (54-208) months, p=0.012 (*Figure 3*).

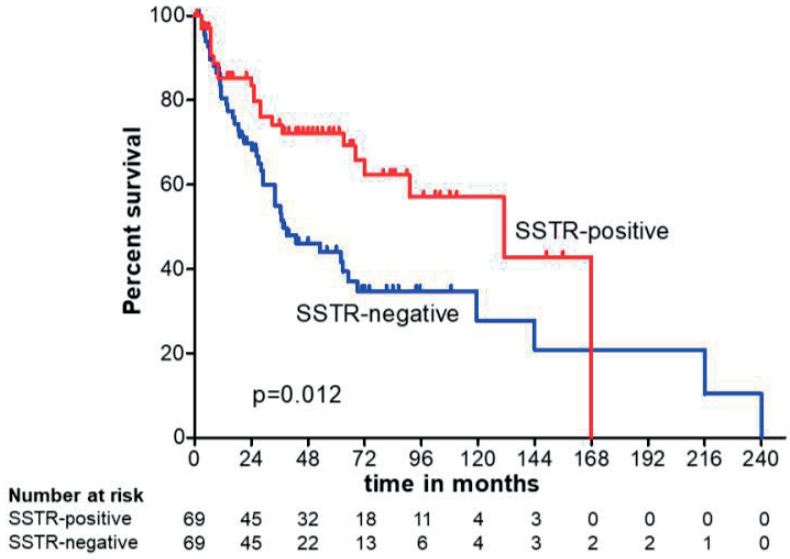

Figure 3 Overall Survival Propensity Score Matched Cohort

Kaplan Meier analysis showing overall survival of the PS-matched cohort, divided into SST-positive (n=69) and SST-negative (n=69) patients.

p-Value indicates difference in survival (Log Rank Test).

PS = propensity score; SSTR = somatostatin receptor

SST-negativity persisted to be associated with worse outcome in multivariate Cox regression analysis with a HR (95% CI) of 2.58 (1.34-4.99), p=0.005. Another independent determinant of mortality was the presence of a grade 2 tumour (HR 2.32 (1.18-4.58), p=0.015), while having a surgical intervention was associated with a lower mortality risk (HR of 0.36 (0.18-0.72), p=0.004) (*Supplementary Table S2*). Again, complete surgical resection and primary tumour site did not influence survival outcome (p=0.30 and p=0.42, respectively). *Supplementary Figure S1* shows the survival curves of the PS-matched SST-negative and -positive patients according to their tumour grade and surgical status.

3.3 Treatment with somatostatin analogues

In a subgroup analysis, we evaluated the outcomes of all patients receiving SSA treatment. This concerned a total of 35 SST-negative NET patients and 152 SST-positive NET patients. The two patient groups differed with regard to the distribution of the site of origin, number of patients having bone metastasis and additional received treatments (*Supplementary Table S3*). Although more SST-negative NET patients in this group received additional chemotherapy and targeted treatment, they continued to have a worse overall survival compared to the SST-positive NET patients (p=0.008, data not shown).

SST-negative NET patients also had a shorter median (95% CI) PFS after the start of SSA treatment of 15 (7-22) months compared to 54 (44-64) months in SST-positive NET patients (p<0.001, *Figure 4A*).

To further validate the above results, SSA-treated patients were PS-matched according to their SST-status, resulting in 35 patients in each group. Patient characteristics were well balanced, with the exception of SST-negative patients receiving more often targeted treatment (*Supplementary Table S4*). In this PS-matched cohort, SST-negative patients tended towards a shorter median overall survival time (39 versus 72 months, p=0.14) and continued to have a significantly reduced PFS (15 versus 47 months, p=0.006). SST-negativity and having a grade 2 tumour remained independent determinants of a shorter PFS in multivariate analysis in all SSA-treated patients as well as in the PS-matched cohort (all SSA-treated patients: p=0.008 and 0.002; PS-matched SSA-treated patients: p=0.004 and 0.002, respectively).

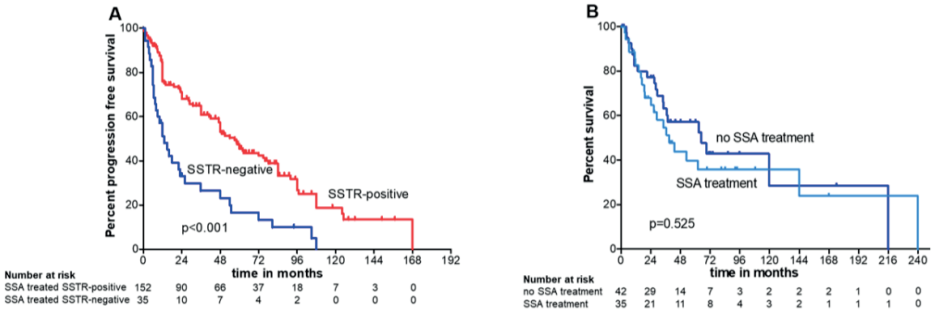

Figure 4 Outcome of Patients Treated with Somatostatin Analogues:
A) Kaplan Meier analysis of progression free survival of all patients treated with SSA according to SST-status (SST positive: n=152, SST-negative: n=35),
B) Kaplan Meier analysis of overall survival of SST-negative patients treated with SSA (n=35) compared to no SSA treatment (n=42).
p-Value indicates difference between groups according to Log Rank Test.
SST = somatostatin receptor; SSA = somatostatin analogues

Finally, we investigated whether SSA treatment leads to a survival benefit in SST-negative NET patients. The two groups were comparable with the exception of SSA-treated SST-negative patients having a higher rate of liver metastases (91% versus 69%, p=0.024). Median overall survival was similar between SST-negative patients receiving SSA and those without SSA treatment (p=0.53, *Figure 4B*). Having received a surgical intervention was the sole independent predictor for a decreased overall mortality risk in this multivariate Cox regression analysis (p=0.016).

4. DISCUSSION

Our study has three major findings: First, we were able to show that SSTR-tumour negativity is an independent determinant of mortality in patients with advanced grade 1-2 NETs. This observation upheld in the PS-matched cohort as well as in multivariate analysis. Second, both a lower tumour grade of the primary tumour and surgical treatment were associated with a better outcome in SSTR-negative NET patients. Third, SSA treatment did not improve progression-free or overall survival in SSTR-negative NET patients.

Our results that SSTR tumour negativity is a negative prognostic biomarker are consistent with previous, more limited studies in gastroenteropancreatic and lung NEN (14-16, 22-24). The main advantage of our analysis is the in vivo definition of SSTR-negativity by insufficient uptake in SSTR-imaging instead of negative immunohistochemistry. SST2 status evaluation on immunohistochemistry is inferior compared to SSTR-imaging, as it evaluates a single lesion and potentially misses interlesional heterogeneous SST2 expression (25, 26). Accordingly, there is no additional benefit of immunohistochemistry compared to SSTR-imaging (27). A further limiting factor of the above mentioned studies was their comparison of patients at all stages and tumour grades, which resulted in high data heterogeneity. Also, with the exception of two studies (22, 24), data were derived from small cohorts with fewer than 100 patients. Most importantly, all studies failed to match the SSTR-negative NET patients to a control group, which is critical given the differences in outcome according to tumour grade and stage (16, 28). We aimed at overcoming those limitations by including only well-differentiated (grade 1-2) advanced stage NETs in our analysis. To further increase comparability, we excluded patients who underwent PRRT, since this treatment option significantly affects prognosis in SSTR-positive NENs (9). Also, we chose to not only adjust results for possible interaction of co-factors, but to create a PS-matched cohort of SSTR-negative and -positive NET patients, thereby optimizing the post-weighting balance of covariates between groups (20, 21).

While our analysis confirmed the worse outcome for SSTR-negative NET patients, the main question remains what causes this survival deficit. A more aggressive tumour biology has been proposed to be the underlying reason for the worse outcome of SSTR-negative tumour patients. Several studies have demonstrated a decrease in the maximum standardized uptake values (SUV) on SSTR-PET imaging with increasing Ki-67 index (29-31). However, since SSTR-negativity remained an independent predictor for worse outcome after correcting for grade via PS matching and multivariate analysis, other mechanisms presumably play a role here. SSTR-negativity could represent an overall state of epigenetic gene silencing, which might be associated with aberrant cellular growth. Modifications of the epigenome via epigenetic drugs which decrease methylation and augment histone acetylation of the

SST2 gene promoter region have been shown to stimulate SST2 expression levels and inhibit cell proliferation in several in-vitro and in-vivo studies (32-37). That SST2-expression levels correlate with prognosis is known (38, 39) however the role of epigenetic treatment for NET-patients remains speculative.

Another essential finding in our analysis was the positive impact of having a surgical intervention on overall survival of patients with advanced stage NETs, which is in line with other studies (40-42). There is a potential bias in patient selection as patients with better performance status or more easily resectable tumours are more frequently operated on. Although no information on performance status was available in our cohort, the radical nature of the operation and primary tumour site had no impact on survival outcome.

Evidence on the use of SSAs as an antiproliferative treatment in NEN has increased over the past years (10, 11). Accordingly, many physicians have also employed SSAs as a treatment for SSTR-negative tumours. An earlier study in 54 small-intestinal NENs reported limited efficacy of this treatment in SSTR-negative tumours with a median PFS of 15.6 months (15), which was similar to the PFS observed in our cohort. However, there was no survival benefit from SSA treatment in the SSTR-negative patient group. Ideally, the effect of SSA treatment in SSTR-negative tumour patients should be compared to patients with a watchful waiting strategy. Unfortunately, such a comparison was not possible in our study due to insufficient patient numbers.

One of the limitations of our study is its retrospective design. However, since data were derived from two carefully managed prospective NEN registries, the resulting relatively large cohort from several different expert centres makes the data more comparable. Having included only well-differentiated grade 1 and 2 tumours in our analysis, no statement can be made about the outcome of SSTR-negative grade 3 NETs. Despite PS-matching, there were more lung NETs and a higher rate of lung metastases in SSTR-negative tumour patients. However, since this had no impact in the multivariate analyses, a significant bias can be excluded. Also, among the SSTR-negative patients, pancreatic NET and NET of unknown origin had the highest mortality rate (data not shown).

Another limitation to our study are the different imaging modalities used. Since SSTR scintigraphy has a lower sensitivity compared to SSTR PET/CT it is theoretically possible that patients scored negative in scintigraphy would have been positive in PET/CT (43). However, for most patients SSTR-negativity was confirmed by immunohistochemistry or PET/CT, leaving only 16 patients with the definition solely based on SSTR scintigraphy. Primary outcomes did not change after exclusion of those 16 patients (data not shown).

Because patients were included over a long study period, different treatment strategies influenced treatment decisions, which could be a reason why nowadays eligible SSTR-positive patients in our cohort did not receive PRRT. However, bias in treatment between the two cohorts were minimized by excluding PRRT-treated patients and a balanced use of SSA treatment among the PS-matched groups.

In summary, we were able to confirm the inferior prognosis of SSTR-negative compared to SSTR-positive NET patients. The results of our study emphasize the clinical unmet need for more effective treatment options in patients with advanced stage SSTR-negative NETs.

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SUPPLEMENTARY APPENDIX

Table S1 Characteristics Propensity Score-Matched Patients

PS-matched cohort	SSTR status negative (n=69)	SSTR status positive (n=69)	p value
Sex (female), n (%)	37 (54)	46 (67)	0.12
Age at diagnosis, years (IQR)	63 (57-68)	64 (56-70)	0.38
Primary tumor site, n (%)			0.98
• Midgut	24 (35)	23 (33)	
• Hindgut	1 (1.4)	2 (3)	
• Pancreas	13 (19)	19 (28)	
• Lung	20 (29)	8 (12)	
• Unknown	11 (16)	17 (25)	
Grade, n (%)			0.73
• 1	29 (42)	31 (45)	
• 2	40 (58)	38 (55)	
Hormone secreting, n (%)	20 (29)	23 (33)	0.78
Chromogranin A, ng/l (IQR)	276 (86-1105)	444 (150-1556)	0.22
Site of metastasis, n (%)			
• Lymphnodes	53 (77)	52 (75)	0.49
• Liver	54 (78)	55 (80)	0.82
• Lung	10 (15)	3 (4)	**0.018**
• Bone	18 (26)	11 (16)	0.13
Treatment, n (%)			
• Somatostatin analogues	38 (55)	40 (58)	0.13
• Chemotherapy	21 (30)	5 (7)	**0.001**
• Targeted therapy	20 (29)	0 (0)	**<0.001**
• Liver directed therapy	6 (9)	7 (10)	0.77
• Surgery	40 (58)	42 (61)	0.73
o *curative*	*16 (40)*	*24 (57)*	
o *palliative*	*24 (60)*	*18 (43)*	
Died, n(%)	41 (59)	22 (32)	**0.001**

Table S1 Patient characteristics of PS-matched cohort according to SST status.

Values are shown as frequencies (%) or median (IQR). p-Value refer to differences between the groups.

PS = Propensity score; SST2SSTR = somatostatin receptor; n = number; IQR = interquartile range

Table S2 Cox Regression Analysis Propensity Score-Matched Cohort

PS matched cohort	Univariate Cox Regression Analysis	Multivariate Cox Regression Analysis	Hazard Ratio (95% CI)
SST negativity	**0.012**	**0.005**	**2.58** (1.34, 4.99)
Sex (female)	0.6		
Age > 65 years	**0.030**	0.21	
Primary tumour site			
• Midgut	**0.027**	0.46	
• Hindgut	0.975		
• Pancreas	**0.003**	0.31	
• Lung	0.180		
• Unknown	**0.009**	0.08	
Grade 2 vs 1	**0.007**	**0.015**	**2.32** (1.18, 4.58)
Site of metastasis			
• Lymph nodes	0.08		
• Liver	**0.009**	0.18	
• Lung	0.40		
• Bone	**0.011**	0.063	
Treatment			
• Somatostatin analogues	0.15		
• Chemotherapy	0.07		
• Targeted therapy	0.17		
• Liver-directed therapy	0.20		
• Surgery	**<0.001**	**0.004**	**0.36** (0.18-0.72)
- curative vs palliative	*0.30*		
- primary tumour site	*0.42*		

Table S2: Cox Regression analysis of the propensity score matched cohort.
Only variables reaching significance in the univariate analysis were included into the multivariate analysis.
SST = somatostatin receptor; 95% CI = 95% confidence interval; vs = versus

Table S3 Characteristics Somatostatin analoga-treated patients

All SSA-treated patients	SST-status negative (n=35)	SST-status positive (n=152)	p Value
Sex (female), n (%)	13 (37)	68 (45)	0.4
Age at diagnosis, years (IQR)	63 (58-66)	67 (62-72)	0.1
Primary tumor site, n (%)			**<0.001**
• Gastro-duodenal	0	3 (2)	
• Midgut	14 (40	110 (72)	
• Hindgut	0	1 (1)	
• Pancreas	4 (11)	16 (11)	
• Lung	9 (26)	6 (4)	
• Unknown	8 (23)	16 (11)	
Grade, n (%)			0.2
• 1	16 (46)	88 (58)	
• 2	19 (54)	64 (42)	
Hormone secreting, n (%)	15 (43)	58 (38)	0.4
Chromogranin A, ng/l (IQR)	464 (83-5107)	444 (236-2077)	0.8
Site of metastasis, n (%)			
• Lymphnodes	28 (80)	134 (88)	0.9
• Liver	32 (91)	135 (89)	0.5
• Lung	3 (9)	12 (8)	0.6
• Bone	10 (29)	19 (13)	**0.012**
Treatment, n (%)			
• Somatostatin analogues	35 (100)	152 (100)	1
• Chemotherapy	7 (20)	5 (3)	**<0.001**
• Targeted therapy	10 (29)	3 (2)	**<0.001**
• Liver directed therapy	1 (3)	12 (8)	0.3
• Surgery	20 (57)	96 (63)	0.5
Time till progression, months (SD)	24 (27)	39 (35)	**0.028**
Died, n(%)	22 (63)	59 (39)	**0.01**

Table S3 Patient characteristics of SSA-treated patients according to SST status. Values are shown as frequencies (%) or median (IQR). p-Value refers to differences between the groups.
SSA = Somatostatin analoga; SST = somatostatin receptor; n = number; IQR = interquartile range

Table S4 Characteristics PS-matched Somatostatin analoga-treated patients

PS-matched SSA-treated patients	SST-status negative (n=35)	SST-status positive (n=35)	p Value
Sex (female), n (%)	13 (37)	13 (37)	1.0
Age at diagnosis, years (IQR)	63 (58-66)	63 (55-69)	0.62
Primary tumor site, n (%)			0.56
• Midgut	14 (40	17 (49)	
• Pancreas	4 (11)	4 (11)	
• Lung	9 (26)	6 (17)	
• Unknown	8 (23)	8 (23)	
Grade, n (%)			1.0
• 1	16 (46)	16 (46)	
• 2	19 (54)	19 (54)	
Hormone secreting, n (%)	15 (43)	11 (31)	0.38
Chromogranin A, ng/l (IQR)	464 (83-5107)	357 (116-756)	0.77
Site of metastasis, n (%)			
• Lymphnodes	28 (80)	29 (83)	0.60
• Liver	32 (91)	31 (87)	0.98
• Lung	3 (9)	3 (9)	0.89
• Bone	10 (29)	4 (11)	0.11
Treatment, n (%)			
• Somatostatin analogues	35 (100)	35 (200)	1
• Chemotherapy	7 (20)	3 (9)	0.18
• Targeted therapy	10 (29)	0 (0)	**0.001**
• Liver directed therapy	1 (3)	3 (9)	0.31
• Surgery	20 (57)	4 (11)	0.81
Time till progression, months (SD)	24 (27)	39 (31)	**0.035**
Died, n(%)	22 (63)	14 (40)	0.06

Table S4 Patient characteristics of PS-matched SSA-treated patients according to SST status.
Values are shown as frequencies (%) or median (IQR). p-Value refers to differences between the groups.
PS = Propensity score; SSA = Somatostatin analoga; SST = somatostatin receptor;
n = number; IQR = interquartile range

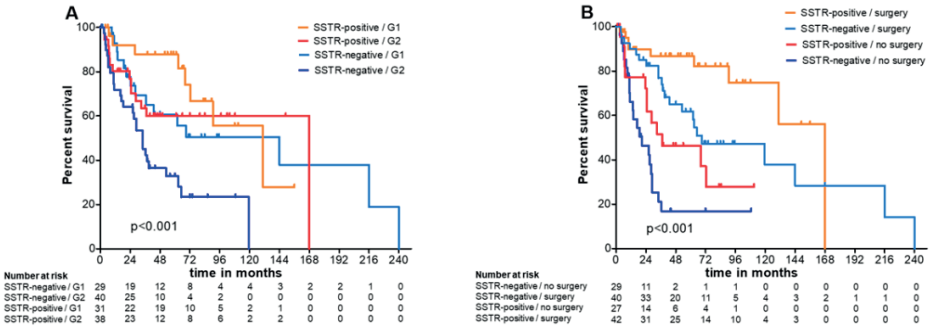

Figure S1 Overall Survival Propensity Score-Matched Cohort according to covariates

Kaplan Meier analysis showing overall survival of the PS-matched cohort, according to

A) SST-status and tumour grade 1 (n=60) or 2 (n=78) and

B) SST-status and having received a surgical intervention (n=82) or not (n=56).

p-Value indicates difference in survival between the groups (Log Rank Test).

PS = propensity score; SST2SSTR = somatostatin receptor subtype 2; G1 = grade 1;

G2 = grade 2;

PART 2

Diagnosis and treatment of neuroendocrine disorders

6 Artifical syndrome of inappropriate antidiuresis model as potential use for diagnostic and therapeutic strategies

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Hormone and Metabolic Research 2017

ABSTRACT

Hyponatremia is the most frequent electrolyte disorder with the syndrome of inappropriate antidiuresis (SIAD) being its predominant cause. Physiological studies in patients with SIAD are difficult to interpret due to usually several comorbidities and polymedication. Therefore, a SIAD model in healthy volunteers would be very helpful to allow insight in this complex disease and to test new therapeutic approaches. The aim of the study was to create a SIAD model with evaluation of subsequent physiological changes. The prospective interventional study on 14 healthy volunteers was carried out at the University Hospital Basel. The intervention was done by induction of hypotonic hyponatremia through hydration and administration of desmopressin. Clinical and laboratory parameters in a SIADH model were the main outcome of the measure. 14 participants (64% males), BMI 23.1 kg/m2 (±2.4), aged 28.6 years (±9), completed the study. Through the intervention, serum sodium level decreased from 140 mmol/l (±1.3) to 132 mmol/l (±2.0) and serum osmolality from 286 mmol/kg (±4.7) to 267 mmol/kg (±3.5). Simultaneously urine osmolality increased to 879 mmol/kg (±97.7) and urine sodium to 213 mmol/l (±51.5) verifying the artificial SIAD model. A significant decrease of copeptin (5 pmol/l (±1.9) to 2.6 pmol/l (±0.5), p 0.002), aldosterone (314.7 pmol/l (±154.1) to 86.7 pmol/l (±23.6), p 0.019), and renin (21.2 ng/l (±26.7) to 3.6 ng/l (3.2), p 0.035) were noted, while NT-proBNP and MR-proANP significantly increased (31.7 ng/l (±18.6) to 50.5 ng/l (±33.0), p 0.001; 48.4 pmol/l (±16.8) to 56.8 pmol/l (±9.0), p 0.003). In conclusion, we were able to induce an artificial SIAD in healthy volunteers and study the changes of various hormonal biomarkers involved. This SIAD model could be helpful in evaluating diagnostic and therapeutic approaches.

1. INTRODUCTION

Hyponatremia is the most frequent electrolyte and fluid disturbance with a prevalence of 15-30% in hospitalized patients [1,2]. The syndrome of inappropriate antidiuresis (SIAD) is the predominant cause of hyponatremia and is characterized by an imbalanced arginine-vasopressin (AVP) secretion leading to water retention and natriuresis [3-5]. Diagnostic criteria for SIAD are defined as hyponatremia with low serum osmolality (<275mmol/kg), inadequate elevated urinary osmolality (>100mmol/kg) and elevated urine sodium (>30mmol/l) [6,7].

Multiple regulatory systems are thought to be involved in the complex pathophysiology of SIAD. Laboratory parameters and biomarkers are helpful to characterize this syndrome in order to draw diagnostic and therapeutic decisions. Copeptin (C-terminal segment of AVP prescursor peptide) – as a reliable surrogate marker of AVP release [8,9] - has become important in the evaluation of AVP-dependent fluid disorders [10]. Mid-regional pro-atrial natriuretic peptide (MR-proANP) derives from the precursor hormone atrial natriuretic peptide (ANP) and is a stable and easily measured marker of hypervolemia [11]. The N-terminal prohormone of brain natriuretic peptide (NT-proBNP) is a widely used biomarker for cardiovascular pressure load [12] and the renin–angiotensin system (RAS) is well known for the regulation of blood pressure and volume changes [13].

So far, only few studies focused on biomarkers in SIAD and most of them evaluated only a single hormone or regulatory system (e.g. copeptin, renine/aldosterone or BNP) [14-17]. Pathophysiological studies in patients with SIAD are difficult to interpret due to confounders such as the multiple comorbidities and polymedication [18,19] in these patients. Therefore, an experimental model in healthy volunteers to study the pathomechanisms as well as therapeutic approaches would be helpful.

Accordingly we aimed to induce an artificial SIAD in healthy volunteers to analyse the dynamics of the hormonal biomarkers involved as well as to create a validated SIAD model for later evaluation of therapeutic approaches.

2. MATERIAL AND METHODS

This study was designed with the aim of creating a valid artificial SIAD model for later evaluation of diagnostic and therapeutic approaches.

2.1 Subjects:

Written informed consent was obtained from 15 healthy volunteers. They had no history of any diseases; renal and hepatic impairment as well as thyroid dysfunction and adrenal insufficiency were excluded through laboratory measurements at baseline. Besides oral anticonception in all females, participants were on no medication during the study period. The study protocol was reviewed and approved by the local ethical committee as well as the national agency for the authorisation and supervision of therapeutic products (swissmedics).

2.2 Procedures:

The procedure and different timepoints are explained schematically in *Figure 1*:

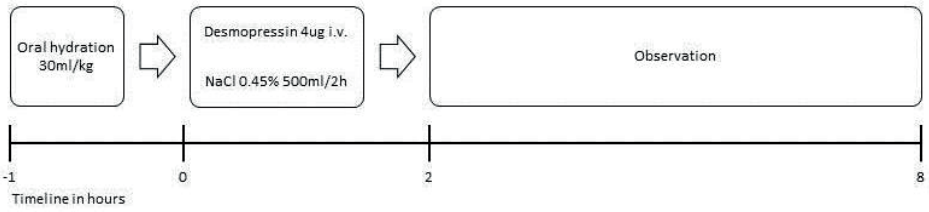

Figure 1: Study procedure

Each subject was obliged to remind fasting after midnight and was admitted to our clinical trial unit between 6.30 to 7a.m. No food was allowed until the end of the observation period. Drinking was only permitted during the oral hydration phase.

At baseline (timepoint -1) possible symptoms of hyponatremia (thirst, vertigo, headache, nausea and malaise), clinical parameters including body weight, blood pressure and heart rate as well as serum and urinary parameters were evaluated and thereafter regularly throughout the study day.

After voiding the bladder, participants were asked to drink 30ml water per kg body weight in one hour replacing fluid loss 1:1 if urinary excretion exceeded 100ml.

After one hour (timepoint 0) desmopressin 4ug i.v. was injected, followed by an infusion of NaCl 0.45% 500ml over 2 hours (end of infusion = timepoint 2).

The primary observation phase was between timepoint 2 and 8. At the end of the observation period, participants received a small meal (sandwich), monitoring was maintained for 2 more hours. Participants were discharged if they were in a clinically good condition and the serum sodium level was above 130mmol/l.

2.3 Laboratory measurements:

Concentrations of sodium, creatinine, urea, uric acid and osmolality were measured at different timepoints during the study in serum and urine. The fractional excretion of urea and uric acid was estimated by the formula: fractional excretion (Ux x Pcreatinine / Ucreatinine x Px), where U=urinary, P=plasma, x=substance to be calculated.

Blood samples for serum copeptin, MR-proANP, NT-proBNP, renin and aldosterone determination were drawn in a supine position, immediately centrifuged and stored at -70°C for batch analysis. Serum copeptin and MR-proANP levels were measured with a commercial sandwich immunoluminometric assay (B.R.A.H.M.S CT-proAVP LIA, Thermo Scientific Biomarkers, Hennigsdorf, Germany) [9]. The copeptin assay had a 0.4pmol/l lower detection limit and <5% (<10%) intra-assay (inter-assay) coefficients of variation for concentrations >2.0pmol/l (>2.5pmol/l)). The MR-proANP assay had a lower detection limit of 6.0pmol/l [20,21].

Aldosterone and renin were measured with a standard chemiluminescent immunoassay (CLIA) with a reference range of 32-654pmol/l and 1.7 - 23.9ng/L respectively in supine position. NT-proBNP was measured with a standard electro-chemiluminescent immunoassay (ECLIA) with a reference range of <127ng/L for males and <177 ng/L for females.

2.4 Statistical analyses

The full analysis set included a total of 14 subjects who completed the whole procedure. One subject withdrew consent during the oral water hydration phase and therefore had to be excluded from further analysis.

All analysis was performed using the non-parametric Wilcoxon signed rank test using stata 12.1, SPSS (SPSS 21, Chicago, IL, USA). P-values < 0.05 were considered to indicate statistical significance.

3. RESULTS

Nine (64%) of the 14 participants were males, the mean age 28.6 years (±9) and body mass index 23.1kg/m2 (±2.4). All parameters measured during the study are presented in *Table 1*.

Table 1: Results are shown according to the different evaluation timepoints:-1 = baseline; 0 = after oral hydration, before desmopressin application; 2 = maximal water load; 8 = end of observation period as mean values with (standard deviation).

	Timepoint				
	-1	**0**	**2**	**8**	**p**
Clinical Evaluations					
blood pressure (mmHg)	125 / 78 (14.1 / 8.3)	129 / 85 (14.4 / 8.9)	124 / 76 (13.6 / 8.2)	126 / 75 (16.3 / 7.9)	0.807
pulse (bpm)	64 (10.4)	57 (9.8)	59 (9.5)	60 (8.5)	0.1
weight (kg)	75.7 (13.1)	77.6 (13.4)	77.9 (13.4)	77.3 (13.2)	**0.001**
Fluid balance					
total volume input (ml)		2359 (404)	2859 (404)	2859 (404)	
urine output (ml) at timepoint		229 (142)	140 (89)	71 (35)	**0.001**
total urine output (ml)		229 (142)	369 (215)	596 (275)	
fluid balance (ml)		2130 (366)	2490 (376)	2263 (399)	
SIAD parameters					
serum sodium (mmol/l)	140 (1.3)	137 (2.1)	133 (2.3)	132 (2.0)	**0.001**
serum osmolality (mmol/kg)	286 (4.7)	281 (6.7)	269 (3.2)	267 (3.5)	**0.001**
urine sodium (mmol/)	107 (38.8)	50 (32.3))	83 (39.5)	213 (51.5)	**0.001**
urine osmolality (mmol/kg)	586 (240.0)	260 (178.0)	468 (151.0)	879 (97.7)	**0.001**
FE Urea (%)	44 (8.3)	50 (6.8)	31 (11.0)	31 (9.0)	**0.002**
FE Uric acid (%)	5.2 (2.2)	6.2 (1.9)	7.6 (2.5)	6.1 (2.3)	**0.026**
Biomarkers					
Copeptin (pmol/l)	5 (1.9)		3.1 (0.7)	2.6 (0.5)	**0.002**
NT-proBNP (ng/l)	31.7 (18.6)		34.4 (21.5)	50.5 (33.0)	**0.001**
MR-proANP (pmol/l)	48.4 (16.8)		59.3 (15.2)	56.8 (9.0)	**0.003**
Aldosterone (pmol/l)	314.7 (154.1)		154.1 (59.0)	86.7 (23.6)	**0.019**
Renin (ng/l)	21.2 (26.7)		8.4 (7.4)	3.6 (3.2)	**0.035**
Symptoms					
thirst (VAS)	12 (4.1)	0 (0)	0 (0)	8 (2.4)	
headache (VAS)	0 (0)	0 (0)	1 (1)	4 (3.3)	
nausea (VAS)	0 (0)	6 (2.3)	2 (1)	1 (3)	
vertigo (VAS)	0 (0)	0 (0)	1 (1)	1 (1)	
general malaise (VAS)	0 (0)	2 (2.5)	1 (1)	1 (1)	

Symptoms are shown as number of participants being symptomatic and average degree of severity according to visual analogue scale (VAS: 0=no symptoms, 10=severe symptoms).
p-value: Wilcoxon signed rank test between timepoint -1 and 8; FE = fractional excretion

There was no change in vital signs throughout the observation phase. Despite the previous water load (mean oral water intake 2359ml (±404); 500ml infusion) urinary volume excretion was decreased to a total of only 596ml (±275), resulting in an average increase in body weight of 1.6kg (±0.5).

Serum sodium and osmolality levels were in the normal range (140mmol/l (±1.3); 286mmol/kg (±4.7)) at baseline. Through hydration and desmopressin application they significantly decreased to 132mmol/l (±2.0) and 267mmol/kg (±3.5) respectively. Urinary sodium and osmolality levels initially decreased after the oral water load (50mmol/l (±32.3); 260mmol/kg (±178.0)), but then increased to a maximum of 213mmol/l (51.5) and 879mmol/kg (±97.7) (*Figure 2*). Patients stayed clinically euvolemic during the whole observation period.

Fractional urea excretion (FEurea) was maximally increased after the oral water load and then decreased below 35%. Fractional uric acid (FEUA) was always below 12% but increased to a maximum of 7.6% (±2.5) at timepoint 2.

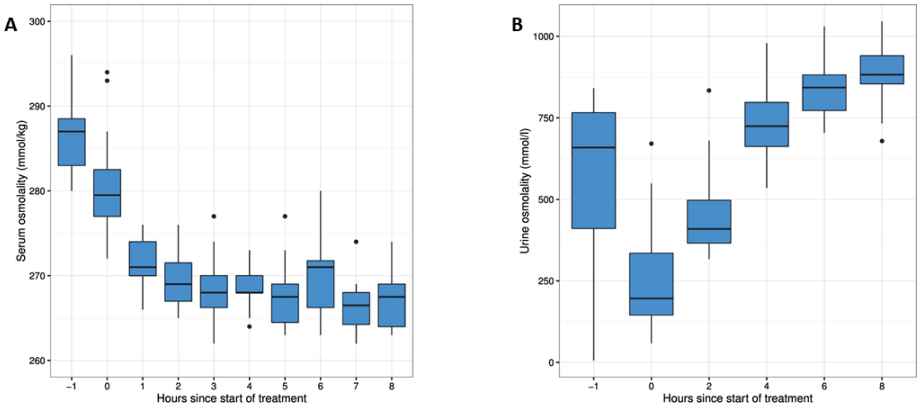

Figure 2: Trajectory of A) serum osmolality and B) urinary osmolality for all subjects pooled at the different timepoints. Serum sodium level mirrored the course of serum osmolality, as did urine sodium level the course of urine osmolality.

There was a significant decrease from baseline to the end of the observation period of copeptin (5pmol/l (±1.9) to 2.6pmol/l (±0.5), p 0.002), aldosterone (314.7pmol/l (±154.1) to 86.7pmol/l (±23.6), p 0.019) and renin (21.2ng/l (±26.7) to 3.6ng/l (±3.2), p 0.035). NT-proBNP and MR-proANP significantly increased (31.7ng/l (±18.6) to 50.5ng/l (±33), p 0.001; 49.2pmol/l (±16.7) to 56.8pmol/l (±8.9), p 0.003), with NT-proBNP showing a delayed reaction to the volume load (*Figure 3*).

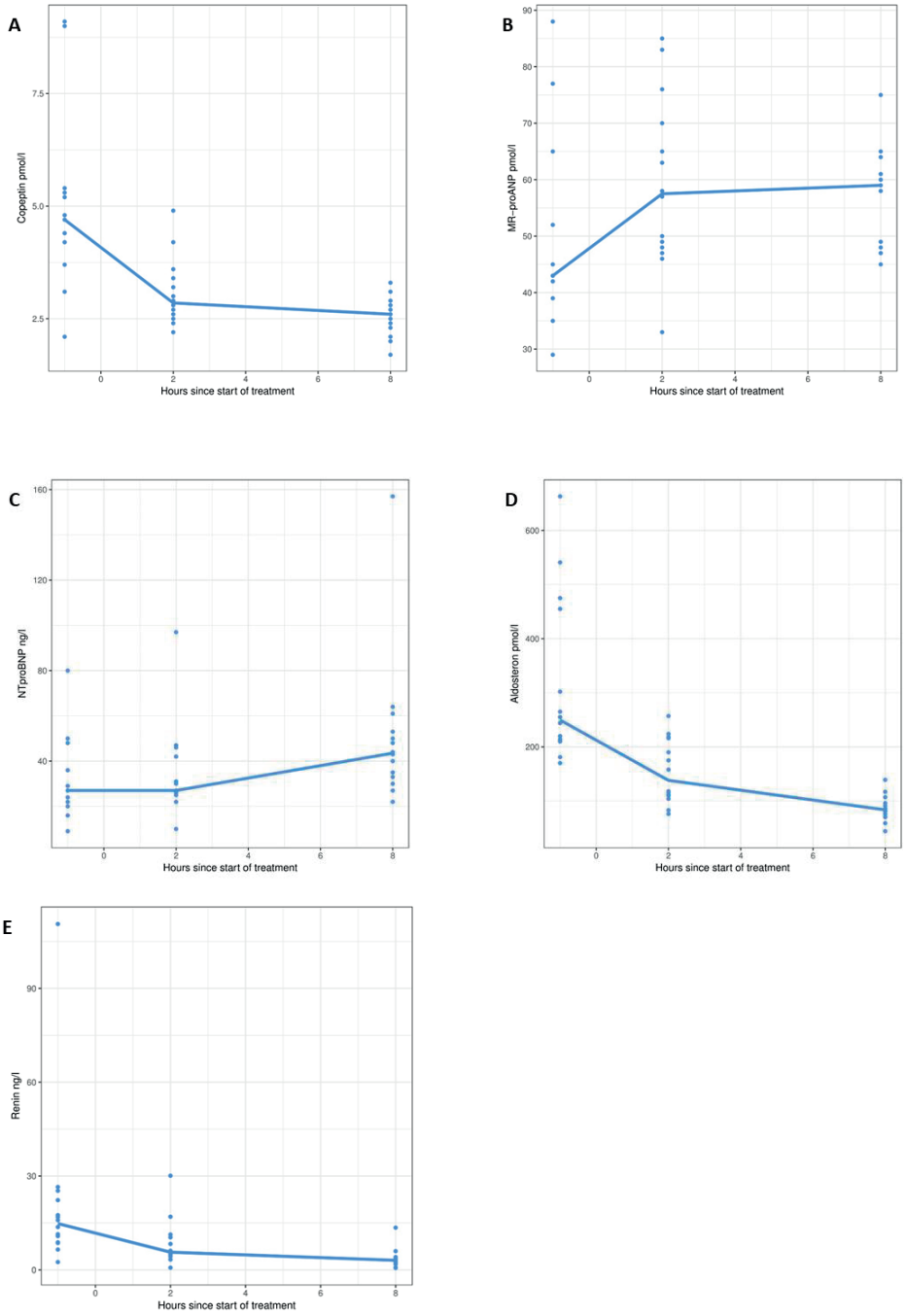

Figure 3 shows plasma levels of different markers of fluid homeostasis:
A) copeptin, B) MR-proANP, C) NT-proBNP, D) aldosterone and E) renin at different timepoints of the study. Points represent individual measurements, the line represents the medians.

Throughout the observation phase, the majority of the participants had none or only mild symptoms with headache and nausea being the most common. Pain medication was given as a symptomatic therapy in addition to total fluid restriction – which was done according to study protocol – in two participants and ameliorated symptoms immediately (*Table 1*).

4. DISCUSSION

Our study has two main findings. First, with overhydration and application of desmopressin we successfully created an artificial SIAD-model, characterized by hypotonic hyponatremia, inadequately elevated urine osmolality and natriuresis [6,7,22]. The SIAD criteria were reached three hours after start of the intervention and maintained for the next six hours. Compatible with the intervention was the low urinary volume excretion despite the water load, leading to an increase in body weight. The induced hyponatremia was only mild with no severe adverse events or side effects. According to those results, we believe that our SIAD-model could be helpful for further diagnostic and therapeutic evaluations. As the diagnostic workup of hyponatremia is difficult, until today it has not been possible to find a biomarker which clearly differentiates between the different aetiologies [11,23,24]. One of the problems in search of a suitable biomarker is that hyponatremic patients usually suffer from several comorbidities and are under multiple medications – i.e. diuretics – influencing the biomarkers involved. As our SIAD model excludes these confounders it could be helpful in validating potential new biomarkers. More importantly, it could be used to investigate therapeutic approaches in SIAD such as urea or V2 receptor antagonists (vaptans) and enable a head-to-head comparison between the different treatment options. Also it could be helpful in the development of urgently needed new treatment options, considering the limited tolerance and efficacy of fluid restriction as well as the high costs and the risk of overcorrection of vaptans.

In contrast to the SIAD parameters mentioned above, fractional urea and uric acid excretion did not reach the cut-offs which have been described as diagnostic parameters in the evaluation of SIAD [23,24]. According to Decaux et al [25] especially chronic hyponatraemia in SIAD leads to a reduced reabsorption of uric acid. Since we induced an acute onset of SIAD and only mild hyponatremia, this could be a possible explanation for the low fractional excretions observed in our study.

Our second main finding is the first description of the dynamics of various hormonal biomarkers involved in the pathophysiology of SIAD. As copeptin is a surrogate marker of AVP release, it's secretion is mainly stimulated by increased serum osmolality and suppressed by decreased osmolality [10]. Therefore, in prolonged hypotonic hyponatremia - as

in our SIAD model - suppressed copeptin levels are expected. Accordingly, we observed a significant decrease of copeptin, but not completely suppressed levels. Similar results where shown in a previous study looking at copeptin levels in hypotonic hypervolemia [26]. As the main antidiuretic effect of AVP as well as desmopressin goes over the stimulation of the AVP receptor 2 [10] leading to an increase in water retention and decrease in plasma osmolality, an interaction on copeptin measurements due to the different vasopressin receptors activation seems unlikely [9,26]. A possible explanation for the lack of full suppression could be the function of AVP in endocrine stress response [10,27]. Although the study subjects did not experience major stress, the fact that the participants had to endure acute hyponatremia and were without food during the whole study could be a possible stress stimulus for AVP release.

NT-proBNP and MR-proANP derive from the precursor hormones BNP and ANP and are two well described biomarkers reflecting volume status. Released in response to distension of cardiomyocytes, their activation leads to increased diuresis and natriuresis [28,29]. In our study NT-proBNP showed a more delayed increase as compared to MR-proANP following the initial volume load. The elevation of MR-proANP persisted throughout the observation period, mirroring the increasing natriuresis. Accordingly, in a population of patients with profound hyponatremia of different etiologies, MR-proANP had shown a better diagnostic performance in identifying patients with SIADH as compared to NT-proBNP [11]. In that study the specificity of MR-proANP-levels below 100.9pmol/l to predict SIADH was 91%. MR-ANP levels were below this cutoff in all our study subjects.

Despite the induction of hyponatremia, we observed a decrease of aldosterone and renin levels. Our findings are confirmed by an older physiological study, which also noted a decrease in aldosterone secretion following hydration and vasopressin administration [30]. A similar study - in severely hyponatremic SIAD-patients and healthy volunteers after water/vasopressin administration - however showed a dissociation with suppressed renin and normal aldosterone levels [16]. Overall, the main regulation of aldosterone and renin seems to be dependent on extracellular volume status, accordingly our results underline the current understanding that SIAD is a result of excess water and not primarily due to sodium deficiency.

The following limitations of our SIAD-model have to be mentioned. First and most importantly an artificial model can never fully mimic the complex mechanisms involved in disorders of fluid and sodium homeostasis. Furthermore, the participants in this study were young, healthy and on no medication, making it difficult to directly transfer the results to usually comorbid elderly patients with SIAD. Also desmopressin is not equivalent to AVP which interacts with different vasopressin receptors [10]. Nonetheless, as the main antidiuretic effect

of AVP as well as desmopressin goes over stimulation of the AVP2 receptor, we believe this difference in the vasopressin receptor stimulation is negligible for our SIAD model.

It is not possible to differentiate the various types (A to D) of SIAD [31] with our model, as for this osmotic stimulation would be needed [32]. However, as the application of desmopressin simulates constant AVP-secretion independently to osmotic threshold, our SIAD model is most comparable to SIAD type A.

An influence of the desmopressin application on the results cannot be excluded, but according to the study by Szinnai et al [26] as well as our own experience no interaction of the application of desmopressin and copeptin measurements have been noted. As the in vivo half-life of copeptin is around twice as long compared to AVP which is around 20-30minutes [33] a carryover effect of the pre-study values on the values 2 hours after induction of artificial SIAD cannot be excluded. However, an influence on the results at the end of the observation period (9 hours after the initial measurement) is unlikely.

In awareness of the mentioned limitations, we believe that our SIAD model will be helpful to further understand this complex disease. Also, due to its standardised and safe protocol, our model could be used to evaluate new diagnostic and therapeutic options for SIAD.

In conclusion, we were able to induce an artificial SIAD in healthy volunteers and study the changes of various hormonal biomarkers involved. This SIAD model could be helpful in evaluating diagnostic and therapeutic approaches.

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7

Empagliflozin increases short-term urinary volume output in artificially induced syndrome of inappropriate antidiuresis

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ABSTRACT

Objective: Syndrome of inappropriate antidiuresis (SIAD) is the predominant cause of hyponatremia, but treatment options are unsatisfying. SGLT2 inhibitors increase urinary glucose excretion with concomitant osmotic diuresis. We therefore hypothesized SGLT2-inhibitors as a novel treatment for SIAD.

Design: Double-blind placebo-controlled randomised crossover study in 14 healthy volunteers.

Methods: We induced an artificial SIAD model by administration of desmopressin and overhydration. Afterwards, empagliflozin 25 mg or placebo was given in random order. The main outcomes were total urinary excretion, glucosuria, and the area under the curve (AUC) of serum sodium concentration. Outcome measures were obtained 2-8 hours after administration of study drug.

Results: 14 participants (64% males), BMI 23 kg/m2 (±2.4), aged 28.6 years (±9), completed the study. Empagliflozin led to significantly increased total urinary excretion (579.3 ml (±194.8) versus 367.3 ml (±158.8); treatment effect 158 ml (CI 48.29, 267.74), $p = 0.017$) due to glucosuria (74.18 mmol (±22.3) versus 0.12 mmol (±0.04); treatment effect (log scale) 2.85 (CI 2.75, 2.96), $p < 0.001$). There was no difference in the AUC of serum sodium concentration (treatment effect 0.2 (CI -7.38, 6.98), $p = 0.96$).

Conclusion: In our SIAD model, empagliflozin increased urinary excretion due to osmotic diuresis. Due to the short treatment duration, serum sodium levels remained unchanged. Real-live studies are needed to further examine empagliflozin as a new treatment for SIAD.

1. INTRODUCTION

The syndrome of inappropriate antidiuresis (SIAD) is the predominant cause of hyponatremia and is characterized by an imbalanced secretion of the antidiuretic hormone arginine vasopressin (AVP) [1, 2, 3]. The impaired AVP regulation leads to a reduction of free water excretion with following hypotonic hyponatremia [4, 5]. Therapeutic options, aside from treating the underlying disease, depend upon the onset of hyponatremia and severity of the symptoms and include primarily fluid restriction or hypertonic saline infusion [4, 6]. Alternative treatment options with loop diuretics, administration of oral urea or vasopressin receptor antagonists (vaptans) are discussed controversially in the literature [4, 6, 7]. Despite those options, there are a considerable number of patients who do not sufficiently respond to treatment [7], making additional therapy necessary.

Empagliflozin is a sodium glucose co-transporter 2 (SGLT2)-inhibitor, which has become a valuable treatment option for type 2 diabetes. The SGLT2 is expressed in the proximal tubule and reabsorbs approximately 90 percent of the filtered glucose [8, 9]. The inhibition of SGLT2 results in pronounced glucosuria with subsequent enhanced water excretion by osmotic diuresis [10]. This mechanism is of major interest in view of new therapeutic options in case of impaired water excretion as in patients with SIAD.

As patients with SIAD are usually older with several comorbidities and multiple medications [11, 12], studies evaluating new treatment options are difficult to interpret. We therefore created an artificial SIAD model in healthy volunteers via administration of desmopressin i.v. and overhydration. We hereby aimed to study the effects of the SGLT2-inhibitor empagliflozin in healthy volunteers in artificially induced SIAD with focus on urinary volume excretion, glucosuria and change of serum sodium level.

2. SUBJECTS AND METHODS

2.1 Study design and subjects:

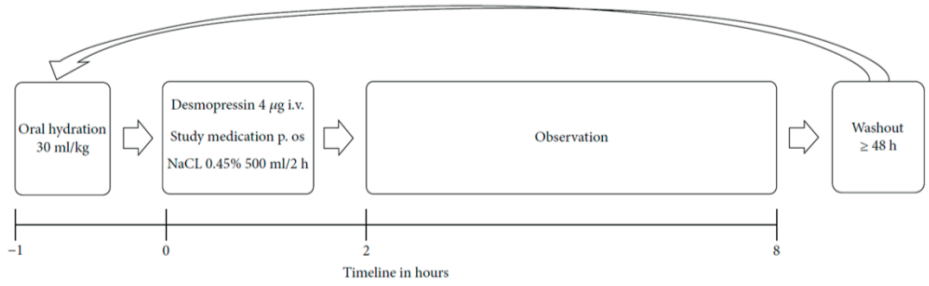
We performed a prospective double-blind placebo controlled randomised crossover study at the University Hospital Basel, Switzerland from March to June 2016. The local ethics committee (EKNZ 2015-00024) as well as the national agency for the authorisation and supervision of therapeutic products (swissmedics 2016 DR 2031) approved the study protocol and study medication. The trial was registered at Clinical Trials.gov (Number: NCT02729766).

Written informed consent was obtained from 15 healthy volunteers. They had no history of any chronic diseases; renal and hepatic impairment as well as thyroid dysfunction and adrenal

insufficiency were excluded through laboratory measurements. Besides oral anticonception in all females, participants were on no medication during the study period.

2.2 Procedures:

The procedure and different timepoints are explained schematically in *Figure 1*:



Each subject underwent two study days receiving empagliflozin or placebo in randomized order with a washout period of at least 48 hours in-between. They reminded fasting after midnight and were admitted to our clinical trial unit between 6.30 to 7a.m. No food was allowed until the end of the observation period. Drinking was only permitted during the oral hydration phase.

On arrival (timepoint -1) clinical symptoms attributed to hyponatremia (vertigo, headache, thirst, nausea and malaise; visual analogue scale (VAS) 0-10), clinical parameters including body weight, blood pressure and heart rate as well as blood and urinary parameters were evaluated and thereafter regularly throughout the study day.

After voiding the bladder, participants were asked to drink 30ml water per kg body weight in one hour (corresponding to 2200ml in average), additionally replacing fluid loss 1:1 if urinary excretion exceeded 100ml within one hour.

After one hour (timepoint 0) desmopressin 4ug i.v. was injected, immediately followed by the application of the study drug (empagliflozin 25mg or placebo) per os. Because empagliflozin and placebo tablets were not identical in colour and shape, participants were blindfolded and the administration was carried out by an independent doctor from the endocrine department who did not belong to the study team. The study team was blinded concerning treatment allocation of the participants. Afterwards an infusion of NaCl 0.45% 500ml over 2hours was started (end of infusion = timepoint 2).

The primary observation phase was between timepoint 2 and 8 (= total 6 hours). At the end of the observation period, participants received a small meal (sandwich), monitoring was maintained for 2 additional hours. Participants were discharged if they were in a clinically good condition and the serum sodium level was above 130mmol/l.

2.3 Laboratory measurements

Concentrations of sodium, glucose, creatinine, urea, uric acid and osmolality were measured at different timepoints in serum and urine. The fractional excretion of urea and uric acid was estimated by the formula: fractional excretion (Uy x Pcreatinine / Ucreatinine x Py), where U=urinary, P=plasma, y=substance to be calculated.

Blood samples for serum copeptin, MR-proANP, NT-proBNP, renin and aldosterone determination were drawn in a supine position, immediately centrifuged and stored at -70°C for batch analysis. Serum copeptin and MR-proANP levels were measured with a commercial sandwich immunoluminometric assay (B.R.A.H.M.S CT-proAVP LIA, Thermo Scientific Biomarkers, Hennigsdorf, Germany) [13, 14]. The copeptin assay had a 0.4pmol/l lower detection limit and <5% (<10%) intra-assay (inter-assay) coefficients of variation for concentrations >2.0pmol/l (>2.5pmol/l). The MR-proANP assay had a lower detection limit of 6.0pmol/l. Aldosterone and renin were measured with a standard chemiluminescent immunoassay (CLIA) with a reference range of 32-654pmol/l and 1.7 - 23.9ng/L respectively in supine position. NT-proBNP was measured with a standard electro-chemiluminescence immunoassay (ECLIA) with a reference range of <127ng/L for males and <177 ng/L for females.

2.4 Statistical analyses

The final analysis set included a total of 14 subjects who completed the whole procedure. One subject withdrew consent during the oral water hydration phase and therefore had to be excluded from further analysis.

The primary endpoint was the area under the curve (AUC, calculated by the trapezoid rule) of the serum sodium concentration between 2-8hours after treatment; glucosuria and urinary volume excretion were further efficacy outcomes of primary interest. All other measurements were defined as secondary outcomes.

Possible symptoms of hyponatremia were averaged over the observation period and compared between empagliflozin and placebo using a paired Wilcoxon's signed rank test. This test was also used for the analysis of secondary outcomes at specific time points between empagliflozin and placebo.

The final outcomes evaluating the observation period between timepoint 2 and 8, were analysed using linear mixed effects models. The models included the corresponding measurement (timepoint 0 for body weight, urinary volume excretion, AUC serum sodium concentration, serum-/urinary osmolality, serum glucose, natriuresis, glucosuria, FEurea, FEuricacid; timepoint -1 for copeptin, MR-proANP, NT-proBNP, aldosterone and renin) as covariate, treatment, treatment-sequence (i.e. empagliflozin-placebo vs. placebo-empagliflozin) and their interaction as predictors (fixed effects), and subject as a random effect. Restricted maximum likelihood (REML) was used. Effect size estimates, 95% confidence intervals based on normal approximation and p-values based on Satterthwaite's approximation are reported. Further, the least-squares means (i.e. the covariate-adjusted model predictions) for each treatment arm are given with 95% confidence intervals. Total glucosuria was log10-transformed in order to meet the assumptions of normally distributed errors. Patient characteristics are summarised as frequencies and percentages or as mean

+- one standard deviation.

Analyses were performed using the statistic program R, version 3.3.1 [15]. All tests were two-sided with p-values <0.05 considered to indicate statistical significance.

3. RESULTS

Main results are shown in *Table 1, 2* and *Figure 2*.

14 participants (9 male (64%)) aged 28.6 years (±9) with a mean body mass index of 23.1kg/m2 (±2.4) completed the study. Laboratory values on arrival were balanced on both study days; in particular there was no difference in serum sodium level (empagliflozin 140mmol/l (±1.5) vs placebo 140mmol/l (±1.3), p=0.9). Through the oral water load, serum and urinary sodium and osmolality decreased at timepoint 0; again with no difference between the two groups. Urinary volume excretion also remained similar (empagliflozin 202ml (±98) vs placebo 229ml (±142), p=0.85).

Table 1: Clinical and laboratory variables, shown as mean (standard deviation) according to the different timepoints.

	Timepoints							
	-1		0		2		8	
	empa	placebo	empa	placebo	empa	placebo	empa	placebo
Clinical Evaluations								
blood pressure (mmHg)	122/76 (13/9)	125/78 (14/8)	125/82(17/8)	129/85 (14/9)	124/75 (13/7)	124/76 (14/8)	127/71 (16/9)	126/75 (16/8)
pulse (bpm)	62 (10)	64 (10)	58 (9)	57 (10)	58 (8)	59 (10)	61 (8)	60 (9)
weight (kg)	75.6 (14)	75.7 (13.1)	77.5 (14.1)	77.6 (13.4)	77.6 (14.1)	77.9 (13.4)	77 (13.9)	77.3 (13.2)
urinary volume excretion (ml)			202 (98)	229 (142)	165 (69)	140 (88.5)	579 (195)*	367 (159)*
Laboratory values								
Serum								
sodium (mmol/l)	140 (1.5)	140 (1.3)	136 (2.1)	137 (2.1)	133 (1.6)	133 (2.3)	132 (1.5)	132 (2)
osmolality (mmol/kg)	286 (2.9)	286 (4.7)	278 (4)	281 (6.7)	269 (3.3)	269 (4)	270 (6.2)	267 (3.5)
glucose (mmol/l)	4.8 (0.4)	5.1 (0.5)	4.7 (0.3)	4.7 (0.4)	4.5 (0.3)	4.8 (0.2)	4.1 (0.5)	4.5 (0.3)
Urine								
sodium (mmol/l)	108 (38)	106 (41)	51 (30)	50 (36)	80 (39)	86 (41)	188 (27)	237 (59)
total natriuresis (mmol)					14.1 (9.7)	12.9 (12.4)	83.3 (42)	64.4 (41)
osmolality (mmol/kg)	678 (249)	586 (240)	302 (175)	260 (178)	536 (121)	468 (151)	823 (60)	879 (98)
glucose (mmol/l)	0.3 (0.2)	0.3 (0.1)	0.1 (0.1)	0.1 (0.1)	67.5 (46.7)	0.2 (0.1)	174.8 (42.1)	0.4 (0.1)
total glucosuria (mmol)					9.5 (5.6)	0.0 (0.0)	74.18 (22.3)	0.12 (0.04)

Table 1: Clinical and laboratory variables, shown as mean (standard deviation) according to the different timepoints. (*continued*)

	Timepoints							
	-1		0		2		8	
	empa	placebo	empa	placebo	empa	placebo	empa	placebo
fractional excretion								
FE urea (%)	44.6 (6.2)	44.3 (8.3)	49.9 (5.3)	50.4 (6.9)	40.8 (7.6)	32.2 (8.5)	42.9 (5.3)	30.6 (9.0)
FE uric acid (%)	5.8 (1.4)	5.2 (2.2)	6.4 (1.7)	6.2 (1.9)	10.2 (1.9)	8.6 (2.4)	10.9 (2.9)	6.4 (1.7)
biomarkers								
Copeptin (pmol/l)	5.9 (3.7)	5 (1.9)			3.1 (0.7)	3.1 (0.7)	3.3 (1.8)	2.6 (0.5)
NT-proBNP (ng/l)	27.1 (14.0)	31.7 (18.6)			30.3 (17.4)	34.4 (21.5)	50.9 (32.2)	50.5 (33.0)
MR-proANP (pmol/l)	48.7 (11.8)	49.2 (16.7)			61.7 (14.8)	59.3 (15.1)	63.1 (18.9)	56.8 (8.9)
Aldosterone (pmol/l)	371.2 (253.1)	314.7 (154.1)			175.6 (93.3)	154.1 (59.0)	128.5 (60.1)	86.7 (23.6)
Renin (ng/l)	14.3 (9.4)	21.2 (26.7)			6.3 (5.3)	8.4 (7.4)	4.9 (5.1)	3.6 (3.2)

⋆ timepoint 8 = cummulative excretion during observation period

Empa=empagliflozin; FE=fractional excretion

At timepoint 2 the artificial SIAD had been induced resulting in hypotonic hyponatremia (overall: serum sodium 133mmol/l (±2.0)); serum osmolality 269mmol/kg (±3.2)), elevated urine osmolality (overall: 502mmol/kg (±138.7)) and natriuresis (overall: urine sodium 83mmol/l (±39.5)). At this time the incipient effect of empagliflozin was already noticeable, with a significant increase in glucosuria (empagliflozin 9.5mmol (±5.6) vs placebo 0.0mmol, p=0.001).

Glucosuria under empagliflozin was maintained during the observation period between timepoint 2 and 8 (total glucosuria 74.18mmol (±22.3) vs 0.12mmol (±0.04); treatment effect (on log scale) 2.85 (CI 2.75, 2.96), p<0.001) with no difference in plasma glucose (empagliflozin 4.1mmol/l (±0.5) vs placebo 4.5mmol/l (±0.3); treatment effect -0.16mmol/l (CI -0.48, 0.17) p=0.367). This led to a significantly increased diuresis under empagliflozin with a total urinary excretion of 579ml (±194.8) versus 367ml (±158.8) in the placebo group (treatment effect 158ml (CI 48.29, 267.74) p=0.017).

No difference was noted in serum sodium levels as well as in the change of the serum sodium concentration (AUC) between empagliflozin and placebo during the observation period (AUC serum sodium concentration 0.2 (CI -7.38, 6.98), p=0.96). There was also no significant difference in total natriuresis calculated according to the excreted volume (empagliflozin 83.3mmol (±42) vs placebo 64.4mmol (±41); treatment effect 8.54mmol/l (CI -14.19, 31.27), p=0.5), however with an increase in the empagliflozin group towards the end of the observation period (*Figure 2*).

Fractional excretion of urea and uric acid did not differ between the two groups until timepoint 2, but increased significantly under empagliflozin by the end of the observation period (FEurea empagliflozin vs placebo 42.9% (±9.3) vs 30.6% (±9.0); treatment effect 8.49% (CI 2.56, 14.41), p=0.016; FEuricacid empagliflozin vs placebo 10.9% (±4) vs 6.4% (±2.3); treatment effect 3.93% (CI 1.01, 6.86) p=0.022).

The course of the different biomarkers was similar in both groups, with a decrease of copeptin, aldosterone and renin and an increase of MR-proANP and NT-proBNP due to the induction of the artificial SIAD. Two copeptin values in the empagliflozin group had to be excluded from the analysis due to extraordinary high values (15.2 and 30.6pmol/l) at timepoint 8, probably due to vomiting.

There was no difference in the average rating of clinical symptoms attributable to hyponatremia (*Table 2*). However, more adverse events were reported under empagliflozin: 6 versus 2 participants required pain medication against headache, 2 versus none suffered from acute vomitus and 1 participant had acute transient hypoglycaemia of 2.7mmol/l.

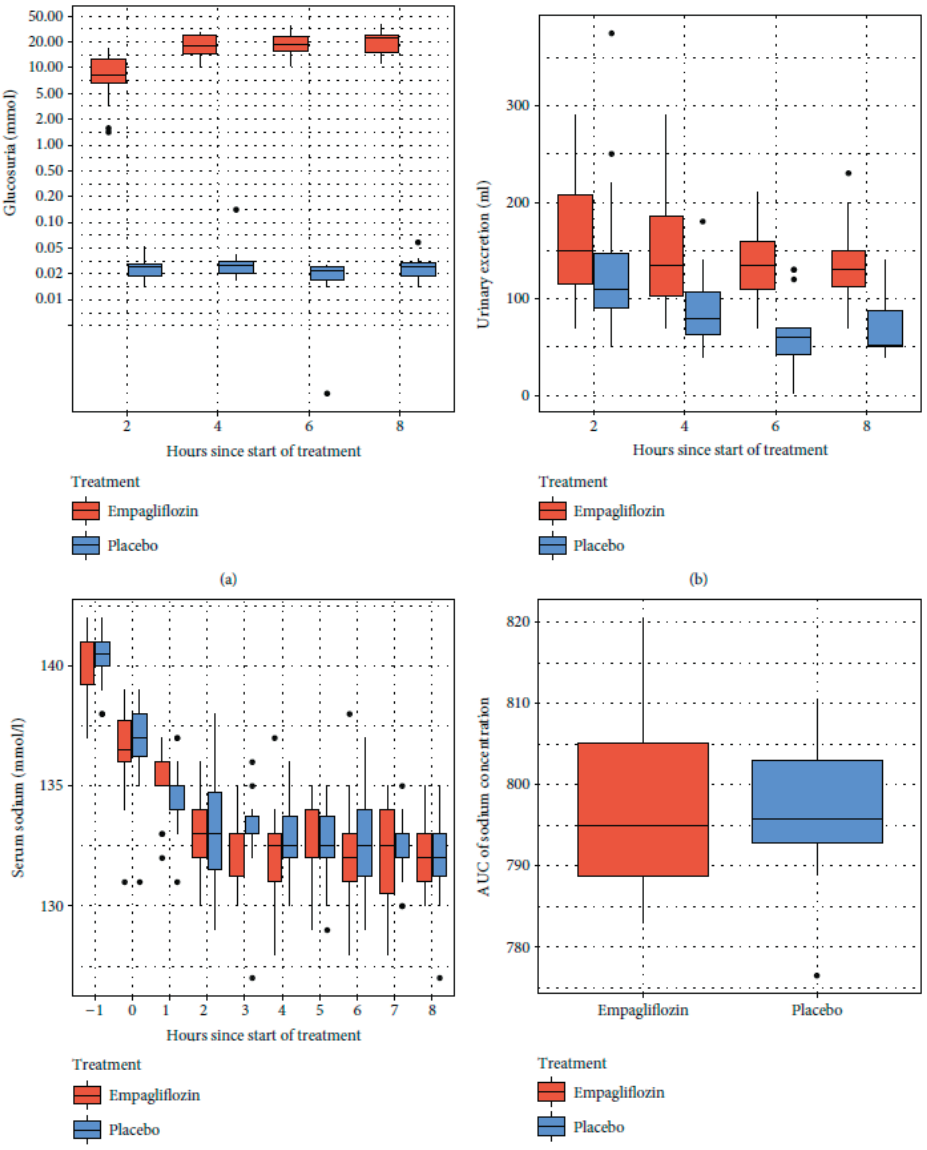


Figure 2: Efficacy outcomes according to treatment (empagliflozin vs placebo); the thick line indicates the median, the box indicates the interquartile range, the whiskers include all points within the range of 1.5x the interquartile range.

Top left: Trajectory of glucosuria (note that y-axis is on log scale)

Top right: Trajectory of urinary excretion

Bottom left: Trajectory of serum sodium

Bottom right: Area under the curve (timepoint 2 to 8) for serum sodium concentration

Table 2: Number of participants who experienced symptoms attributable to hyponatremia during the study with the according average score of symptom severity on visual analogue scale in brackets (range 0=no symptoms, to 10=severe symptoms).

Symptoms	Empagliflozin	Placebo	p
thirst	9 (2.4)	7 (2.9)	0.51
headache	10 (2.4)	11 (1.7)	0.75
nausea	7 (3.1)	6 (3.0)	0.67
vertigo	6 (2.6)	3 (2.5)	0.5
general malaise	7 (2.8)	5 (3.0)	0.94

4. DISCUSSION

The main finding of our study is that compared to placebo the SGLT2-inhibitor empagliflozin leads to significantly increased water excretion in healthy volunteers with artificially induced SIAD. This is a novel finding, as according to available data in healthy volunteers and type 2 diabetic patients, no difference in total urine output when compared to placebo has been described [8, 16, 17]. The enhanced water excretion is promoted by osmotic diuresis as reflected by the increased glucosuria but also due to a direct modulation of the uric acid transporter [18]. Osmotic diuresis is well described in patients with type 2 diabetes under treatment with empagliflozin [9], however we here show for the first time that this effect also works in SIAD - a condition with a strong antidiuretic impact. The assumption that osmotic diuresis is the cause of the elevated urinary volume excretion is also supported by the increase of fractional urea and uric acid secretion [16]. As SIAD is characterised by pathological water retention, the acquaretic properties of empagliflozin may be effective as shown for other therapeutic strategies targeting water excretion [4, 6] vasopressin receptor antagonists (vaptans) [17] and oral urea [18, 19]. However, vaptans are expensive and bear the risk of sodium overcorrection whereas oral urea is usually not well tolerated. Empagliflozin could be a safe and effective alternative. Given that our participants were not allowed to eat anything during the study, the therapeutic effect of empagliflozin could arguably be even more pronounced in real life situation as the amount of glucose excreted in urine depends on the level of glycaemia [9].

Serum sodium levels did not differ between the two treatment arms at the end of the observation period. The lack of an effect of empagliflozin on serum sodium levels is possibly due to the short observation period of only six hours and the consequently relative small difference in urinary volume excretion of 200 ml. An extrapolation of those results to one day with further increase in total urinary volume excretion and thereafter change in serum sodium levels is tempting, but highly speculative. Nevertheless, keeping the pharmacodynamics of empagliflozin in mind with increasing urinary glucose excretion before reaching a plateau in the first 24 hours [16], this assumption cannot be excluded. Previous studies in healthy

volunteers and diabetic patients are of limited value in regard of serum sodium levels [23, 24], as those populations do not suffer from free water retention which is characteristic for SIAD [2].

A longer treatment period to detect a difference in serum sodium levels may also be needed because of the initial increase in natriuresis described under treatment with SGLT2-inhibitors [25]. The transient nature of this effect was shown by a recent study in type 2 diabetic patients with standardised food-, fluid- and salt-intake and treatment with empagliflozin 25mg for 5 days [26]. Natriuresis increased after the first empagliflozin dosage and normalized after multiple dosing [26]. Empagliflozin outcome studies including poorly controlled type 2 diabetic patients showed no events of hyponatremia, which also argues against a persistent natriuretic effect [27].

Because of the glucosuria and transient natriuresis under empagliflozin, calculation of the free water clearance and the electrolyte free water clearance was not helpful to estimate the potential effect of empagliflozin on serum sodium levels.

There was an increased rate of adverse events under treatment with empagliflozin compared to placebo. The majority of reported symptoms are most likely due to the acute effects of hyponatremia; an aggravation through administration of empagliflozin is possible. This would argue against the use of empagliflozin in acute hyponatremia. As however most patients with SIAD suffer from chronic hyponatremia which is often clinically less symptomatic [11], the tolerability of empagliflozin should be tested in patients with chronic hyponatremia.

Some additional findings of our study are noticeable. First, the role of copeptin as a stress marker [28, 29, 30] can nicely be illustrated with our results: the levels of the two participants with vomitus were extraordinary elevated despite persisting hypotonic hyponatremia. Second, there was an episode of transient mild symptomatic hypoglycaemia (2.7mmol/l) despite intact liver function in one of the participants. This has so far not been described in studies evaluating the effect of empagliflozin in healthy volunteers [17]. As the hypoglycaemia occurred after a prolonged fasting period of 17 hours, the combination of urinary glucose loss and fasting may have led to the symptomatic decrease in glucose in this lean patient.

The following limitations have to be mentioned. First the effect of empagliflozin was measured in an artificial SIAD model, which can never fully mirror the complex pathomechanisms of sodium and fluid homeostasis in clinical reality. Also this was a small study involving 14 healthy participants who received only one dose of treatment and who do not represent the usually comorbid elderly patients with SIAD. However, our crossover design allowed us to study the physiological reaction without any confounding factors. Second, the observation

time of the treatment effect was limited to six hours, making it difficult to extrapolate the results to a longer treatment phase. Unfortunately, the observation period in the proposed model cannot be prolonged due to side effects described above.

The strength of our study is its prospective double blind placebo controlled randomised crossover design – with every participant being his own control any physiological changes can be easily detected and analysed. Therefore, given the rapid and significant increase in urinary volume excretion under empagliflozin, we hypothesize that SGLT2-inhibitors could be a promising new treatment option for patients with SIAD.

In conclusion, in healthy volunteers with artificially induced SIAD, empagliflozin increased volume excretion due to osmotic diuresis. Due to the short treatment duration serum sodium levels remained unchanged. Additional studies in real live setting are needed to further examine the possible role of empagliflozin as a new treatment option for SIAD.

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8 Treatment effect of the SGLT2-inhibitor empagliflozin on chronic SIAD – results of a double-blind, randomized, crossover, placebo-controlled trial

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ABSTRACT

Background: The syndrome of inappropriate antidiuresis (SIAD) is characterized by a reduction of free water excretion with consecutive hypotonic hyponatremia and is therefore challenging to treat. The sodium-glucose cotransporter 2 (SGLT2) inhibitor empagliflozin promotes osmotic diuresis via urinary glucose excretion, resulting in electrolyte-free water excretion.

Material and Methods: In this double-blind, randomized, crossover, placebo-controlled trial we compared 4-week treatment with empagliflozin 25mg/day to placebo in outpatients with chronic SIAD-induced hyponatremia. At baseline and after both treatment cycles, patients underwent different assessments including neurocognitive testing (Montreal Cognitive Assessment (MoCA)). The primary endpoint was the difference in serum sodium levels between treatments.

Results: 14 patients, 50% female, median (IQR) age 72 years (65-77) completed the trial.

Median (IQR) serum sodium level at baseline was 131mmol/L (130-132). Following treatment with empagliflozin, median (IQR) serum sodium level rose to 134mmol/L (132-136), while no increase was seen with placebo (130mmol/L (128-132)), corresponding to a serum sodium increase of 4.1mmol/L (95% CI 1.7 to 6.5, p=0.004). Exploratory analyses showed that treatment with empagliflozin led to improved neurocognitive function with an increase of 1.16 (95% CI 0.05 to 2.26) in the MoCA score. Treatment was well-tolerated; no serious adverse events were reported.

Conclusion: The SGLT-2 inhibitor empagliflozin is a promising new treatment option for chronic SIAD-induced hyponatremia, possibly improving neurocognitive function. Larger studies are needed to confirm the observed treatment effects.

1. INTRODUCTION

The syndrome of inappropriate antidiuresis (SIAD) leads to a reduction of free water excretion with consecutive hypotonic hyponatremia (1, 2). The causes of SIAD are diverse and include various organ dysfunctions, but also stress, such as chronic pain, or secondary to medications (3).

Treatment options for SIAD, in addition to treating the underlying cause, are limited (4, 5). The first-line treatment option is fluid restriction (6-8), which is burdensome and unlikely to be sustained over an extended period, especially in chronic SIAD-induced hyponatremia. Urea effectively treats hyponatremia through the induction of osmotic diuresis (9, 10). While a recent study showed its safety also over an extended treatment period (11), its poor palatability affects compliance(12). Vaptans correct hyponatremia through the induction of aquaresis, but they are costly and bear the risk of serum sodium overcorrection (5, 13, 14). As a result, treatment often remains inadequate (15). This is concerning given the reported adverse effects of hyponatremia, particularly neurocognitive deficits and gait disturbances (16-18). Despite these associations, intervention studies showing reversibility of these symptoms are largely lacking with only one study indicating improvement of a subdomain of neurocognition upon treatment with vaptans (7).

We recently reported the efficacy of a four-day treatment with the sodium glucose co-transporter 2 (SGLT2) inhibitor empagliflozin in correcting hyponatremia in hospitalized patients with SIAD-induced hyponatremia (19). The therapeutic effect of empagliflozin is the induction of marked glucosuria leading to osmotic diuresis with subsequent increased electrolyte free water clearance (20, 21). Given its good tolerability and positive effects on cardiovascular and renal outcomes (22, 23), empagliflozin could be an ideal treatment for outpatients with chronic SIAD. However, its efficacy in these patients is unknown.

The aim of this randomized, double-blind, placebo-controlled crossover trial was to investigate whether four weeks of treatment with the SGLT2 inhibitor empagliflozin leads to a greater increase in serum sodium levels than placebo in outpatients with chronic SIAD. As secondary objectives, we investigated whether hyponatremia correction by treatment with empagliflozin leads to improvement in neurocognitive deficits and gait disturbances.

2. METHODS

2.1 Trial design and participants

This prospective randomized, double-blind, crossover, placebo-controlled trial was performed at the University Hospital Basel, Switzerland from 12/2017 to 08/2021. The local ethics committee (EKNZ 2017-00701) as well as the national agency for the authorization and supervision of therapeutic products (swissmedics 2017DR2127) approved the study protocol and study medication. The trial was registered at ClinicalTrials.gov (NCT03202667). Written informed consent was obtained from all participants.

Eligible patients were 18 years of age or older and had chronic SIAD-induced hyponatremia <135 mmol/L defined as: euvolemia according to clinical assessment, serum osmolality <275 mmol/kg, urine osmolality >100 mmol/L, urine sodium >30 mmol/L, exclusion of hypothyroidism and hypocortisolism (24). Patients with acute or transient hyponatremia, severe symptomatic hyponatremia in need of hospital treatment, diabetes mellitus type 1, renal insufficiency (glomerular filtration rate (GFR) <45 ml/min), heart failure, known liver cirrhosis or acute hepatic impairment (ALAT / ASAT > 3x upper limit), pregnancy or breastfeeding or patients under treatment with SGLT2 inhibitors, lithium chloride, urea or glitazone were excluded.

2.2 Trial procedures and assessments

Outpatients with hyponatremia due to chronic SIAD from our institution as well as patients referred from endocrinologist in practice were asked to participate. After fulfilling eligibility criteria and providing informed consent, a medical questionnaire including evaluation of general well-being and possible symptoms and effects of hyponatremia was compiled. Routine physical examination and baseline diagnostics were conducted including blood and urine sampling which were processed according to standardized operation procedures. Participants then completed the following tests:

- EQ-5D-5L test (25, 26): a standardized test assessing quality of life consisting of two parts. The first part was a visual analog scale (VAS) ranging from 0 (= worst health) to 100 (= best health) on which the patients rated their current health. In the second part, the patients were asked to attribute a level (no problems = 1 point, slight problems = 2 points, moderate problems = 3 points, severe problems = 4 points, extreme problems/unable to = 5 points) to each of the following five dimensions: mobility, self-care, usual activities, pain/discomfort, anxiety/depression.
- Montreal Cognitive Assessment (MoCA) (27): The MoCA is a 30 point test (≥ 26 defined as normal) used to assess the cognitive domains visuospatial, executive function, naming, memory, attention, language, abstraction, delayed recall and orientation (to time and place). The MoCA was chosen because it is a sensitive screening test for the detection of

mild cognitive impairment and was superior to the MiniMental State Examination(28). A special focus was set on the subtest executive function, ranging from 0 to 5 points.

- Grip strength test: This was measured using a hand dynamometer(29). The best score of three trials of the dominant hand was reported.
- Gait analysis: A quantitative analysis of spatiotemporal gait parameters using the GAITRite electronic walkway system (30) was performed at the Basel Mobility Center of the University Department of Geriatric Medicine FELIX PLATTER. Gait was assessed from one trial of self-paced, habitual walking and included the variables walking speed, step width, single support time (indirect marker of dynamic balance), gait regularity (measured by cycle time variability)

Participants were randomly assigned to undergo the first treatment period in the empagliflozin or – with equal chance - the placebo group. Treatment involved 1 capsule per day (empagliflozin 25 mg or placebo respectively) for 28 days. Further treatment included limitation of daily fluid intake to ≤1.5L/day. Participants were asked to maintain their fluid intake during the observation period and to record their daily fluid intake.

After baseline, weekly examinations including a medical questionnaire, clinical parameters and blood and urine sampling were performed. At the end of each treatment cycle (i.e. 4 weeks after starting study medication), participants additionally performed the above described quality of life, neurocognition, grip strength and gait assessments. A wash out period of at least 2 weeks between the treatment cycles and a follow up visit 30 days after completion of the second treatment phase was scheduled.

2.3 Trial outcomes

The primary outcome was the difference in serum sodium levels (mmol/L) after 4 weeks of treatment with empagliflozin 25 mg as compared to placebo. Secondary endpoints included serum sodium levels after 1, 2 and 3 weeks of treatment; derived serum sodium area under the curve (AUC), serum and urinary electrolytes, osmolality and glucose after 1, 2, 3 and 4 weeks of treatment; course of hyponatremia symptoms as assessed in the medical questionnaire and clinical parameters after 1, 2, 3 and 4 weeks of treatment; change from baseline to end of treatment in EQ-5D-5L test, MoCA, grip strength test and gait analysis. In addition, adverse events, defined as any new medical issue or exacerbation of an existing medical issue according to Common Terminology Criteria for Adverse Events v4.0, were recorded. Seriousness and severity of each event was documented and its relation to the study intervention assessed.

2.4 Laboratory measurements

Serum and urine concentrations of sodium, glucose, creatinine, urea, uric acid and osmolality were measured by the central laboratory of the University Hospital Basel. Serum sodium levels were analyzed by indirect ion selective electrode method (cobas® 8000 modular ana-

lyzer, Roche diagnostics). Serum and urinary osmolality were measured using the freezing point depression osmometer method. To ensure the double-blind design of the study, results from the urinary diagnostics after administration of the first study drug were blinded until the end of the study.

2.5 Sample size estimation

Assuming a baseline value of 127 mmol/L (SD 3 mmol/L) (16) and a within-patient correlation of $\rho = 0.70$, we estimated that a sample of 13 evaluable patients would provide the trial with 90% power to detect a difference of 3 mmol/l in serum sodium levels after 4 weeks of treatment at a significance level of 5%. The hypothesis test was based on a linear model with treatment as categorical predictor and the baseline value as covariate. Considering a drop-out rate of 20%, we planned to recruit 3 additional patients.

2.6 Patient flow and analysis sets

A total of 17 patients were included in the study and randomized (full analysis set - FAS) to the sequence empagliflozin–placebo (n = 8) or placebo–empagliflozin (n = 9) (Figure 1). Of these, 3 patients were excluded from the analyses: 2 patients withdrew their consent within/after the first week of treatment during the first treatment phase (1 randomized to empagliflozin–placebo and 1 randomized to placebo–empagliflozin), and 1 patient – randomized to placebo–empagliflozin – was excluded after the first treatment phase due to initial incorrect diagnosis (transient hyponatremia).

The remaining 14 patients defined the modified intention-to-treat analysis set (ITTS). All patients in the ITTS took at least one dose of study medication for both empagliflozin and placebo treatments and received the treatments in the sequence as randomized. Of these, 1 patient (placebo–empagliflozin) took only 18 pills of a total of 28 pills and was not adherent to the treatment as defined per protocol (predefined as 75% of pills taken, i.e. 21/28). The remaining 13 patients defined the per-protocol analysis set (PPS).

2.7 Statistical analyses

We used linear mixed regression models to analyze the primary outcome serum sodium levels at the end of treatment, with treatment (empagliflozin – placebo) and baseline levels (visit 0) as fixed effects. In order to account for the cross-over design, patient was included as random effect (random intercept). Since preceding analyses revealed no evidence for either carry-over or sequence effect, treatment-sequence and study phase were not included in the primary analysis model (c.f. Supplementary Appendix).

The primary analysis was performed as modified intention-to treat analysis (ITTS) and repeated as per protocol analysis (PPS). We performed adjusted analyses by adding serum

and urinary sodium and osmolality, and the fractional excretions of urinary sodium, urea and uric acid as additional covariates (separate models, also including the interaction term covariate x treatment). The adjusted mean treatment effect is presented with 95% confidence interval (CI).

Secondary endpoints were analyzed analogously, using mixed linear (continuous outcomes) or logistic regression models (binary outcomes). To evaluate the impact of reaching normonatremia (serum sodium level at end of treatment ≥135 mmol/L), separate mixed-effects linear regression models were fitted with the change in MoCA, grip strength and gait parameters, from baseline to end of treatment as dependent variable, baseline value (covariate) and sodium status at the end of treatment as fixed effects, and patient as random effect.

Secondary analyses were performed on the ITTS. Adverse events are reported for the FAS.

Sodium measurements for baseline and week 4 were available for all patients for both treatment phases, however several patients missed visits in-between, due to comorbidities as well as the ongoing COVID19 pandemic. To derive the serum sodium AUC, missing values were imputed by multiple imputation using chained equations (c.f. Supplementary appendix). For all other secondary endpoints analyses are based on the available data.

All analyses were predefined and conducted using the statistical software package R versions 3.6.3 (2020-02-29) and 4.1.2 (2021-11-01)(31). No adjustment was made for multiple testing.

3. RESULTS

Baseline Characteristics

Fourteen patients completed both treatment cycles and were included into the analysis (see Study Flow Chart *Figure 1*).

Median (IQR) age was 71.5 years (64.5, 76.8), with 50% of the participants being female. Main comorbidities were arterial hypertension (79%), cerebrovascular and psychiatric disorders (both 36%) (*details in Table 1*).

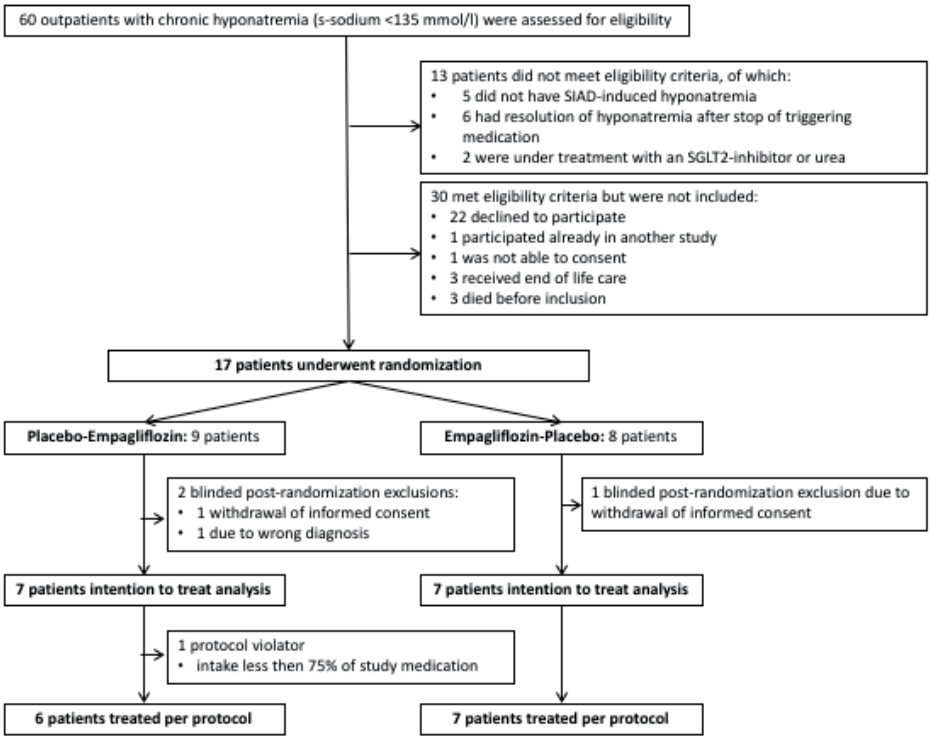

Figure 1 Study Flow Diagram

Table 1: Summary statistics of patient characteristics according to the intention to treat analysis set. Categorical variables are shown as frequencies (%), numerical variables as median [interquartile range]

	Participants n=14
Age, years [IQR]	71.5 [64.5, 76.8]
Sex, female (%)	7 (50.0)
BMI, kg/m2 [IQR]	24.4 [21.6, 27.6]
Comorbidities	
Arterial hypertension, n (%)	11 (79)
Cerebrovascular disease, n (%)	5 (36)
Pulmonary disease, n (%)	4 (29)
Psychiatric disease, n (%)	5 (36)
Diabetes mellitus type 2, n (%)	2 (14)
Central nervous system disorders, n (%)	2 (14)
Malignancy, n (%)	1 (7)
Chronic infectious disorders, n (%)	1 (7)

Table 1: Summary statistics of patient characteristics according to the intention to treat analysis set. Categorical variables are shown as frequencies (%), numerical variables as median [interquartile range] (*continued*)

	Participants n=14
Drugs	
Antihypertensive, n (%)	9 (64)
Statins, n (%)	4 (29)
Antiepileptic / Multiple sclerosis treatment, n (%)	4 (29)
Asthma / COPD inhalativa, n (%)	4 (29)
Proton pump inhibitors, n (%)	4 (29)
Hormonal replacement, n (%)	4 (29)
Anti-inflammatory, n (%)	4 (29)
Metformin, n (%)	3 (21)
Antipsychotic / antidepressants, n (%)	2 (14)
Diuretics, n (%)	2 (14)
Pain medication, n (%)	2 (14)
Antiretroviral, n (%)	1 (7)
other, n (%)	9 (64)
Causes of SIAD	
Central nervous system disorders, n (%)	2 (14)
Stress (chronic pain), n (%)	1 (7)
Drug-induced, n (%)	4 (29)
Pulmonary disease, n (%)	3 (21)
Idiopathic, n (%)	4 (29)
Duration of hyponatremia, months (IQR)	45.5 [15.8, 57.3]

Median (IQR) serum sodium level at baseline was 131 mmol/L (130, 132) (*Table 2*). The etiology of chronic SIADs ranged from drug-induced (antiepileptic (n=3) and antidepressants (n=1) which could not be stopped), to pulmonary (n=3) or central nervous system disorders (n=2) to stress induced due to chronic pain (n=1). In four patients, the etiology remained idiopathic. Hyponatremia duration ranged from a minimum of 4 months to a maximum of 90 months.

Efficacy

After four weeks of treatment with empagliflozin, serum sodium levels rose to a median (IQR) of 134 mmol/L (132, 136), while no improvement was seen under placebo (130 mmol/L (127.5, 132); *Table 2*).

Table 2 Summary statistics and treatment effect for clinical parameters, laboratory values and assessment outcomes assessed in 14 patients (unless otherwise indicated: [a] = 13 patients, [b] = 12 patients, [c] = 10 patients). Summary statistics are shown as median [interquartile range] or mean (standard deviation) for descriptive purpose.

	Baseline	End of treatment			
		Placebo	Empagliflozin	Treatment effect [95% CI]	p value
Clinical parameters					
Body weight (kg), IQR	72.0 [69.0, 80.4]	73.5 [70.1, 79.6]	72.8 [66.4, 78.4]	-1.65 [-2.75, -0.55]	0.009
Systolic blood pressure (mmHg), IQR	149.0 [134.5, 160.2]	141 [135.3, 152.5]	140.5 [132, 146.5]	-5.34 [-14.05, 3.38]	0.231
Diastolic blood pressure (mmHg), IQR	80.0 [68.8, 86.0]	79 [75, 93]	80.5 [72.3, 88]	-1.74 [-6.94, 3.47]	0.505
Heart rate (bpm), IQR	66.0 [60.8, 72.2]	64 [60.3, 66]	64.5 [60, 68]	0.03 [-6.27, 6.32]	0.993
Laboratory values					
S-sodium (mmol/L), IQR	131 [130, 132]	130 [127.5, 132]	134 [132, 136]	4.09 [1.68, 6.49]	0.004
S-glucose (mmol/L), IQR	5.0 [4.8, 5.8]	5 [4.73, 5.88]	5.1 [4.8, 5.8]	-0.41 [-0.81, -0.02]	0.052
S-creatinine (µmol/L), IQR	61.0 [57.5, 64.5]	61 [53.3, 70.3]	72.5 [59, 78.3]	7.76 [3.88, 11.64]	0.001
GFR (ml/min), IQR	87.0 [85.0, 101]	88 [82.3, 100.5]	81.5 [68.3, 95]	-7.09 [-10.97, -3.21]	0.003
S-urea (mmol/L), IQR	3.70[a] [3.40, 4.40]	3.75 [3.3, 4.92]	4.75 [3.73, 5.68]	0.50 [-0.24, 1.24]	0.186
S-uric acid (mmol/L), IQR	213.0[a] [160.2, 332.8]	200.5 [149.5, 304.75]	192 [134.75, 271]	-20.09 [-52.39, 12.21]	0.222
S-osmolality (mosm/kg), IQR	271.5 [264.0, 281.0]	268.5 [261.75, 280.75]	281 [274.5, 284.75]	17.16 [0.38, 33.93]	0.057
U-sodium (mmol/L), IQR	97.5 [77.0, 130.0]	81.5 [70, 101.25]	94 [57.75, 107.5]	3.26 [-16.08, 22.61]	0.733
U-glucose (mmol/L), IQR	0.2 [0.2, 0.27]	0.25 [0.2, 0.38]	131.7 [90.58, 155.88]	6.19 [5.79, 6.59]	<0.001
U-urea (mmol/L), IQR	143.0 [109.8, 187.8]	155.5 [97.25, 208.5]	160 [123, 216]	-1.82 [-36.51, 32.88]	0.915
U-uric acid (µmol/L), IQR	2072 [1532, 2393]	1989 [1332.25, 2583.5]	2009.5 [1578.5, 2640.75]	78.31 [-563.97, 720.59]	0.804
U-osmolality (mosm/kg), IQR	468.0 [361.2, 567.0]	494 [337.75, 592.75]	601 [502.5, 676.3]	131.11 [70.61, 191.62]	<0.001

Table 2 (*continued*)

	Baseline	End of treatment			
		Placebo	Empagliflozin	Treatment effect [95% CI]	p value
Assessments					
EQ-5D-5L score					
EQ-5D-5L (VAS score), IQR	70[a] [60, 85]	75[a] [60, 80]	65[a] [60, 87]	-0.38 [-9.02, 8.26]	0.929
EQ-5D-5L (unit score), IQR	0.87[a] [0.72, 0.94]	0.8[a] [0.72, 0.86]	0.87[a] [0.72, 1]	n.d.	n.d.
MoCA score	n=12	n=12	n=12		
MoCA total score, SD	22.7[b] (5.1)	24.6[b] (4.4)	25.8[b] (4.2)	1.16 [0.05, 2.26]	0.042
MoCA executive function, SD	3.0[b] (1.5)	3.0[b] (1.4)	3.4[b] (1.7)	0.36 [-0.02, 0.74]	0.064
Grip strength					
Grip strength (kg), IQR	25.5[b] [17.4, 34.8]	25[b] [16.5, 33.5]	21.5[b] [18, 34]	-0.57 [-4.14, 2.99]	0.680
Gait tests					
Gait speed normal walk (cm/s), SD	99.7[b] (35.5)	100.6[b] (32.3)	106.0[c] (25.9)	-0.8 [-5.9, 4.3]	0.770
Step width normal walk (cm), SD	9.9[b] (4.2)	10.9[b] (4.1)	10.0[c] (5.0)	-0.8 [-2.2, 0.5]	0.280
Cycle time variability normal walk (%), SD	3.0[b] (1.3)	3.0[b] (1.6)	2.3[c] (0.6)	-0.21 [-0.78, 0.35]	0.480
Single support time normal walk (s), SD	0.45[b] (0.11)	0.44[b] (0.06)	0.41[c] (0.03)	-0.0062 [-0.0138, 0.0015]	0.158

For inferential purpose, the treatment effect is indicated by the estimated difference in the outcome (estimate [95% confidence interval]: empagliflozin – placebo), derived from linear mixed effects models fit by maximum likelihood. Models included the baseline levels as covariate and patient as random effect. Each outcome was analyzed by a separate statistical model.

Please note that the treatment effect results from the individual, within-patient differences empagliflozin – placebo and is adjusted for baseline levels; the treatment effect cannot be derived directly from the summary statistics. n = number; n.d. = not done; S = serum; U = urinary;VAS = visual analogue score;

This resulted in an estimated increase of 4.09 mmol/L (95% CI 1.68 to 6.49) under empagliflozin as compared to placebo, (intention-to-treat analysis, p=0.004; *Figure 2)*. This finding was confirmed by the per protocol analysis: 4.44 mmol/L (95% CI 1.87 to 7.00), p=0.004. While serum sodium levels rose already within the first week of treatment and stayed constantly increased for the remaining treatment period, no notable change was seen under placebo (*Figure 2*); difference in AUC week 1 to 4: 9.08 mmol/L per three weeks (95% CI 2.78 to 15.39), p = 0.013.

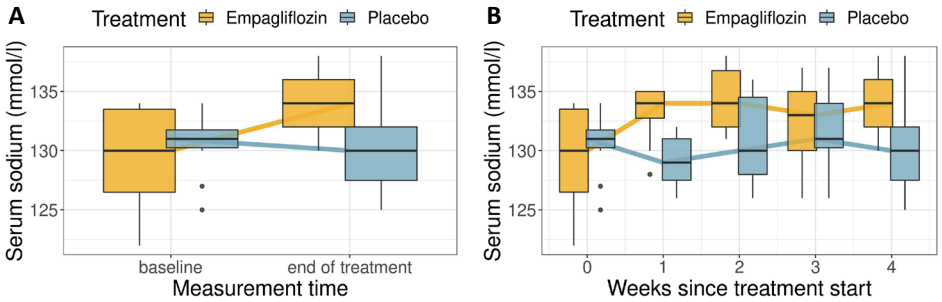

Figure 2: Course of serum sodium levels from baseline to end of treatment

A Serum sodium levels before (baseline) and after (week 4) treatment with empagliflozin or placebo.

B Course of serum sodium from baseline to end of treatment according to treatment phase

Boxes contain the 25 through 75% quantiles (spanning the interquartile range), the thick horizontal line is the median. Whiskers indicate the most extreme values lying within the box-edge and 1.5 times the interquartile range. All eventual further values are plotted as individual points (outliers).

Under treatment with empagliflozin, 36% (5/14) of the patients reached normonatremia, compared to 2/14 (14%) of the patients under placebo. One patient reached normonatremia under both treatments. Six patients had moderate hyponatremia (serum sodium < 130 mmol/L) at the start of treatment with empagliflozin, with two of these patients also having moderate hyponatremia at the start of placebo treatment. At the end of the treatment period, no patients on empagliflozin had moderate hyponatremia compared to six patients on placebo. Empagliflozin was effective in increasing serum sodium levels independently of the randomization sequence, there was no carry-over effect (*Supplementary Figure S1*). At the 30 day follow up visit 79% (11/14) of the patients showed persistent hyponatremia.

Fluid intake remained stable during the active study phase with no relevant difference between the treatment cycles (median (IQR): fluid intake week 1 empagliflozin 1.5 liters (1.3, 1.6) versus placebo 1.6 liters (1.4, 1.9); week 4 empagliflozin 1.5 liters (1.2, 1.5) versus placebo 1.5 liters (1.1, 1.5)).

Patients with a higher fractional excretion of urea at baseline tended to have a higher increase in serum sodium at the end of the treatment period, irrespective of the treatment (Estimate: 0.122 mmol/l (95% CI 0.007 to 0.236), p=0.059). No evidence for an association was found for outcome or treatment effect with serum or urine osmolality, urine sodium or fractional excretion of sodium or uric acid (*Supplementary Figure S2*) as well as for age (data not shown).

Secondary outcomes

Course of clinical and laboratory parameters as well as the different assessments are summarized in *Table 2*.

Clinical and laboratory parameters

As expected, a strong increase in urine glucose and osmolality was observed after four weeks of treatment with empagliflozin as compared to placebo (*Figure 3 and Supplementary Figure S3)*.

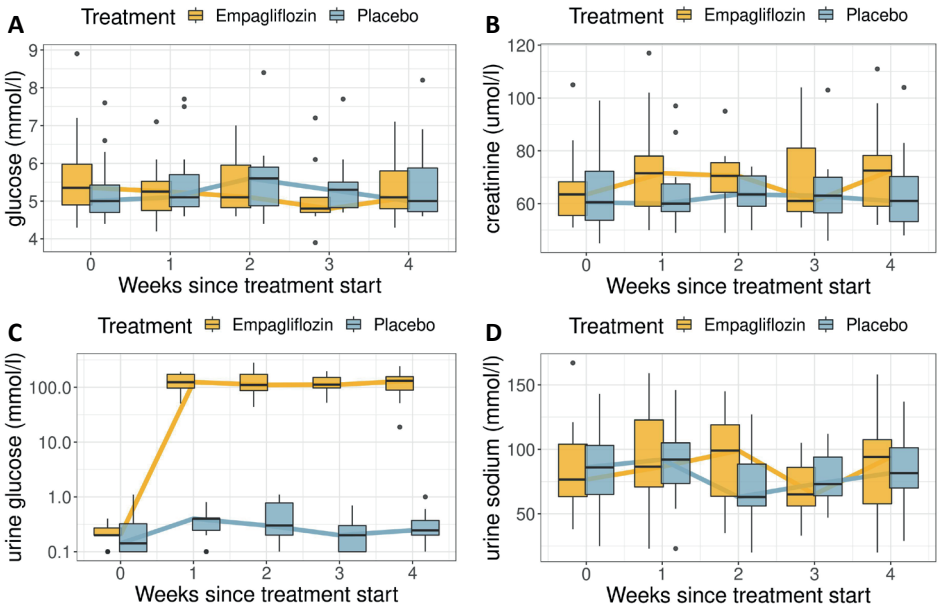

Figure 3 Time course of different parameters under treatment with empagliflozin or placebo:
A) serum glucose, B) serum creatinine, C) urine glucose, D) urine sodium.
Boxes contain the 25 through 75% quantiles (spanning the interquartile range), the thick horizontal line is the median. Whiskers indicate the most extreme values lying within the box-edge and 1.5 times the interquartile range. All eventual further values are plotted as individual points (outliers).

Additionally, treatment with empagliflozin compared to placebo led to an increase of serum osmolality and mild decrease of serum glucose and body weight. Our data further showed a slight increase in serum creatinine under treatment with empagliflozin. Changes during the treatment phase occurred within the first week and were maintained until the end of treatment (*Figure 3*). No effect of empagliflozin was seen for the other clinical or laboratory parameters.

Assessments

The patients rated their general health state as rather good according to the EQ-5D-5L questionnaire. Compared to baseline, the majority of patients indicated an improved overall health after both treatment cycles, with no relevant difference between treatments.

The mean (SD) score in the MoCA at baseline was 22.7 (5.1). After 4 weeks of treatment with empagliflozin, the mean (SD) score rose to 25.8 (4.2) compared to 24.6 (4.4) under placebo. This resulted in a difference of 1.16 (95% CI 0.05 to 2.26) under empagliflozin compared to placebo. A similar observation was made for the MoCA executive function subscore with a difference of 0.36 (95% CI -0.02 to 0.74). Visualization and evaluation of the data showed no signs of a possible learning effect (p=0.29) or sequence effect (p=0.21).

Test scores after both treatment phases of patients reaching normonatremia (number of observations: 7) were compared to those of patients with persistent hyponatremia (number of observations: 17). While no effect of sodium normalization on MoCA total score was seen (0.29, 95% CI -1.45 to 2.02), a beneficial effect was seen for the MoCA executive function subscore: 0.77 (95% CI 0.16 to 1.38).

No notable change from baseline or difference between empagliflozin and placebo was seen in grip strength (*Table 2)*, independently of whether treatment led to normonatremia or not (data not shown).

Baseline gait analysis showed slightly slowed walking speed with a healthy step width and single support time as well as regular gait, as measured by the cycle time variability. Although some improvements were observed under empagliflozin resulting in faster gait, reduced cycle time variability and shorter single support time, no difference was seen between treatments. Also, no effect of sodium normalization could be shown.

Tolerability and safety

Treatment with empagliflozin was generally well tolerated (*Table 3*). Of the evaluated symptoms, thirst was indicated in about half of all patients under both treatments and at any measurement time. Few patients reported headache and vertigo, nausea was only reported under placebo.

No events of sodium overcorrection, hypoglycemia, hypotension, urinary tract or genital infection occurred during the observation period under empagliflozin. Of the 7 reported adverse events, 5 were potentially related to empagliflozin treatment. Under placebo, 2 of the reported 7 adverse events were judged to be potentially related to the study intervention. No adverse events were recorded for the two participants who withdrew their consent. There were no serious adverse events during the observation period.

Table 3: Symptoms and adverse events occurring during observation phase.

	Placebo		Empagliflozin	
	Week 0	**Week 4**	**Week 0**	**Week 4**
Symptoms (yes / no)				
Thirst, n (%)	8 (57)	6 (43)	6 (43)	9 (64)
Vertigo, n (%)	1 (7)	1 (7)	1 (7)	3 (21)
Headache, n (%)	1 (7)	2 (14)	5 (39)	3 (21)
Nausea, n (%)	0 (0)	2 (15)	0 (0)	0 (0)
Adverse events				
All adverse events	**7**		**7**	
Serious adverse events	0		0	
Potentially study related	2		5	
Specific adverse events				
Mild headache	1		1	
Potentially study related	*1*		1	
Viral / bacterial / fungal infection	4		1	
Potentially study related	*0*		*0*	
Gastrointestinal disorders	0		2	
Potentially study related	*0*		*2*	
Dry mouth	0		1	
Potentially study related	*0*		*1*	
Hypertensive episode	1		0	
Potentially study related	*0*		*0*	
Tiredness	0		1	
Potentially study related	*0*		*1*	
Gait insecurity (heavy legs)	0		1	
Potentially study related	*0*		*0*	
Exanthema	1		0	
Potentially study related	*1*		*0*	

n = number of patients

4. DISCUSSION

We here show that treatment with the SGLT2-inhibitor empagliflozin leads to a relevant increase in serum sodium levels compared to placebo in outpatients with chronic SIAD. This treatment led to an improved neurocognitive function. Treatment was safe and well tolerated.

In addition to the findings of our proof of concept study consisting of a four-day treatment period in hospitalized patients with SIAD (19), we here show that empagliflozin is efficient as a long-term treatment option for chronic SIAD-induced hyponatremia. The rise in serum sodium levels was similar to the one of two recent intervention studies, evaluating the efficacy of fluid restriction of <0.5-1 liter/day (8, 32). However, such a pronounced fluid restriction is a burdensome treatment that is unlikely to be sustained over an extended period. Meanwhile, the effect of empagliflozin was evident within one week of treatment with sodium levels remaining consistently elevated until the end of the treatment cycle, supporting the efficacy of a long-term treatment effect. Possibly, the treatment effect of empagliflozin could be increased by a mild fluid restriction analogous to our previous study (19).

Having a higher fractional excretion of urea was found to be a predictive marker for empagliflozin treatment response in our study. Since this ratio is usually used to distinguish between SIAD and hypovolemic hyponatremia (33), a higher value likely characterizes patients with more substantial water excess which therefore benefit from osmotic diuresis. Interestingly all patients for which serum sodium levels did not increase under placebo (change ≤0) showed a clear increase in serum sodium levels following empagliflozin treatment. This observation further strenghtens the role of empagliflozin as a new treatment option in patients with chronic SIAD.

One safety concern of the proof-of concept study (19) was the transient decrease in renal function observed in four cases in the empagliflozin group. A mild 6% decrease in glomerular function was again observed in the current study. However, several large outcome studies investigating SGLT2-inhibitors in diabetic and non-diabetic patients (34-36) showed that a decrease in glomerular filtration rate of up to 30% of baseline after initiation of treatment is to be expected and was associated with nephroprotective effects. Other factors associated with improved outcome were glucosuria and mild weight loss, which were also present in our cohort. Accordingly, we consider treatment with empagliflozin to be safe in patients with chronic SIAD-induced hyponatremia, as also reflected by the low number of only mild adverse events.

In addition to being a novel well-tolerated and efficient treatment option for chronic SIAD-induced hyponatremia, exploratory analyses also suggest a possible treatment-induced

improvement in neurocognitive function. Several studies report neurocognitive deficits in hyponatremic patients, regardless of the severity of hyponatremia (16-18). This was confirmed in our cohort with mild to moderate hyponatremia, where patients achieved a median MoCA score of 22.7 (normal ≥26 points) indicating mild to moderate impairment. However, despite one tolvaptan trial showing improvement in the psychomotor speed domain – a subtest combining neurocognitive and gait function (7) - no interventional study has yet demonstrated reversibility of neurocognitive impairments following treatment of hyponatremia (37-40). The MoCA test is very sensitive for mild cognitive impairment, which could explain why we were able to detect an effect. We consider the observed improvement to be significant, because most intervention studies - even in other disorders outside the area of hyponatremia -, showed no effect on MoCA scores or only in patients with severe neurocognitive impairment (41-44). One study evaluating physical exercise was able to show a 2-point increase after 3 months of intensive training (45). Normonatremic patients in our study reached a higher score on the executive function subtest of the MoCA. This suggests the increase in sodium levels as underlying mechanism for improvement of neurocognitive function. While a drug-specific effect of empagliflozin cannot be excluded, it seems unlikely in view of several observational studies showing improvement in neurocognitive function following treatment, especially in those patients reaching normonatremia (16, 18, 46).

With daily treatment cost of empagliflozin being similar to urea (approximately 2 USD versus 4 USD), but being 1/40th of the daily cost of tolvaptan (approximately 80 USD), this would also be a cost-effective treatment option. Considering the cardiovascular and renal benefits of SGLT2 inhibitors, a chronic treatment with empagliflozin can be seen as a holistic approach in patients with chronic hyponatremia, who are usually older and have a high burden of comorbidities.

Chronic hyponatremia has been associated with gait instability leading to increased falls (18, 47, 48) and two observational studies have shown gait normalization after hyponatremia correction (18, 47). The baseline gait parameters of this study population showed only mild gait disturbances, primarily manifested with reduced walking speed. While some improvements in gait were noted after treatment, no differences were seen between the interventions. The fact that no clinically relevant improvement was observed in our cohort could be due to the good baseline situation. Another explanation could be that, compared to the two observational studies, only a minority of our patients achieved normonatremia. Interestingly, however, a post- versus pre-treatment reduction in gait cycle time variability was observed, suggesting improved gait regularity. Since deficits in executive function are associated with increased gait cycle time variability (49), this finding is in line with the improvement in the MoCA executive function subscore.

The strength of our study lies in the prospective, double-blind randomized crossover design and the novelty of the treatment approach. The observed treatment effect of empagliflozin is convincing and should prompt further evaluations of empagliflozin as an efficient treatment option in SIAD-induced hyponatremia.

A limitation of our study includes the small patient population and that not all patients were able to perform the planned assessments (due to comorbidities and the ongoing COVID19 pandemic), thereby inducing a possible power issue for detecting a difference in test performance. Also, while our data suggest an increase in electrolyte free water excretion as explanation for the therapeutic effect of empagliflozin on serum sodium levels, this cannot be verified since urine output and urine potassium were not measured.

In conclusion, this study shows that the SGLT-2 inhibitor empagliflozin is a promising treatment option for outpatients with chronic SIAD-induced hyponatremia, possibly leading to an improvement of neurocognitive function. Larger studies in in- and outpatient setting are needed to confirm the observed treatment effects.

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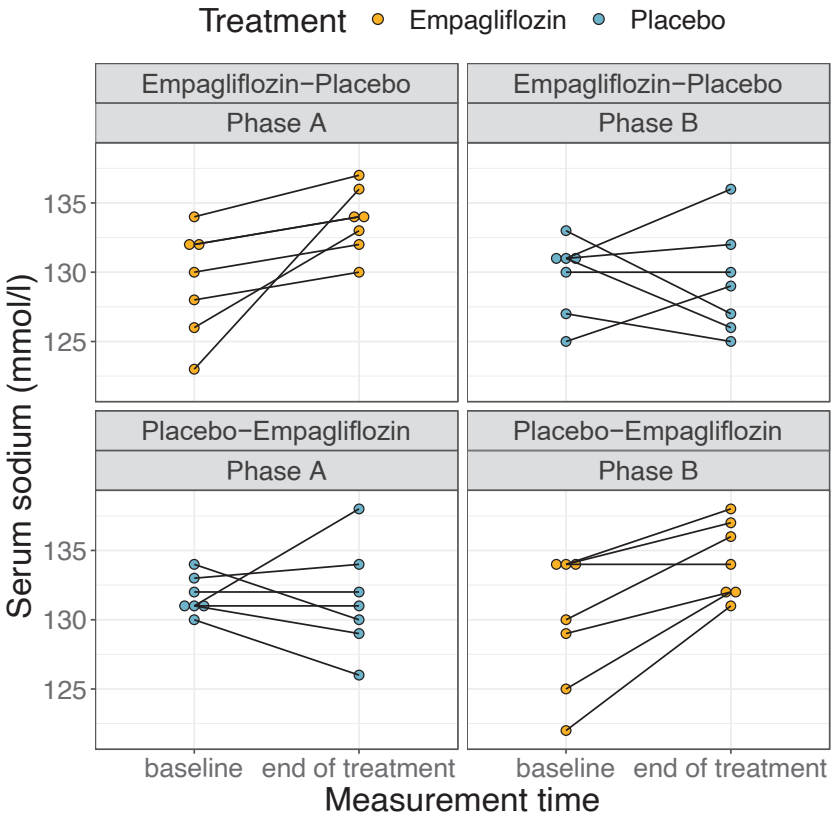
SUPPLEMENTARY MATERIAL TABLE OF CONTENTS

Statistical analyses

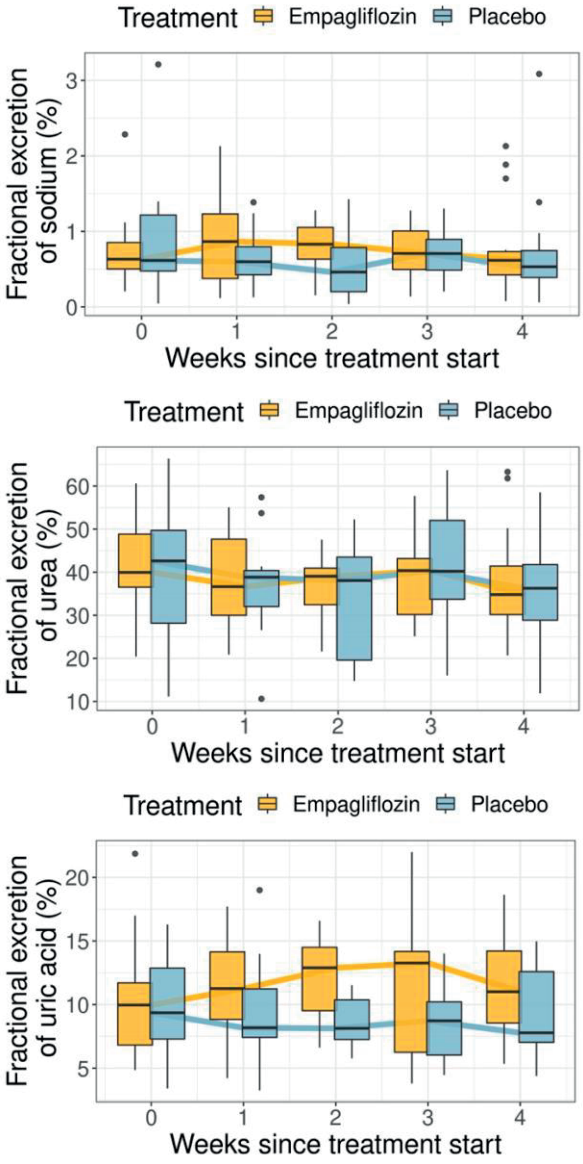
Carry-over/sequence effects: We assumed no carry-over effects and hence no differences in the treatment effect between the two treatment sequences (placebo/verum vs. verum/placebo). These assumptions were examined by a) fitting a model with sequence and the interaction term treatment x sequence as additional fixed effects; and b) calculating for each patient the difference between verum and placebo in the baseline levels and testing whether this difference differs between the two sequences using a two-sample t-test. Since there was no evidence for a sequence effect (primary endpoint: p-value interaction term = 0.666, p-value t-test = 0.435), neither the main effect nor the interaction term of treatment-sequence was included in the statistical analysis model.

Missing data: Serum sodium measurements for week 0 and 4 were complete for all patients for both treatment phases. However, several patients skipped one or several in-between visits (week 1–3) – mainly because of the COVID-19 pandemic. Thus, several serum sodium measurements and other secondary outcomes are missing. In order to derive a serum sodium AUC for all patients, the missing values were imputed by multiple imputation using chained equations (R package mice van Buuren & Groothuis-Oudshoorn (2011)) creating 50 imputation sets. All available serum sodium measurements and the treatment arm were included in the imputation model. For each imputed data set, the serum sodium AUC was then derived. Statistical analyses were performed on each imputed data set separately and results were pooled according to Rubin's rule.

Missing values in secondary endpoints were not imputed and all analyses are based on the respective data available (indicated in the summary tables).



Supplementary Figure S1 Individual serum sodium measurements at baseline and at the end of treatment for each patient under each treatment, grouped by the two randomization sequences.



Supplementary Figure S2 Time course of fractional excretion of sodium, urea and uric acid under treatment with empagliflozin or placebo.

Boxes contain the 25 through 75% quantiles (spanning the interquartile range), the thick horizontal line is the median. Whiskers indicate the most extreme values lying within the box-edge and 1.5 times the interquartile range. All eventual further values are plotted as individual points (outliers).

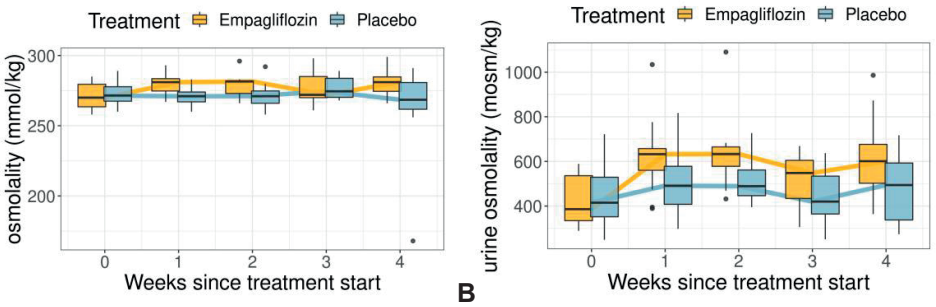


Figure S3 Time course of serum osmolality (A) and urine osmolality (B) under treatment with empagliflozin or placebo.

Boxes contain the 25 through 75% quantiles (spanning the interquartile range), the thick horizontal line is the median. Whiskers indicate the most extreme values lying within the box-edge and 1.5 times the interquartile range. All eventual further values are plotted as individual points (outliers).

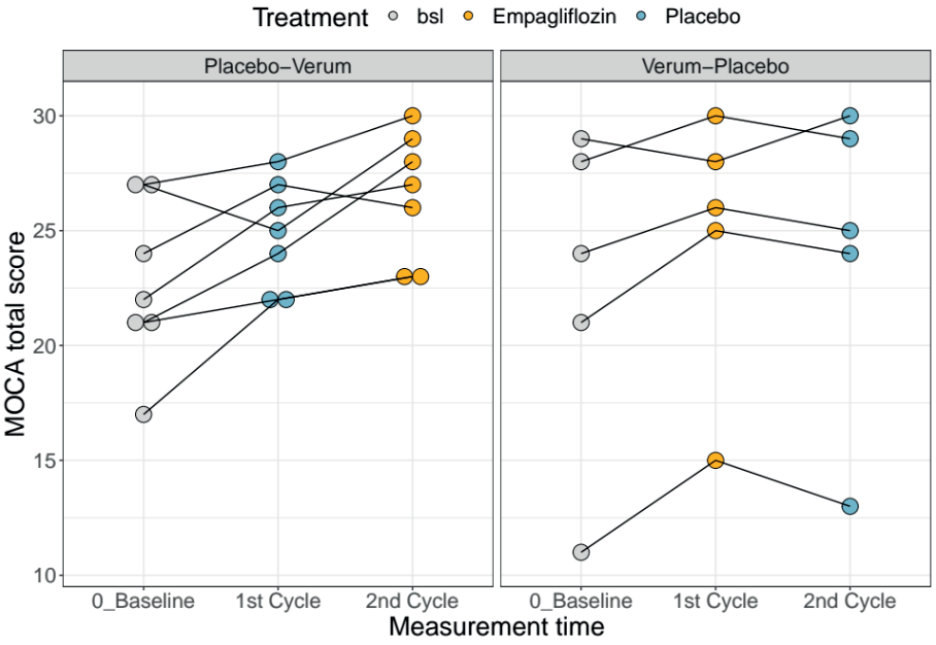


Figure S4 Visualization of the MOCA total score according to the two treatment cycles

bsl = baseline, Verum = Empagliflozin

9 Epigenetic regulation of SST2 expression in small intestinal neuroendocrine tumors

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ABSTRACT

Background

Somatostatin receptor type 2 (SST_2) expression is critical for the diagnosis and treatment of neuroendocrine tumors and is associated with improved patient survival. Recent data suggest that epigenetic changes such as DNA methylation and histone modifications play an important role in regulating SST_2 expression and tumorigenesis of NETs. However, there are limited data on the association between epigenetic marks and SST_2 expression in small intestinal neuroendocrine tumors (SI-NETs).

Methods

Tissue samples from 16 patients diagnosed with SI-NETs and undergoing surgical resection of the primary tumor at Erasmus MC Rotterdam were analysed for SST_2 expression levels and epigenetic marks surrounding the SST_2 promoter region, i.e. DNA methylation and histone modifications H3K27me3 and H3K9ac. As a control, 13 normal SI-tissue samples were included.

Results

The SI-NET samples had high SST_2 protein and mRNA expression levels; a median (IQR) of 80% (70-95) SST_2-positive cells and 8.2 times elevated SST_2 mRNA expression level compared to normal SI-tissue ($p=0.0042$). In comparison to normal SI-tissue, DNA methylation levels and H3K27me3 levels were significantly lower at five out of the eight targeted CpG positions and at two out of the three examined locations within the SST_2 gene promoter region of the SI-NET samples, respectively. No differences in the level of activating histone mark H3K9ac were observed between matched samples. While no correlation was found between histone modification marks and SST_2 expression, SST_2 mRNA expression levels correlated negatively with DNA methylation within the SST_2 promoter region in both normal SI-tissue and SI-NETs ($p=0.006$ and $p=0.04$, respectively).

Conclusion

SI-NETs have lower SST_2 promoter methylation levels and lower H3K27me3 methylation levels compared to normal SI-tissue. Moreover, in contrast to the absence of a correlation with SST_2 protein expression levels, significant negative correlations were found between SST_2 mRNA expression level and the mean level of DNA methylation within the SST_2 promoter region in both normal SI-tissue and SI-NET tissue. These results indicate that DNA methylation might be involved in regulating SST_2 expression. However, the role of histone modifications in SI-NETs remains elusive.

1. INTRODUCTION

Recent DNA sequencing studies have shown a very low mutation rate for well-differentiated neuroendocrine tumors (NETs) of all origins (1, 2). Accordingly, epigenetic changes are likely the principal pathological drivers in the development and progression of NETs, especially in small intestinal NETs (SI-NETs) (3, 4). Epigenetic changes affect gene expression without changing the DNA sequence and consist of DNA methylation and various histone modifications (3). DNA methylation is a process in which cytosine residues within CpG islands, which are often located in gene promoter regions, are methylated, resulting in gene silencing. Histone modifications can lead to both transcriptional repression and transcriptional activation, depending on the type of epigenetic mark and its precise location, e.g., the activating histone mark H3K9Ac and the repressive histone mark H3K27me3 (5).

Several studies have uncovered a possible prognostic role for epigenetic marks in SI-NETs. For example, promoter methylation of the RASSF1A and CTNNB1 genes was associated with extensive disease and poor overall survival in SI-NETs (6-8). Another study was able to identify a panel of 21 genes with an altered DNA methylation profile resulting in changes in gene expression levels in the majority of the SI-NETs, thereby enabling to discriminate SI-NETs from other gastrointestinal tract malignancies and normal gastrointestinal tissue (2). Histone modifications also contribute to tumorigenesis, with a small study demonstrating high expression of dimethylation on H3K4 in 93% of primary intestinal neuroendocrine carcinomas (9).

In accordance with the importance of epigenetic changes in tumorigenesis of NETs, research has also been focused on epigenetic drugs to improve diagnosis and therapy of NETs. As no genetic mutations in the somatostatin receptor subtype 2 (SST_2) gene have been described, it has been suggested that the epigenetic machinery is strongly involved in regulating SST_2 expression. SST_2 is the most important molecular marker for NETs as functional imaging with radiolabeled somatostatin analogues is crucial for tumor staging. Furthermore, sufficient SST_2 expression is the key element for treatment with unlabeled or radiolabeled somatostatin analogues (10). Several in vitro and in vivo studies showed an increase in SST_2 expression levels by decreasing DNA methylation and augmenting histone acetylation levels of the SST_2 gene promoter region in human NET cell lines (11-17). Although the majority of these studies have been performed using pancreatic NET cell lines, similar effects were also observed in the SI-NET cell line GOT-1. Accordingly, one would expect correlations between epigenetic marks and SST_2 expression levels in SI-NET tissues, i.e. inverse correlations of both DNA methylation levels and/or inhibiting histone marks with SST_2 expression levels, and a positive correlation of SST_2 expression with activating histone marks near the SST_2 promoter region. However, so far, no such data have been described on SI-NETs. Therefore,

the aim of this study was to investigate the role of DNA methylation as well as repressive and activating histone modifications (i.e. H3K27me3 and H3K9ac, respectively) in the regulation of SST_2 expression of SI-NETs.

2. METHODS

2.1 Samples

The selected samples consisted of fresh frozen tissue (FFT) material and formalin-fixed paraffin-embedded (FFPE) material of patients diagnosed with SI-NETs who underwent surgical resection of the primary tumor at the Erasmus MC Rotterdam, the Netherlands, and for which the diagnostic evaluation had been completed. Patients could refuse the use of their material, however, no specific consent was needed as long as patient anonymity is guaranteed.

In total, 21 SI-NET and 13 normal SI-tissues samples were collected for evaluation. Whereas FFPE material was used for SST_2 immunohistochemistry, FFT material was used for all other analyses. Prior to analyses, FFT was cut according to standard protocol, and hematoxylin and eosin staining was performed for quality control. Based on this staining, tumor cell content was measured by counting the number of cell nuclei and, subsequently, the tissues with less than 50% tumor cell content (n=5) were excluded. Of the remaining 16 SI-NET samples, 9 had matching normal SI-tissue available.

2.2 Immunohistochemistry

SST_2 immunostaining was performed on 4 μm thick whole slide sections from FFPE embedded tissue blocks, on a validated and accredited automated slide stainer (Benchmark ULTRA System, VENTANA Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. Briefly, following deparaffinization and heat-induced antigen retrieval (pH 9.0), the tissue samples were incubated with rabbit anti-SST2A antibody (Biotrend; NB-49-015-1ML, dilution 1:25) for 32 min at 37 °C, followed by Optiview detection (#760-500 & #760-700, Ventana). Counterstain was done by hematoxylin II for 12 min and a blue colouring reagent for 8 min. Stained slides were scanned with the NanoZoomer 2.0 HT (Hamamatsu Photonics, Hamamatsu City, Japan) and both the percentage of SST_2 positive cells and the intensity per area (intensity/area) were assessed using the CellProfiler software (version 4.0.7, ww.cellprofiler.org) as previously described (18).

2.3 SST_2 mRNA analysis

Tissues were lysed and incubated with Dynabeads oligo(dT)25 (Invitrogen, Breda, The Netherlands) to isolate poly-A+ mRNA, as described previously (17). H2O (23 μL) was

added for elution, and 10 μL poly-A+ mRNA was used in the next steps. Poly-A+ mRNA was converted into cDNA using the commercial RevertAid First Strand cDNA synthesis kit (Thermo Scientific, Breda, The Netherlands). cDNA was also prepared without the addition of RevertAid Reverse Transcriptase to exclude possible DNA contamination. Subsequently, samples were diluted by adding 180 μL H2O. Afterwards 5 μL sample was mixed with 7.5 μL Taqman Universal PCR mastermix (Applied Biosystems, Breda, The Netherlands) supplemented with primers and probes. SST_2 expression was determined relative to the three housekeeping genes (HKGs) GUSB, HPRT1 and ACTB. Primer information can be found in *Supplementary Table 1*. For analysis, the QuantStudio 7 Flex RT-qPCR system with QuantStudio Real-Time PCR software v1.5 was used. The number of copies for SST_2 and all HKGs was calculated by the efficiency factor to the power of ΔCt (i.e., 40 minus measured Ct). Subsequently, the relative SST_2 expression was calculated by dividing the number of SST_2 copies by the geometric mean of all HKGs.

2.4 DNA isolation, bisulfite treatment and pyrosequencing

DNA was isolated from the FFT samples according to protocol of the Genome Wizard DNA isolation kit (Promega Corporation, Madison, USA). For bisulfite conversion 1000 ng DNA was used with the Zymo Research EZ DNA Zymo kit according to the manufacturer's protocol (Zymo Research Corporation, Irvine, USA). Primer design was done with PyroMark Assay Design 2.0 (Qiagen N.V., Venlo, the Netherlands). Bisulfite treated DNA was aliquoted and stored at -20°C.

Pyrosequencing of bisulfite treated DNA was performed with the primers listed in *Supplementary Table 1*. PCR products were analysed on the PyroMark Q24 (Qiagen) with PyroMark Gold Q24 reagents (Qiagen) according to manufacturer's protocol. The eight CpG sites present in the SST2 promoter region were targeted, as these loci had been shown to be involved in the regulation of SST2 expression (19).

2.5 Chromatin immunoprecipitation

Chromatin immunoprecipitation (CHIP) analysis was performed on 11 SI-NET samples and 13 normal SI-tissue samples, of which seven samples were matched, measuring H3K27me3 and H3K9ac enrichment at three positions of the SST_2 promoter region, i.e. the transcription start site (TSS) and two regions upstream of this location, allocated as -2 and -1. The fold-enrichment was calculated using the following formula: efficiency factor ^ (CT input adjusted – CT immunoprecipitation) x 100%, and subsequently divided by the fold-enrichment obtained with the IgG antibody. The used efficacy factors are 1.96, 1.99 and 2.00 for -2, -1 and TSS, respectively. A detailed protocol can be found in the *Supplementary Appendix*.

2.6 Statistics

Categorical data are presented as frequencies and percentages; quantitative data are reported as mean ± standard deviation (SD) or as median and interquartile range (IQR). To test for normality, the D'Agostino and Pearson test was used. For differences between SST_2 expression levels in SI-NET and normal SI-tissue, a paired parametric t-test was performed. For differences in epigenetic marks, a Friedman test (matched, non-parametric One-Way ANOVA) was performed with a Dunn's multiple comparisons test. For correlation analysis, the data was log transformed to stabilize the variance, followed by Spearman correlation analysis. To test for uniform epigenetic modifications across the SST_2 promoter region, a Spearman correlation matrix was performed on log-transformed data, using an adjusted p-value based on a Bonferroni correction. Differences were considered statistically significant at $p<0.05$. Statistical evaluation was performed using GraphPad Prism version 9.0.0 (GraphPad Software).

3. RESULTS

3.1 Patient characteristics

Of the included 16 SI-NET samples, 9 (56%) came from male patients. Median age (IQR) was 61 years (54-66) at the time of tumor resection. The majority of samples were grade 1 tumors (12, 75%), while the remaining samples were low-grade 2 tumors. Nine (56%) patients had stage IV disease with lymph node metastases in 13 (81%), liver metastases in 8 (50%), bone metastases in 2 (13%) and peritoneal metastases in 4 (25%) patients. Ten (63%) patients suffered from hormonal syndrome, with 4 (25%) patients being pre-treated with somatostatin analogues of which 2 (13%) were also treated with peptide receptor radionuclide therapy using ^{177}Lu-DOTATATE.

3.2 Immunohistochemistry and mRNA analyses

Overall, the SI-NET samples showed high SST2 expression, with a median (IQR) percentage of positive cells of 80% (70-100) and an intensity/area of 0.262 (0.192-0.424) based on SST2 IHC, and a SST2/HKG ratio of 0.10 (0.05-0.14) based on the RT-qPCR analysis, *Figure 1A-D*. Results for one sample had to be excluded from SST2 IHC quantification due to insufficient eosinophilic counter-staining, hampering automated analysis. Analysis of the nine matched samples showed that SST2 mRNA expression levels of the SI-NET tissues were on average 8.2 times higher compared to that of normal SI-tissue with a median (IQR) SST2/HKG ratio of 0.05 (0.02-0.10) and 0.007 (0.005-0.009), $p=0.0042$, for SI-NETs and normal SI-tissue, respectively, *Figure 1E*. No underlying factor such as gender, grade or stage for the wide range in expression could be identified. Also, no significant differences in SST2 mRNA or protein expression levels between treatment-naïve versus pretreated patients were observed (data not shown).

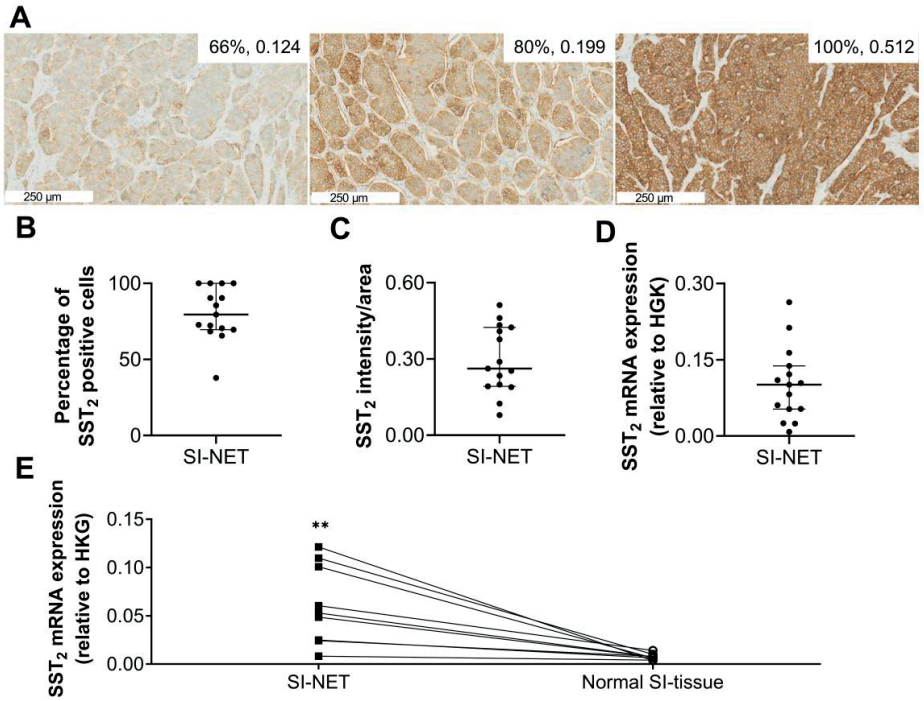

Figure 1 (A) Representative images of immunohistochemical SST2 staining. The numbers in the right upper corner represent the number of SST2 positive cells and the SST2 intensity/area. (B) The percentage of SST2 positive cells, (C) the SST2 intensity/area and (D) SST2 mRNA expression levels measured in small intestinal neuroendocrine tumor tissue, and (E) SST2 mRNA expression levels in small intestinal neuroendocrine tumor samples compared to paired normal small intestinal tissue. Data in B-D are presented as median with interquartile ranges. **p<0.01

SI-NET = small intestinal neuroendocrine tumor, normal SI-tissue = normal small intestinal tissue, SST2 = somatostatin receptor subtype 2

3.2 Epigenetic profiles of SI-NET samples

Using the matched tissue samples, it was demonstrated that the epigenetic profiles of SI-NET tissues differ compared to normal SI tissues. In general, DNA methylation levels of the SST_2 gene promoter of the SI-NET samples were relatively low and significantly lower at five out of the eight targeted CpG positions compared to what was observed in the normal SI-tissue, *Figure 2a*. For SI-NET samples, we observed a uniform DNA methylation across the SST_2 promoter region, with each location, except position –1, showing a significant positive correlation with at least three other locations (*Supplementary Table 2*). Interestingly, position -1 showed a significant positive correlation with four positions in normal SI-tissue, whereas location 6 was not characterized by any significant correlation (*Supplementary Table 3*).

In addition to DNA methylation of the SST_2 promoter region, differences were also found in histone methylation profiles. The enrichment of repressing epigenetic mark H3K27me3 was significantly lower in two out of the three locations in SI-NET tissue compared to the matched normal SI-tissue, *Figure 2b*. No differences in the activating histone mark H3K9ac position were observed between matched samples, *Figure 2c*.

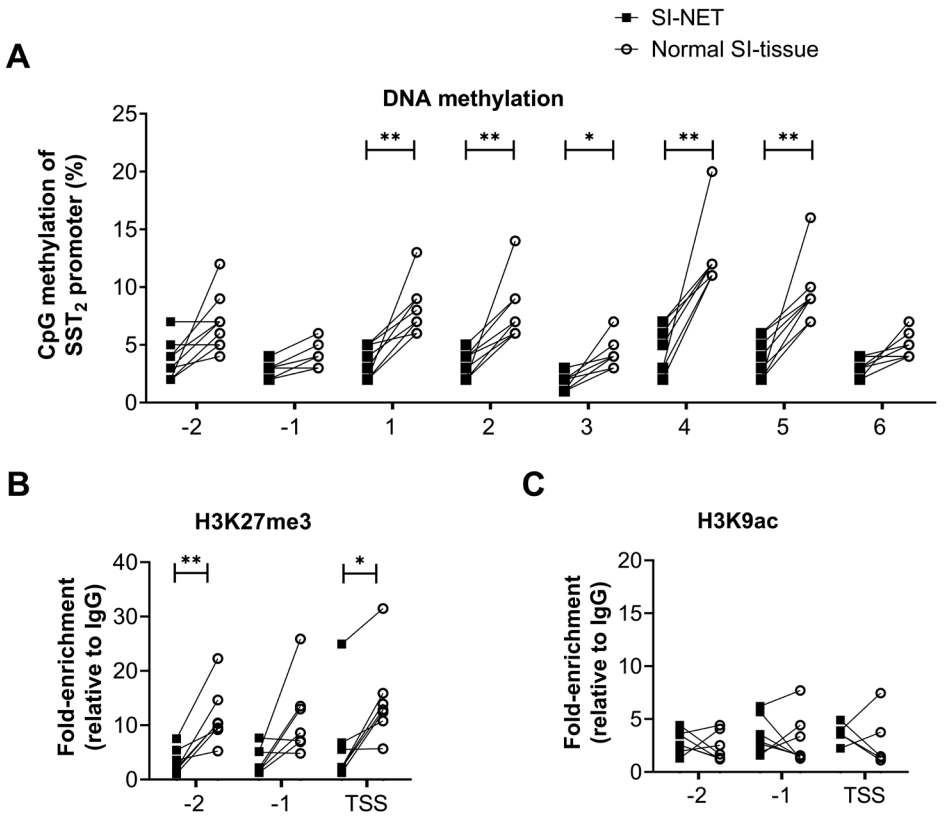

Figure 2

(A) Percentage of DNA methylation levels at different CpG positions of the SST_2 gene promoter of small intestinal neuroendocrine tumor samples compared to matching normal small intestinal tissue.

(B, C) Enrichment of H3K27me3 and H3K9ac on three locations in the SST_2 promoter region (i.e. -2, -1 and TSS) in small intestinal neuroendocrine tumor samples compared to the matching normal small intestinal tissue. Data is presented as fold enrichment relative to IgG controls and log-transformed. *$p<0.05$, **$p<0.01$. SI-NET = small intestinal neuroendocrine tumor, normal SI-tissue = normal small intestinal tissue, SST_2 = somatostatin receptor subtype 2

Similar to the pattern observed for the DNA methylation profile, a uniform epigenetic profile was also demonstrated for the histone marks, i.e. a significant positive correlation between -2, -1 and TSS for both histone methylation and acetylation, *Supplementary Table 4* and *5*.

3.3 Epigenetic profiles and SST_2 expression

To further evaluate the role of the epigenetic marks in regulating SST_2 expression, the epigenetic modifications were correlated with the percentage of SST_2 positive cells, the SST_2 intensity/area and SST_2 mRNA expression levels. SST_2 mRNA expression levels correlated negatively with the mean level of DNA methylation of the SST_2 promoter in the normal SI-tissue samples (p=0.006, *Figure 3A*), reaching statistical significance (adjusted p-value of 0.006) for the individual CpG positions 1, 2 and 4 (rS = -0.79, -0.81 and -0.74; p=0.002, 0.001 and 0.005, respectively). For the SI-NET samples, a statistically significant negative correlation was also found for SST_2 mRNA expression levels and the mean level of DNA methylation of the SST_2 promoter (p=0.04), *Figure 3B*.

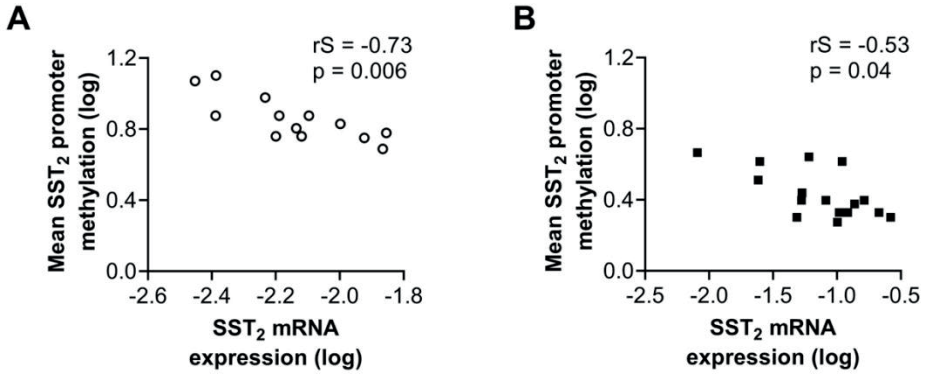

Figure 3 Correlation of the mean level of DNA methylation at CpG positions in the SST_2 promoter region with SST_2 mRNA expression levels in
(A) normal small intestinal tissue and (B) small intestinal neuroendocrine tumor samples. Data are log-transformed.
rS = Spearman r, SST_2 = somatostatin receptor subtype 2

However, using the adjusted p-value, no individual location showed a significant correlation, but a trend towards negative correlations was observed for location 1, 3, 4 and 5 (rS = -0.59, -0.58, -0.52 and –0.61; p=0.019, 0.019, 0.040 and 0.013, respectively). No statistically significant correlations between the mean level of DNA methylation and the number of SST_2 positive cells (p=0.41) nor SST_2 intensity/area (p=0.21) were demonstrated in SI-NET tissues (*Supplementary Figure 1*).

A similar correlation analysis was performed with the mean level of histone mark enrichment on the three examined locations within the SST_2 promoter region. In contrast to the correlation found between the level of DNA methylation and SST_2 mRNA expression in both normal SI-tissue and SI-NETs, no correlations were found in SI-NET samples between histone mark enrichment and SST_2 mRNA expression levels (p=0.33 and p=0.43 for H3K27me3 and H3K9ac, respectively), *Figure 4*, nor with the percentage of SST_2 positive

cells (p=0.54 and p=0.89 for H3K27me3 and H3K9ac, respectively, data not shown) or the SST_2 intensity/area (p=0.19 and p=0.71 for H3K27me3 and H3K9ac, *Supplementary Figure 2*). Whereas correlations using the mean level of enrichment were lacking, correlations were also not found focusing for each individual location.

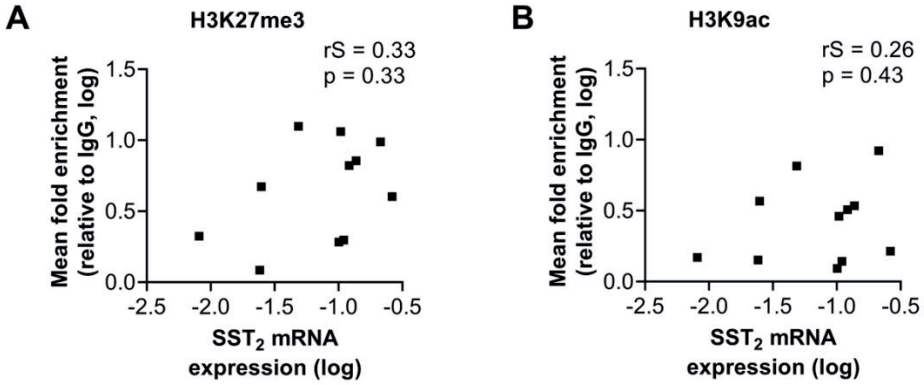

Figure 4 Correlation of SST_2 mRNA expression levels with the fold enrichment of (A) H3K27me3 and (B) H3K9ac calculated as the mean enrichment on three locations within the SST_2 promoter (i.e. -2, -1 and TSS) in the small intestinal neuroendocrine tumor samples.
All data are presented as fold enrichment relative to IgG and data are log-transformed. rS = Spearman r, SST_2 = somatostatin receptor subtype 2

4. DISCUSSION

The aim of the current study was to investigate the association between DNA methylation, histone modifications and SST_2 expression in SI-NET tissues. We show that the SI-NET tissues had lower DNA and histone methylation levels compared to normal SI-tissue. Moreover, significant negative correlations were found between SST_2 mRNA expression level and DNA methylation levels within the SST_2 promoter region for both normal SI-tissue and SI-NETs.

Our results confirm that DNA methylation may play a role in SI-NET tumorigenesis. DNA methylation levels are significantly lower in the SI-NET samples compared to the adjacent normal SI-tissue, suggesting tumor induced changes in the epigenetic profile of the SST_2 promoter region. In addition, we were able to show a clear negative correlation between the mean level of DNA methylation within the SST_2 promoter and SST_2 mRNA expression level in SI-NETs. Although significance was not reached after correcting for multiple testing, locations 1, 3, 4 and 5 seemed to be mostly involved in regulating SST_2 expression. It cannot be excluded that a higher sample size might have led to significant results in one or multiple of these individual locations. Moreover, the heterogeneous character of SI-NET tissues could

have complicated the analysis (20). The observed negative correlation is in line with previous research using NET cell lines showing compelling results demonstrating upregulation of SST_2 expression following epigenetic treatment, and more specifically, DNA methyltransferase inhibitors (12, 21). Moreover, a significant inverse correlation was found between DNA methylation – measured within an CpG island containing an upstream TSS for SST_2 – and SST_2 mRNA expression levels in a panel of 11 cell lines (19). We did not only demonstrate a correlation between DNA methylation and SST_2 mRNA in SI-NETs, a correlation was also found in normal SI-tissue. Surprisingly, location -1 was not correlated with any other positions in SI-NETs, whereas this was position 6 in normal SI-tissue. It is therefore possible that the epigenetic machinery responsible for DNA methylation is activated differently in normal SI-tissue and SI-NET tissue. Nevertheless, it should be considered that for a true comparison enterochromaffin cells should have been analysed instead of the normal SI-tissue.

While a correlation between DNA methylation and SST_2 mRNA expression was found in the SI-NET tissues, this correlation was not found between DNA methylation levels and the percentage of SST_2 positive cells, nor with the intensity/area. This might be due to the analyses performed; whereas mRNA and DNA methylation levels were determined based on the entire tumoral tissue including other cell types (e.g. fibrotic cells, endothelial cells), quantification of the SST_2 IHC was purely based on the analysis of tumor cells. Also, while mRNA and DNA methylation were both studied from FFT, protein expression was quantified on FFPE samples, possibly introducing a sample bias. It would therefore have been of interest to perform western blot analysis on FFT material as well. Unfortunately, this analysis could not be performed due to the scarcity of tissue, and no statement can be made about possible correlations.

In contrast to the correlations found between SST_2 mRNA and DNA methylation, no correlations were found between two widely studied histone modifications, i.e. activating (H3K9Ac) and repressive (H3K27me3) histone marks, and SST_2 expression levels. Possibly other epigenetic histone modifications are involved that can alter SST_2 gene expression, e.g. histone methylation at H3K9me2/3 (repressing), or at H3K4me1/2/3 and H3K36me3 (activating). Moreover, several lysine residues can be acetylated resulting in activation of gene transcription (22). Accordingly, the use of antibodies for panacetylation on either histone 3 or histone 4 might be of interest, thereby evaluating histone modifications in a broader view.

Research is currently focusing on upregulating SST_2 in NETs to improve diagnosis and treatment, but the available clinical data is ambiguous. Based on our findings, we would expect epigenetic drugs targeting the DNA methylation profile to be more effective in upregulating SST_2 than drugs targeting the histone modifications. However, one trial involving nine patients with NETs from different origin and low baseline SST_2 expression showed no SST_2

upregulation upon epigenetic treatment with DNA methyltransferase inhibitor hydralazine combined with histone deacetylase (HDAC) inhibitor valproic acid (23). Meanwhile, another small clinical trial involving five well-differentiated SI-NET patients with sufficient SST_2 expression showed a minor but significant increase in radiolabelled somatostatin analogue uptake after treatment with the HDAC inhibitor vorinostat (24). As discussed above, different histone marks could play a role in SST_2 upregulation, thereby enabling SST_2 upregulation in response to vorinostat. The opposing outcomes of these two clinical studies could also be due to differences in intratumoral drug levels or differences in tumor biology between NETs with low and high SST_2 expression (25).

Our current study only focused on SI-NETs, and it is therefore unknown if our findings would have been similar in NETs of other origins. In line with our results, a correlation was found between the level of DNA methylation in the SST_2 promoter and SST_2 expression levels in pancreatic NETs (26). In contrast, the direct role of histone marks in regulation SST_2 in pancreatic NETs remains unclear. In vitro experiments using pancreatic NET cell lines, e.g. BON-1 and QGP-1, showed convincing effects of HDAC inhibitors on SST_2 expression (17, 21, 23). Moreover, elevated HDAC expression levels have been described in pancreatic NET tissues (27), together suggesting a possible role of histone acetylation in regulating SST_2 expression in pancreatic NETs. However, despite these data, evidence for a direct association is lacking.

In conclusion, our study showed that well-differentiated SI-NETs have lower DNA and histone methylation levels on the SST_2 promoter region compared to normal SI-tissue. A statistically significant correlation between SST_2 mRNA expression and DNA methylation within the SST_2 promoter region was observed in both normal SI-tissue and SI-NETs. Thus, while epigenetic factors seem to play an important role in SI-NET tumorigenesis, it is mainly DNA methylation that seems to be involved in regulating SST_2. However, the role of histone modifications in regulating SST_2 expression remains to be further elucidated.

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SUPPLEMENTARY DATA

Chromatin immunoprecipitation

Snap frozen tissue was crushed and washed with 950 µL PBS supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF, Thermo Scientific), followed by incubation with formaldehyde for fixation (final concentration of 1%, 10 minutes, room temperature (RT)). To quench the reaction, glycine was added (final concentration of 0.125 M) and the sample was incubated (5 minutes, RT). The sample was washed twice with 950 µL PBS supplemented with 1 mM PMSF (2000 RPM, 5 minutes, 4°C), and the pellet was then resuspended in 500 µL lysis buffer (1 % sodium dodecyl sulphate (SDS), 50 mM tris hydrochloride (Tris-HCl, pH 8.1) and 10 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0) supplemented with 1 mM PMSF). After incubation on ice (10 minutes), the sample was sonified on ice for 5 - 11 times for 20 seconds at 600 microns. The sample was centrifuged (13000 RPM, 10 minutes, 4°C) and the supernatant was used for further analysis. To include a sample for further analysis, the right DNA concentration and fragment size was confirmed. To do so, 50 µL of the supernatant was incubated (30 minutes 37°C) with 100 µL H_2O, 6 µL 5 M NaCl and 1 µL RNase A (20 mg/mL). Then 2 µL proteinase K (20 mg/mL) was added and the sample was incubated again (4 hours, 65°C). The DNA was purified with the QIAquick PCR purification kit (Qiagen) according to manufacturer's protocol and subsequently heated (10 minutes, 65°C). The quality of the sample was then confirmed with the NanoDrop 8000 spectrophotometer (Thermo Scientific) and the 4200 TapeStation system (Agilent) (*Supplementary Figure 3*). For this, 1 µL of 50 ng/µL DNA was loaded into the Genomic DNA ScreenTape (Agilent).

Per antibody of interest, 5 µg chromatin was diluted in CHIP dilution buffer (1.1% Triton X-100, 0.01% SDS, 167 mM NaCl, 16.7 mM Tris-HCl (pH 8.1) and 1.2 mM EDTA supplemented with 1x SIGMAFAST protease inhibitor (Sigma)) to a total volume of 500 µL. Then rabbit anti-mouse IgG antibody (ab46540, Abcam) was added and the mixture was incubated (1 hour, 4°C) to preclear the chromatin, followed by the incubation (2 hours, 4°C) with Dynabeads Protein-G (Invitrogen). Of the precleared chromatin sample, an input sample (10%) was collected. Then, 2.5 µg antibody (H3K27me3 (ab6002, Abcam), H3K9ac (ab4441, Abcam) or IgG (ab46540, Abcam)) was added to each 5 µg chromatin sample, followed by an overnight incubation step (4°C). Dynabeads Protein-G was added followed by an incubation step (2 hours, 4°C). The sample was washed: (1) three times with 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1 % Triton X-100 and 150 mM NaCl, (2) once with 20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1 % Triton X-100, 0.1% SDS and 500 mM NaCl, (3) once with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.25 M lithium chloride (LiCl), 0.5% IGEPAL and 0.5% sodium deoxycholate and (4) once with 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Then, 150 µL elution buffer (25 mM Tris-HCl (pH 7.5) + 10 mM EDTA + 0.5% SDS) was added and the chromatin was eluted by incubation (30 minutes, 65°C, 1200rpm). To both the input

sample and the eluted chromatin, 6 μL 5 M NaCl and 2 μL proteinase K (20 mg/ml) were added and the samples were incubated (4 hours, 65°C). The resulting DNA was purified with the QIAquick PCR purification kit (Qiagen) and the enrichment was measured by RT-qPCR. For this, 2 μL DNA was mixed with 10.5 μL primer mix consisting of 3.5 μL H_2O, 0.375 μL 10 μM reverse primer, 0.375 μL 10 μM forward primer and 6.250 μL 2x SensiFAST SYBR Lo-ROX mix (Meridian Bioscience). Primer information can be found in *Supplementary Table 6*. To obtain the CT-values, a threshold of 0.030 was used for all three primer sets (i.e. -2, -1 and TSS).

Supplementary Table 1. (A) Primer sequences for RT-qPCR. Primers for housekeeping genes (Thermo Fisher Scientific) were diluted twenty times. For SST2, final concentrations were 0.5 pM for both the forward and reverse primer, and 0.1 pM for the SST2 probe (Sigma). (B) PCR and sequencing primer sequences for pyrosequencing. (C) Primer sequences for CHIP analysis (11).

	Primer information	**Efficiency Factor**
(A) RT-qPCR		
GUSB	Hs00939627_m1	1.95
HPRT1	Hs02800695_m1	1.97
B-Actin	Hs01060665_g1	1.96
SST_2	Forward: 5'-TCGGCCAAGTGGAGGAGAC-3' Reverse: 5'-AGAGACTCCCCACACAGCCA-3' Probe: 5'-FAM-CCGGACGGCCAAGATGATCACC-TAMRA-3'	1.91
(B) Pyrosequencing		
PCR	Forward: 5'-[Btn]GGGTTGGTTGGGTTAGTTTT -3' Reverse:	
Sequencing	5'-ATTCCTAACTCCTCCACCCTCTT-3' Reverse strand: 5'-ACCTCAAACTAAAACTCTA-3'	
(C) CHIP analysis		
-2	Forward: 5'-TGCTGACTGACGTGGCTACA-3' Reverse: 5'-CGCACCTGGAGTCCAAGATT-3'	1.96
-1	Forward: 5'-GTCCTTGCCATGAGTCTTGA-3' Reverse: 5'-CAGGCAGAGCTTACAGACAG-3'	1.99
TSS	Forward: 5'-AGCGAAGCCGCTGTGACGTA-3' Reverse: 5'-TCTGGGCGCTGGTGGTCTTG-3'	2.00

Supplementary Table 2 Spearman R values of the correlation analyses between the eight examined CpG positions of the SST2 promoter in small intestinal neuroendocrine tumors samples, demonstrating a uniform DNA methylation profile, except for location -1 which did not correlate with any other location. To correct for multiple testing, results were considered statistically significant at $p<0.002$ and are shown in bold.

	-2	-1	1	2	3	4	5	6
-2		0.06	**0.79**	0.72	**0.75**	**0.74**	**0.75**	0.72
-1			0.29	-0.18	0.27	0.08	0.08	-0.30
1				**0.75**	**0.92**	**0.82**	**0.88**	0.68
2					0.67	**0.79**	**0.88**	**0.82**
3						**0.83**	**0.85**	0.64
4							**0.90**	**0.81**
5								**0.83**
6								

Supplementary Table 3 Spearman R values of the correlation analyses between the eight examined CpG positions of the SST2 promoter in normal small intestinal tissue samples, demonstrating a uniform DNA methylation profile, except for location 6 which did not correlate with any other location. To correct for multiple testing, results were considered statistically significant at $p<0.002$ and are shown in bold.

	-2	-1	1	2	3	4	5	6
-2		0.79	**0.80**	**0.83**	0.74	**0.89**	**0.80**	0.74
-1			**0.90**	0.73	**0.92**	**0.87**	**0.82**	0.69
1				**0.86.**	**0.96**	**0.95**	**0.88**	0.69
2					**0.80**	**0.89**	**0.91**	0.57
3						**0.90**	**0.81**	0.63
4							**0.90**	0.68
5								0.75
6								

Supplementary Table 4 Spearman R values of the correlation analyses between the three examined locations within the SST2 promoter region (i.e. TSS, -2 and -1) in small intestinal neuroendocrine tumor samples, demonstrating a uniform H3K27me3 profile. To correct for multiple testing, results were considered statistically significant at $p<0.017$ and are shown in bold.

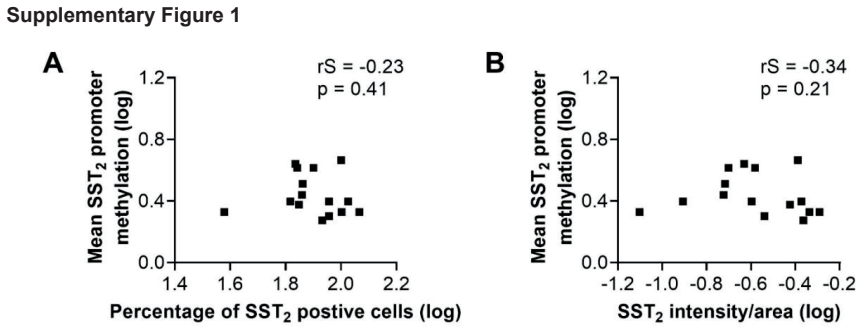
	TSS	-2	-1
TSS		**0.96**	**0.87**
-2			**0.95**
-1			

Supplementary Table 5. Spearman R values of the correlation analyses between the three examined locations within the SST2 promoter region (i.e. TSS, -2 and -1) in small intestinal neuroendocrine tumor samples, demonstrating a uniform H3K9ac profile. To correct for multiple testing, results were considered statistically significant at $p<0.017$ and are shown in bold.

	TSS	-2	-1
TSS		**0.89**	**0.89**
-2			**0.92**
-1			

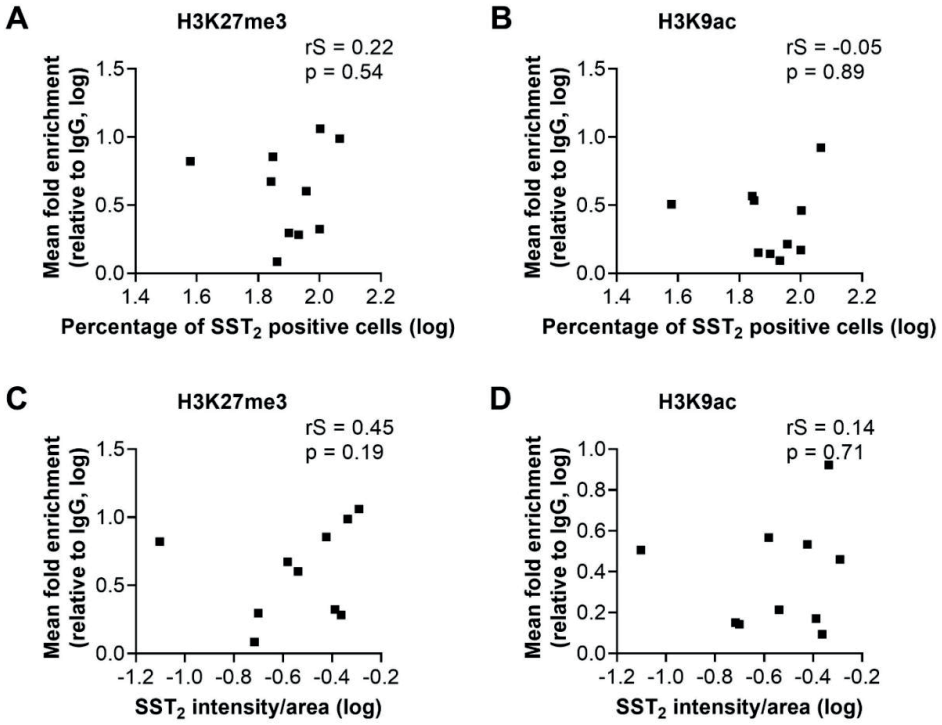
Supplementary Figure 1

	TSS	-2	-1
TSS		**0.89**	**0.89**
-2			**0.92**
-1			



Supplementary Figure 1 Correlation of the mean level of DNA methylation at CpG positions in the SST_2 promoter region with (A) the percentage of SST_2 positive cells and (B) the SST_2 intensity/area in small intestinal neuroendocrine tumor samples. Data are log-transformed.
rS = Spearman r, SST_2 = somatostatin receptor subtype 2

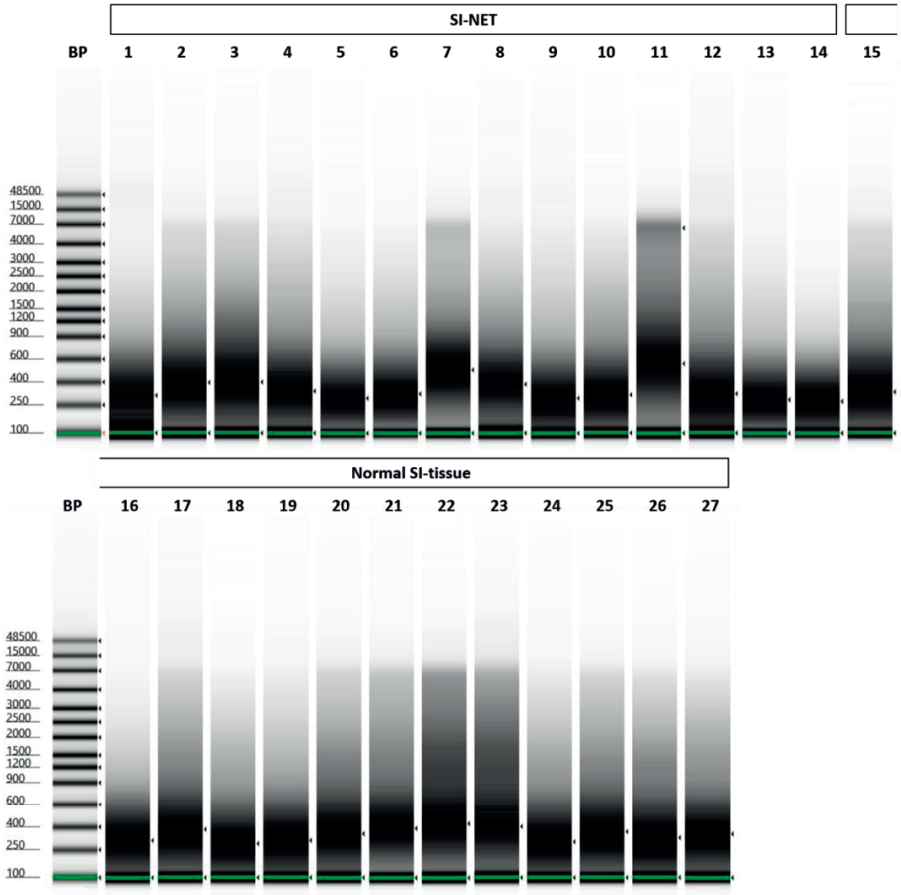
Supplementary Figure 2



Supplementary Figure 2 Correlation of (A, B) the percentage of SST_2 positive cells and (C, D) the SST_2 intensity/area with the fold enrichment of (A, C) H3K27me3 and (B, D) H3K9ac calculated as the mean enrichment on three locations within the SST_2 promoter (i.e. -2, -1 and TSS) in the small intestinal neuroendocrine tumor samples. All ChIP data are presented as fold enrichment relative to IgG and data are log-transformed.

rS = Spearman r, SST_2 = somatostatin receptor subtype 2

Supplementary Figure 3



Supplementary Figure 3 DNA fragment size of small intestinal neuroendocrine tumor samples and normal small intestinal tissue, demonstrating that the majority of the DNA fragments has the desired fragment size between 200 and 1000 base pairs. SI-NET = small intestinal neuroendocrine tumor, Normal SI-tissue = normal small intestinal tissue, BP = base pairs.

10 Effect of epigenetic treatment on SST_2 expression in neuroendocrine tumor patients

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Dear Editor,

Several preclinical studies have uncovered that epigenetic drugs can upregulate somatostatin receptor subtype 2 (SST_2) expression in neuroendocrine tumor (NET) models (1, 2), which could be of eminent importance for NET patients with low tumoral SST expression. In a prospective clinical proof-of-concept trial involving nine advanced NET patients with low SST expression, we were able to show that epigenetic treatment with the histone deacetylase (HDAC) inhibitor valproic acid and the DNA methyltransferase (DNMT) inhibitor hydralazine did not lead to an increase in tumor-uptake of 68Ga-DOTATATE, contradicting the in vitro data.

A prerequisite for the treatment of advanced NETs with (radiolabeled) somatostatin analogues (SSA) is the expression of SST_2 on the tumor cell surface, providing rationale for the inferior outcome in patients with low uptake on functional SST imaging (3). Several previous in vitro studies and one in vivo study achieved stimulation of SST2 expression levels and binding of SSAs by increasing histone acetylation levels and reducing DNA methylation of the SST2 gene promoter region in NET cells by epigenetic drugs (1, 2, 4). Despite these promising results, there is only data from one study showing limited increase of 68Ga-DOTATOC uptake by HDAC inhibitor vorinostat in five NET patients already expressing SST at baseline (5).

In the present study, which was approved by the ethics committee of the Erasmus Medical Center Rotterdam and registered at the Netherlands Trial Register (NL7726), nine patients with advanced NETs (*Table 1*) and low SST expression at baseline on ^{68}Ga-DOTATATE/PET, defined as tumor uptake below or equal to the physiological uptake in the liver, were included and provided written informed consent.

Table 1 Baseline characteristics of the neuroendocrine tumor patients included in the clinical trial. Values are shown as median (IQR) or number (%).

Patient characteristics	**Total** (n=9)
Age, years (IQR)	67 (54, 75)
Sex (male), n (%)	5 (56)
Origin	
Pancreas NET, n (%)	2 (22)
Small intestinal NET, n (%)	1 (11)
Lung NET, n (%)	4 (44)
Rectum NET, n (%)	1 (11)
Thymus NET, n (%)	1 (11)
Metastases	
Lymph nodes, n (%)	9 (100)
Liver, n (%)	5 (56)
Mesenterial, n (%)	1 (11)
Bone, n (%)	3 (33)
Lung, n (%)	1 (11)
Other, n (%)	4 (44)
Ki67-Index	
0-2%, n (%)	3 (33)
5-10%, n (%)	4 (44)
30%	1 (11)
unknown	1 (11)
Grading	
G1, n (%)	4 (44)
G2, n (%)	4 (44)
G3, n (%)	1 (11)
Previous treatments	
Surgery, n (%)	3 (33)
Somatostatin analogue, n (%)	2 (22)
Chemotherapy, n (%)	1 (11)
Other, n (%)	3 (33)

Bpm=beats per minute, N=number, IQR=interquartile range, SUV=standard uptake values.

Patients were treated for 14 days simultaneously with the HDAC inhibitor valproic acid (30mg/kg body weight/day, max. 3000mg/day) and the DNMT inhibitor hydralazine (150mg/day). One week after start of treatment, valproic acid dosage was adjusted to target a serum concentration of 75-120 μg/ml (6). Hydralazine dosage remained unchanged unless adjusted for tolerability. Treatment effect was evaluated after two weeks by the change in ^{68}Ga-DOTATATE uptake on PET/CT. The last two patients (lung NET, rectum NET) completed the trial without hydralazine due to emerging insights from the in vitro studies, which were performed simultaneously in three human NET cell lines BON-1 (pancreatic NET), GOT1 (small intestinal NET) and NCI-H727 (lung NET). Here, effects of valproic acid sodium salt (VPA) and hydralazine on SST_2 mRNA and protein levels as well as ^{111}In-DOTATATE uptake were assessed (details in *Supplementary Appendix*).

At the end of the 2-week epigenetic treatment period, none of the NET patients had an increase in ^{68}Ga-DOTATATE uptake grade (*Table 2*).

Table 2 Change in study parameters of neuroendocrine tumor patients at baseline and after 1 and 2 weeks of epigenetic treatment.

Clinical parameters	**Baseline**	**Week 1**	**Week 2**	**p Value**
Weight, kg (IQR)	76 (68, 86)	77 (68, 88)	77 (69, 88)	0.05
Blood pressure systolic, mmHg (IQR)	147 (130, 155)	139 (129, 151)	135 (126, 148)	0.14
Heart rate, bpm (IQR)	69 (62, 81)	77 (67, 109)	76 (65, 96)	0.34
Laboratory parameters				
Hemoglobin, mmol/L (IQR)	8.5 (8.1, 9.2)	8.5 (7.7, 9.2)	8.1 (7.6, 8.6)	0.05
Thrombocytes, x10`9/l (IQR)	247 (195, 282)	233 (173, 255)	177 (148, 271)	0.11
Creatinine, umol/L (IQR)	73 (58, 90)	74 (54, 86)	76 (56, 89)	0.72
ASAT, U/L (IQR)	27 (23, 32)	23 (21, 30)	28 (24, 36)	1
ALAT, U/L (IQR)	26 (17, 35)	17 (16, 25)	21 (13, 26)	0.09
GGT, U/L (IQR)	65 (19, 98)	46 (19, 82)	48 (18, 115)	0.16
Valproic acid drug level, ug/ml (IQR)	NA	102 (84, 126)	95 (90, 117)	NA
Study medication				
Valproic acid dosage, mg/day (IQR) (n=9)	NA	2300 (1900, 2500)	1900 (1763, 2000)	NA
Hydralazine dosage, mg/day (IQR) (n=7)	NA	150 (150, 150)	150 (100, 150)	NA
Tumor Uptake of ^{68}Ga-DOTATATE				
none, n (%)	6 (67)		6 (67)	1
below liver, n (%)	3 (33)		3 (33)	1
Peak Uptake				
Primary tumor, SUV (IQR)(n=6)	8.1 (3.0, 11.4)		6.8 (2.8, 9.9)	0.17
Lymph node metastases, SUV (IQR) (n=5)	4.8 (3.1, 9.0)		5.8 (2.6, 7.8)	0.35
Liver metastases, SUV (IQR) (n=5)	7.5 (5.0, 7.9)		7.3 (4.5, 8.4)	0.29
Bone metastases, SUV (IQR) (n=4)	4.1 (2.6, 5.1)		4.2 (2.7, 5.2)	0.47
Intestinal metastases, SUV (IQR) (n=2)	9 (7.5, 10.5)		8.7 (6.7, 10.6)	0.67
Skin metastases, SUV (IQR) (n=1)	3.5		3.7	NA
Liver, SUV (IQR)	10.5 (8.3, 12.6)		10.7 (8.3, 12.3)	0.95
Kidneys, SUV (IQR)	16.3 (14.3, 19.2)		20.7 (16.1, 26.0)	**0.02**
Spleen, SUV (IQR)	25.9 (22.7, 32.7)		27.8 (22.0, 31.9)	0.68

Values are shown as median (IQR) or number (%) in 9 patients, unless otherwise indicated.
N=number, NA=not applicable, IQR=interquartile range, SUV=standard uptake values. Bold writing signifies significance.

No change in median ^{68}Ga-DOTATATE uptake in any NET sites was observed, there was even a tendency for reduced uptake in primary tumors (*Figure 1, Supplementary Figure S1*). These findings were independent of tumor etiology, metastatic location or drug treatment. Meanwhile, a significant median (IQR) increase of 27% (4.1, 46.4) in uptake was observed in the kidneys, p=0.02, independent of the study medication.

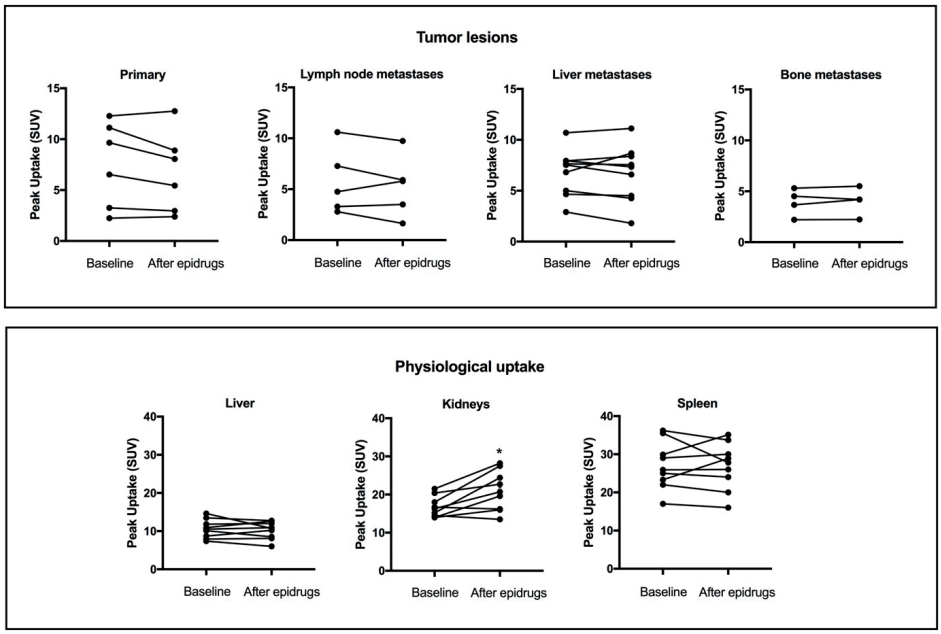

Figure 1 Change in peak uptake of ^{68}Ga-DOTATATE on PET/CT at baseline and after 2-week epigenetic treatment in patients with neuroendocrine tumors with low somatostatin receptor expression. The upper panel showing changes in tumor lesions, the lower panel showing changes in physiological uptake.

Patients were prepared according to our local protocol, which includes the drinking of 1 liter of water in the two hours before injection. Imaging was performed from scull base to thighs after median (IQR) 60 minutes (59-65) injection with an activity of 118 MBq (103-121) ^{68}Ga-DOTATATE. For each patient, at least two tumor target lesions, including the primary if applicable, were defined on the initial ^{68}Ga-DOTATOC PET/CT. Peak SUV was calculated for every lesion as well as for the liver, kidneys and spleen.

IQR = interquartile range; SUV = standard uptake value; *p<0.05 according to Wilcoxon signed Ranks Test

A limitation of our study is the restricted patient number, but given the lack of effects in any of the patients with different NET origin this protocol is unlikely to affect tumoral SST_2 expression in vivo. All patients reported known side effects of the study medication (details in *Supplementary Appendix*), no serious adverse events occurred during the study.

In all cell lines tested, treatment with valproic acid led to significant increase of SST2 mRNA levels and 111In-DOTATATE uptake, p<0.001 respectively (*Figure 2*).

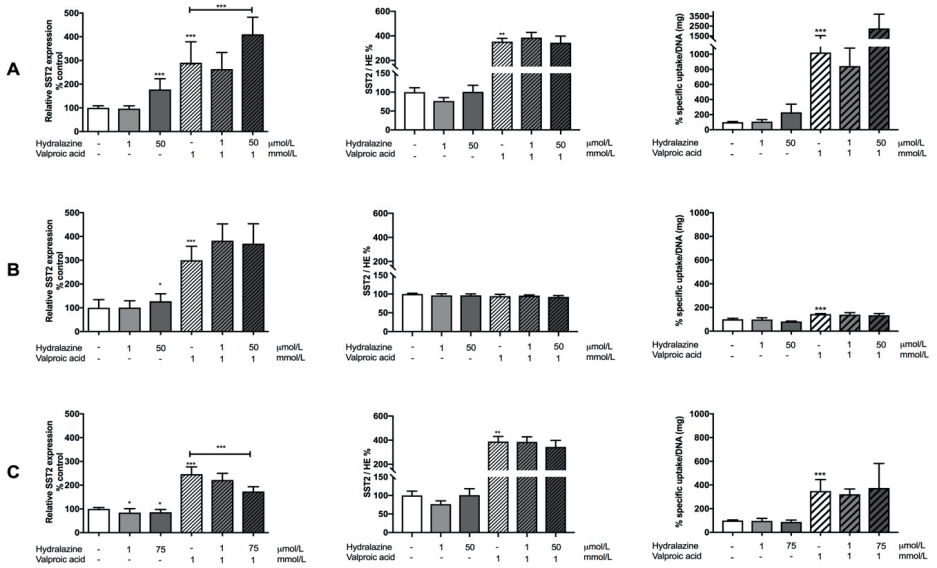

Figure 2 Effect of epigenetic treatment with valproic acid and hydralazine on the human neuroendocrine cell lines A) BON-1, B) GOT1 and C) NCI-H727.

Graphs show SST_2 mRNA expression levels, SST_2 protein levels and uptake of radiolabeled ^{111}In-DOTATATE as percentage increase or decrease compared to control cells.

DNA quantification (as a measure for cell amount in cell growth experiments) was performed with Hoechst 33256 for BON-1 and NCI-H727, while Quant-iT PicoGreen dsDNA reagent (Invitrogen, Breda, The Netherlands) was used for GOT1.

For mRNA-analysis Taqman Universal PCR mastermix (Applied Biosystems, Breda, The Netherlands) supplemented with primers and probes was used. SST_2 expression was determined relative to three housekeeping genes (*GUSB, HPRT1* and *ACTB*) using the QuantStudio 7 Flex RT-qPCR system with QuantStudio Real-Time PCR software v1.5.

Immunohistochemistry was performed using rabbit monoclonal anti-SST_2 IgG (NB-49-015, 1:25 dilution, NeoBiotech, Nanterre, France). Stained cells were visualized with the NanoZoomer 2.0 HT (Hamamatsu Photonics, Hamamatsu City, Japan) and SST_2 staining intensity per cell was assessed using the CellProfiler software (version 4.0.7, www.cellprofiler.org).

Internalization studies were performed with ^{111}In-DOTATATE. $^{111}InCl_3$ (Curium Pharma, Petten, The Netherlands) was used to radiolabel DOTATATE (Bachem AG, Bubendorf, Switzerland) with a molar activity of 50 MBq/nmol.

Data are shown as mean with standard deviation of three (mRNA expression levels and radiolabeled ^{111}In-DOTATATE uptake) or two (immunohistochemistry) independent experiments. Data were normalized to control values, all set at 100%.

*p<0.05, **p<0.01, ***p<0.001 according to one-way ANOVA analysis with Tukey post-hoc test after log transformation of data.

HE = hematoxylin eosin, SST_2 = somatostatin receptor type 2

An increase in the SST_2 staining intensity per cell was observed in BON1 and NCI-H727 cells ($p<0.01$), but not in GOT1 cells, possibly due to the high baseline SST_2 expression levels (Supplementary Figure S2). Meanwhile, an increase in SST_2 mRNA levels was seen only for the stronger hydralazine dose in BON-1 cells ($p<0.001$) and GOT1 cells ($p<0.05$), but hydralazine decreased mean (SD) SST2 mRNA expression levels in NCI-H727 cells by 15% (13), $p<0.05$. No changes in SST_2 protein expression and ^{111}In-DOTATATE uptake were seen following incubation with hydralazine in all cell lines. Combined treatment of valproic acid with the stronger hydralazine dosage led to an additional mean (SD) increase in SST_2 mRNA expression levels in BON-1 cells of 120% (72), $p<0.001$, while no additional effect was seen for GOT1 cells and even an inhibitory mean effect (SD) of 73% (34), $p<0.001$, was observed in NCI-H727. No synergistic or antagonistic effect on ^{111}In-DOTATATE uptake and SST_2 staining intensity per cell was observed for the combined treatment.

Our study shows, for the first time, that contrary to the promising *in vitro* and *in vivo* data on epigenetic upregulation of SST2 expression, epigenetic treatment did not translate into stimulation of ^{68}Ga-DOTATATE uptake in NET patients with low baseline SST expression.

This appears to be in contrast to the study with five SST-positive patients who received vorinostat-treatment for 4 days (5), but their observed change in the maximum standard uptake value (SUVmax) of 1.3 could lack clinical relevance. Combined these studies might imply that either epigenetic upregulation of SST_2 expression is only effective in patients with sufficient baseline ^{68}Ga-DOTATATE uptake, or the epigenetic effect depends on the epidrugs used or the drug levels achieved in patients are not sufficient to induce upregulation. The importance of choice and dosage of the epidrugs was shown by the effect of the DNMT inhibitor hydralazine, exhibiting only mild effects in pharmacologically unreachable dosages despite good efficiency observed in other tumors (7, 8). A possible future limitation for epigenetic treatment in NETs could also be the observed non-specific effect of increased renal uptake in our patients. Although change in uptake measures of up to 25% SUVmax between two scans have been described (9), the increase in renal uptake was seen in 78% of our patients in the second PET/CT. Since all patients underwent the same hydration protocol before the scan and no changes in kidney function were noted, this could signify that epigenetic treatment is not tumor specific and also activates basal expression of SST_2 in renal tissue (10).

In conclusion, short-term epigenetic treatment with valproic acid and hydralazine had no stimulating effect on ^{68}Ga-DOTATATE uptake in nine patients with well-differentiated NETs of various origin with low baseline SST expression, contradicting preclinical findings. Clinical trials with alternative epigenetic drugs or in patients with positive baseline SST_2 expression may be able to clarify whether epigenetic treatment has a role in the treatment of NETs, however a potential increase in renal uptake should be closely monitored.

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SUPPLEMENTARY APPENDIX

METHODS

1. Clinical study

Study design and participants

This prospective proof-of-concept study was performed at the Erasmus Medical Center Rotterdam, the Netherlands from 07/2019 until 06/2021. Eligible patients were ≥18 years of age, had an inoperable or metastatic NET with well-differentiated histology grade 1, 2 or 3 and low SST uptake on ^{68}Ga-DOTATATE PET scan. Patients with hypotension (systolic blood pressure <90 mmHg), heart failure NYHA III-IV, creatinine clearance <50 ml/min, liver transaminases >3 times upper normal range, uncontrolled hormonal symptoms including severe diarrhea, serum albumin concentration <25 g/L, epilepsy or existing drug treatment which could not be stopped and interacted with the study medication were excluded.

Study procedures and assessments

Patients were recruited from the NET clinic at the ENETS Center of Excellence, Erasmus MC in Rotterdam. After signing the informed consent, screening included a medical questionnaire, physical examination and blood sampling. If ^{68}Ga-DOTATATE PET/CT had been performed in a different institute or >3 months before inclusion, it was repeated during screening.

The primary endpoint was the percentage of patients with an increase in uptake of ≥1 point ^{68}Ga-DOTATATE in the tumor lesions according to a predefined uptake scale. Grade 1 uptake was below the liver, grade 2 similar to the liver, grade 3 higher than the liver and grade 4 higher than uptake in spleen/kidneys. Pre-specified secondary endpoints included the change in tumoral ^{68}Ga-DOTATATE uptake as well as physiological uptake of liver, kidneys and spleen as measured by peak standardized uptake value (SUV), and impact of epigenetic treatment on clinical and laboratory parameters.

Adverse events were registered according to Common Terminology Criteria for Adverse Events (CTCAE) version 5.0.

Imaging and radiological assessment

^{68}Ga-DOTATATE was prepared locally in our institute. PET images were acquired on a Siemens Biograph mCT PET/CT scanner (Siemens Healthineers, Erlangen, Germany).

Quantitative assessment of lesions and physiological uptake was performed on Hermes Hybrid Viewer software (V 2.6D Hermes medical solutions, Stockholm) software.

2. In vitro experiments with NET cell lines

For the cell line experiments, the human pancreatic NET cell line BON-1 (kind gift of Dr. Townsend, University of Texas, Medical branch, Galveston, TX, USA), the human midgut NET cell line GOT1 (kind gift of Ola Nilsson, Sahlgrenska Cancer Center, University of Gothenburg, Sweden) and the human pulmonary carcinoid cell line NCI-H727 (ATCC CRL-5815) were used.

Cell Culture

BON-1 cells were cultured in DMEM/F-12 (1:1) supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 1.25 mg/L fungizone, and 100 U/ml penicillin; GOT-1 cells were cultured in RPMI medium 1640 supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1.0 g/L insulin, 0.55 g/L transferrin, and 67 μg/L selenite; NCI-H727 cells were cultured in RPMI medium 1640 + L-glutamine supplemented with 10% (v/v) FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Once a week, BON-1 and NCI-H727 cells were trypsinized using 0.05% (v/v) trypsin + 0.53 mM EDTA and fresh medium was added on day four. GOT1 cells were trypsinized every two weeks using 0.05% (v/v) trypsin + 0.53 mM EDTA supplemented with DNAse (2 U/mL) with medium refreshment after one week.

Epigenetic treatment and evaluation

Valproic acid sodium salt (VPA; Sigma-Aldrich, Zwijndrecht, The Netherlands) and hydralazine (Hydralazine HCl; Selleckchem.com) were dissolved in the according cell line culture media. Dose-response studies were performed based on a 7-day treatment schedule. One day before the start of the epigenetic treatment, cells were plated in 24-well plates.

Epigenetic treatment: Cells were plated in T75 flasks on day zero. VPA and hydralazine, alone or in combination, were added on day 1 at their IC_{50} growth inhibitory concentrations and at the maximum treatment dosage used in patients (equal to IC_{50} dosage for VPA, lower dosage for hydralazine). Medium without or with drugs was refreshed on day 3. On day 5, cells were trypsinized and plated for further analysis. Exactly 4 hours after cell plating, the drugs were added again. On day 7, samples were collected for RT-qPCR analysis (24-well plates) and for internalization studies (12-well plates). For immunohistochemistry, cells were plated in chamber slides after pre-treatment with poly-L-lysine.

mRNA-Analysis: After lysis, cells were incubated with oligo$(dT)_{25}$ dynabeads (Invitrogen, Breda, The Netherlands) to isolate poly-A+ mRNA, as described previously(1). 23 μL H_2O

was added for elution, and 10 µL poly-A+ mRNA was used in the next steps. Poly-A+ mRNA was converted into cDNA using the commercial RevertAid First Strand cDNA synthesis kit (Thermo Scientific, Breda, The Netherlands). cDNA was also prepared without the addition of RevertAid Reverse Transcriptase to exclude DNA contamination. Samples were diluted by adding 180 µL H_2O. Afterwards 5 µL sample was mixed with 7.5 µL Taqman Universal PCR mastermix (Applied Biosystems, Breda, The Netherlands) supplemented with primers and probes. SST_2 expression was determined relative to three housekeeping genes (HKGs). For analysis, the QuantStudio 7 Flex RT-qPCR system with QuantStudio Real-Time PCR software v1.5 was used. The number of copies for SST_2 and all HKGs was calculated by the efficiency factor to the power of ΔCt (i.e., 40 minus measured Ct). Subsequently, the relative SST_2 expression was calculated by dividing the number of SST_2 copies by the geometric mean of all HKGs.

Immunohistochemistry: Cells were fixed with 4% paraformaldehyde for 20 minutes, before incubating them with 50% methanol for 3 minutes and 100% methanol for 3 minutes. Then, cells were permeabilized (0.1% triton X100 detergent in 1x PBS) for 15 minutes, and blocked (1% BSA) for 1 hour at room temperature (RT). Rabbit monoclonal anti-SST2 IgG (NB-49-015, 1:25 dilution, NeoBiotech, Nanterre, France) was added (overnight, 4° C). Finally, the cells were incubated for 30 min at RT with HRP/anti-Rabbit/Mouse (Dako Detection System). Bound antibodies were visualized by incubation with freshly prepared DAB (Dako Detection System). For negative controls, primary antibody was omitted. Slides were counterstained with hematoxylin and mounted. Five locations per slide were used to assess the SST_2 staining intensity per cell, using a 10x magnification and the CellProfiler software (version 4.0.7, www.cellprofiler.org).

^{111}In-DOTATATE radiolabeling and internalization studies: DOTATATE (Bachem AG, Bubendorf, Switzerland) was radiolabeled with $^{111}InCl_3$ (Curium Pharma, Petten, The Netherlands) as previously described(2). Internalization studies were performed as previously described(3). Cells were incubated with internalization medium (DMEM (1x)–GlutaMAX-I, 1% (wt/v) BSA, and 20 mM HEPES (pH 7.4)) supplemented with 10^{-9} M ^{111}In-DOTATATE (50 MBq/nmol), with or without 10^{-6} M unlabeled DOTATATE, for 4 hours. Following incubation, the excess of unbound radiotracer was removed, and the membrane-bound and internalized radioactivity were determined. The protocol was adjusted for GOT1 cells due to insufficient cell adherence and included the collection of non-adherent cells (pelleted by centrifugation). For GOT1 cells, the total uptake was determined. Cell pellets of additional wells were collected and DNA content was measured as described above, to correct for possible differences in cell numbers.

3. Statistical analysis

This proof-of concept clinical trial aimed to include 10 patients. Twelve patients were enrolled in the study. Two patients failed screening because of sufficient uptake on ^{68}Ga-DOTATATE PET and other tumor diagnosis than NET, respectively. Ten patients started study treatment, but one patient withdrew from the study after one week due to adverse events.

Descriptive statistics were used to characterize clinical, laboratory and radiological data, summarized by median and inter-quartile-range (IQR) or mean and standard deviation (SD), categorical variables by frequency and percentages.

Statistical analyses were performed using SPSS version 25.0 (IBM Corp., Armonk, NY) and GraphPad Prism7. A two-sided significance level of 0.05 was set for every analysis.

RESULTS

Safety

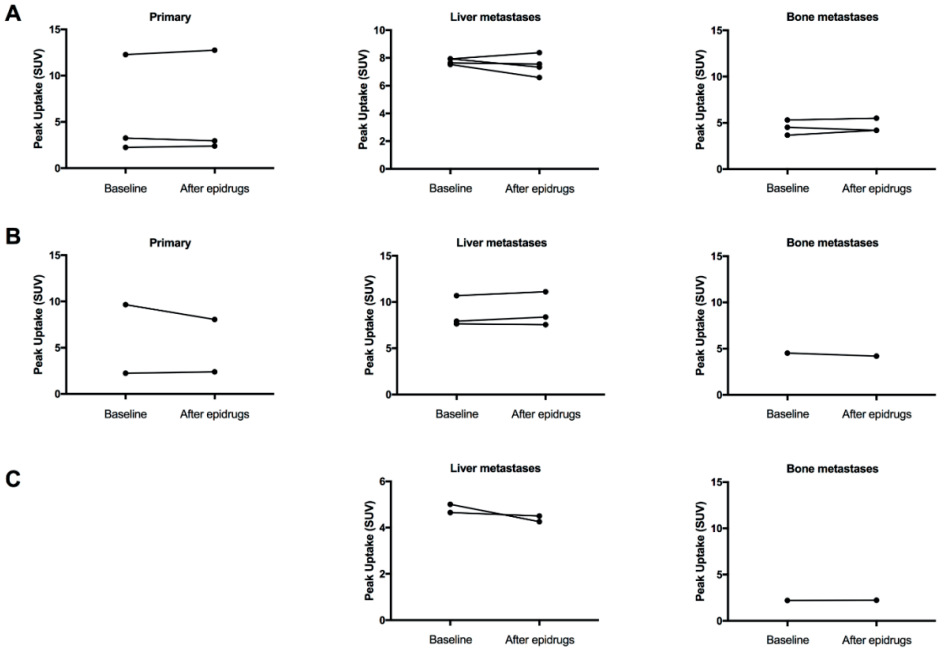
A total of 18 adverse events occurred during the observation phase in ten patients of the intended to treat analysis set of which 14 were judged to be related to the study intervention. Five events were known effects of valproic acid treatment and involved neurocognitive symptoms, tiredness and/or nausea. Five other adverse events were classified as hydralazine-related and involved palpitations, hypotonia and/or water retention. One patient developed an exanthema and one suffered from glucose dysregulation, requiring adjustment of insulin treatment. The patient who stopped treatment due to side effects suffered from nausea with vomitus, headaches and generalized aches.

Cell line experiments

Basal SST_2 expression, SST_2 protein levels and ^{111}In-DOTATATE uptake

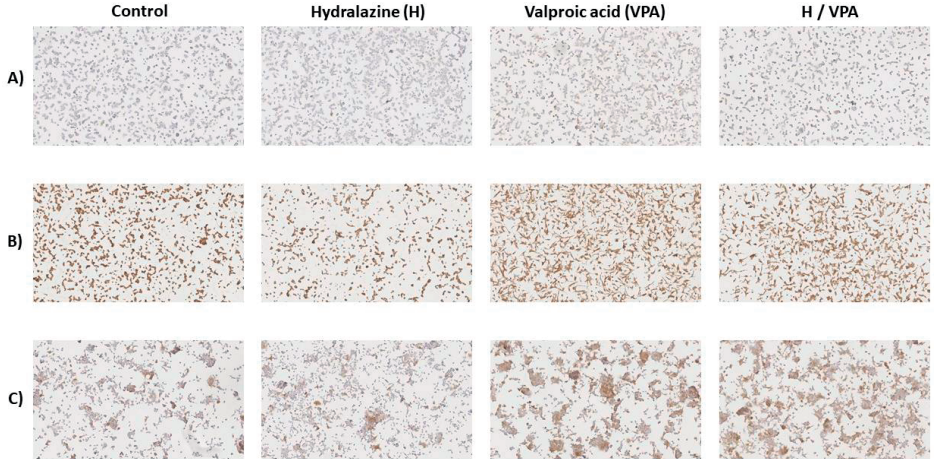
BON-1 cells showed the lowest mean (SD) SST_2 expression level of 0.0046 (0.001) (corrected for the geometric mean of three HKGs), followed by the NCI-H727 cells (0.0054 (0.001)) with the highest levels being measured in the GOT1 cells (0.142 (0.025)). Mean (SD) SST_2 staining intensity per cell was also lowest for BON-1 cells with 7.95 (7.61), again followed by the NCI-H727 cells with 13.43 (4.72) and showing the highest levels for GOT1 cells with 91.83 (6.89), *Figure S2*. A similar trend was observed for the mean (SD) uptake of ^{111}In-DOTATATE, which was 4.23 (1.86), 21.51 (6.09) and 327.0 (20.95) % added dose per milligram DNA for the three cell lines, respectively.

Supplementary Figure S1



Supplementary Figure S1 Change in peak uptake of ^{68}Ga-DOTATATE on PET/CT at baseline and after 2-week epigenetic treatment in patients with neuroendocrine tumors with low somatostatin receptor expression according to their origin: A) lung, B) pancreas, C) small-intestine.

Supplementary Figure S2



Supplementary Figure S2 Immunohistochemistry of somatostatin receptor subtype 2 in A) BON-1, B) GOT1, C) NCI-H727 shown in an untreated control group (control) and following epigenetic treatment with hydralazine (50 μmol/L), valproic acid (1 mmol/L) or the combined treatment of hydralazine / valproic acid (H / VPA).
Pictures were taken at 100x magnification.

PART 3

Summary and general discussion

11 General Discussion

GENERAL DISCUSSION

Neuroendocrine cells are characterized by their ability to synthesize and secrete hormones and peptides in response to neuronal stimulation, thereby linking the nervous and endocrine systems (1). They are distributed throughout the body and form major organs such as the pituitary gland, but are also scattered throughout the gastrointestinal tract and lungs, forming the diffuse neuroendocrine system (2). Neuroendocrine cells are intimately involved in the regulation of hormonal pathways, with disruptions leading to various symptoms and disorders (1). Among the most important are disorders of the hypothalamic-pituitary axis and tumors arising from neuroendocrine cells, so-called neuroendocrine neoplasms (NENs). NENs are commonly localized in the pituitary, gastroenteropancreatic and bronchopulmonary system (3, 4).

Due to the diversity and complexity of neuroendocrine disorders, their diagnostic evaluation and treatment are often inadequate. In the following, opportunities for improvement are discussed.

The interplay of neuroendocrine disorders and water-salt homeostasis

Although hyponatremia is the most common electrolyte disorder, it is often considered clinically irrelevant. This was disturbingly highlighted by a registry analysis in 2015 (5), which showed that appropriate laboratory tests to assess etiology were performed in less than half of patients hospitalized with hyponatremia, and the majority of patients were still hyponatremic at discharge. That chronic hyponatremia is not benign has been demonstrated in several observational studies that showed an association with various clinical complications such as attention deficits, gait disturbances and falls (6, 7) as well as bone loss and fractures (8, 9). New evidence on this matter has been added with the study discussed in **chapter 2**. Here, it was again confirmed that even mild chronic hyponatremia leads to clinical symptoms as well as neurocognitive and neuromuscular impairment (10). Surprisingly, short-time treatment of hyponatremia failed to improve neuromuscular function and only improved neurocognition in sodium-normalized patients. This is probably due to the adaptive mechanisms of cells during hyponatremia. With the aim of counteracting hypotonicity, they adjust their intracellular solute content, causing them to lose osmolytes such as glutamate and taurine (11). This protects the cells from swelling, but also makes them vulnerable to neuronal impairment. To reverse these changes, the cells need to accumulate osmolytes again, which takes time and may explain why the improvement was seen only in the sodium-normalized patients.

In addition to hyponatremia leading to multiple symptoms, there is also a striking association with poorer prognosis in multiple diseases, higher mortality and hospital readmissions

(12-14). That hyponatremia also affects the outcome of NENs was shown in **chapter 3**. Here, the median overall survival of 57 NEN patients suffering from hyponatremia, before or during treatment with peptide receptor radionuclide therapy (PRRT), was significantly decreased compared to the 512 normonatremic patients (25 months compared to the 55 months) (15). While this result was expected based on the available evidence, the analysis in NEN patients with hypernatremia revealed the opposite. These 80 patients had a longer median overall survival of 94 months. Further analyses could not provide an explanation for this contradictory outcome. The hypothesis that PRRT-induced tumor lysis (16) may have led to urea-induced osmotic diuresis could not be confirmed, as no difference was found between groups in terms of treatment response. Dedicated studies investigating this possible PRRT-related effect are needed to further elucidate this issue.

Although there is a growing body of evidence linking hyponatremia to poorer patient outcomes, the data are mainly based on observational studies. Accordingly, the debate as to whether hyponatremia contributes directly to mortality or is more likely a surrogate marker for severity of the underlying disease is still open. The only way to address this question is to conduct a randomized intervention trial comparing the effect of targeted hyponatremia treatment with standard care - which, it has been shown, is often no treatment (5) - on hard endpoints such as 30-day mortality and rate of readmissions. With the support of an international study group involving nine tertiary hospitals, we are currently undertaking this challenging task with the goal of including 2278 hyponatremic patients (17). The first results are expected in 2024 and will have major implications for future hyponatremia guidelines. If the intervention results in a reduction in mortality and rehospitalization rates, awareness of hyponatremia will increase significantly and hopefully lead to better management. However, if the intervention shows no effect, hyponatremia may actually be a marker of disease severity rather than its cause.

Characteristics of neuroendocrine neoplasms influencing their outcome

In addition to dysnatremia, there are a number of other factors that are associated with the outcome of NENs. One of them is hyperammonemia, as discussed in **chapter 4**. Hyperammonemia results from inadequate hepatic clearance of ammonia (18). The liver is a crucial organ in NEN, as these mainly slow-growing tumors tend to develop liver metastases (19). Although these metastases are often quite extensive, reports of liver failure in NEN patients are rare. However, hyperammonemia may still occur, and few are aware of this complication. That hyperammonemia in NEN is a complication to be aware of was confirmed by the retrospective single center review of 44 NEN patients with documented hyperammonemia (20). This analysis revealed hyperammonemia presence to be a high-risk factor for death, with a median survival of only 1.7 months after diagnosis. Further analysis showed that the

scoring system used to classify the severity of liver disease - the Child-Pugh score - takes into account all relevant risk factors in these patients and could be used for early-on risk stratification. However, to really assess if early detection of hyperammonemia leads to outcome improvement in advanced stage NEN patients, a prospective study would be needed. In such a study, NEN patients with known liver metastases would be regularly assessed using the Child-Pugh score, with a high value triggering ammonia measurement. Because the degree of hyperammonemia does not determine the severity of symptoms (20), any hyperammonemia should be treated and affected patients should be carefully evaluated for possible signs of hepatic encephalopathy. In addition, the diagnosis of hyperammonemia should be followed by a diagnostic evaluation of its cause and an evaluation of its treatment. Based on current data, liver-specific procedures may be superior, but more data on different interventions and outcomes are needed.

Another important prognostic factor for the diagnosis and treatment of NENs is the sufficient expression of somatostatin receptors (SST) on the tumor cell surface. That NEN patients with insufficient SST expression (further on referred to as SST-negative) have a worse outcome has often been attributed to the fact that they are not eligible for PRRT and are less frequently treated with somatostatin analogues (SSA). However, the retrospective analysis of two large NEN databases described in **chapter 5** has shown that there is likely a difference in tumor biology (21). While the 77 SST-negative advanced NEN patients had a shorter median overall survival of 53 months compared to 131 months in the 248 SST-positive patients, this difference persisted after the two populations were matched using a propensity score approach correcting for age, gender, tumor grade and site of origin. These results imply that SST-negative tumors should be treated differently from SST-positive NENs. On the one hand, an earlier, more aggressive approach could counteract their worse outcomes. On the other hand, treatments targeting their biological differences could reverse their SST negativity. Accordingly, special attention should be paid concerning the regulation of SST expression in order to define new treatment targets. Such an approach is described in the **chapters 9** and **10**.

Improving diagnostic and therapeutic options for the syndrome of inappropriate antidiuresis

The syndrome of inappropriate antidiuresis (SIAD, formerly referred to as SIAD) is not only one of the main causes of hyponatremia, it is also the most difficult cause to diagnose. This is because several urine and blood tests are needed to make the diagnosis. In addition, the typical SIAD patient is elderly, polymorbid, and receiving various medications (5, 22) that can confound said parameters. This makes the development and evaluation of new treatments challenging. Accordingly, a new approach by means of generating an artificial SIAD model in healthy volunteers is discussed in **chapter 6**. This SIAD model enabled the study of changes

in biomarkers involved in water-salt homeostasis (23). Perhaps more importantly, however, it also allowed us to test the effect of a new therapeutic approach using the antidiabetic drug empagliflozin (**chapter 7**). Through the inhibition of the sodium glucose cotransporter 2 (SGLT2) in the proximal tubule of the kidneys, empagliflozin led to a significantly higher urine output in the 14 healthy volunteers with artificially induced SIAD (24). These results supported the hypothesis that empagliflozin-induced osmotic diuresis may indeed be a new therapeutic option for SIAD, which is characterized by an excess of free water. However, the observation period was too short to draw firm conclusions, and the intervention did not increase serum sodium levels. Because the observation period of the SIAD model could not be extended, which is a limitation of this approach, transfer to the clinic was warranted.

The outcome of this transfer is described in **chapter 8**, which discusses the results of a randomized, placebo-controlled, cross-over study in 14 patients with chronic SIAD-induced hyponatremia (22). In this study, a 4-week treatment with empagliflozin 25mg lead to a significant increase of 4.1 mmol/l in serum sodium levels compared to placebo. Importantly, treatment effect was not only limited to increasing sodium levels, but also improved neurocognitive impairment in the exploratory analyses. Interestingly, similar to the findings discussed in **chapter 2**, no major improvement in the neuromuscular function – assessed via gait analysis – was found. Again, only a minority of patients reached normonatremia. Empagliflozin thus appears to be a promising and safe treatment option for SIAD-related hyponatremia, but much remains to be done. First, empagliflozin treatment should be tested in a larger cohort of SIAD patient and as a treatment for any type of hypotonic hyponatremia. Second, the treatment effects on neurocognition should be confirmed. Third, the missing treatment effect on neuromuscular function should be further investigated. Ideally, a future intervention study would repeat the neuromuscular function test only after hyponatremia has been successfully corrected. Using this approach, improvements were demonstrated in the observational study by Renneboog et al. (6). We are currently addressing points 1 and 2 in the new multicenter intervention study EMPOWER (ClinicalTrials.gov Identifier: NCT04447911), in which hospitalized and outpatients with hyponatremia are randomized to receive treatment with empagliflozin or placebo for 30 days. In addition to studying the effect of empagliflozin on hypotonic hyponatremia of any cause, we can hopefully confirm its beneficial effect on neurocognition.

Improving diagnostic and therapeutic options for neuroendocrine neoplasms

As discussed in **chapter 5**, special research focus should be put on the mechanisms behind SST subtype 2 (SST_2) expression in well-differentiated NENs, called neuroendocrine tumors (NETs). According to preliminary data in pancreatic NETs, DNA methylation and histone acetylation play a key role there, with SST_2 promoter methylation showing an inverse cor-

relation and the histone mark H3K9Ac showing a positive correlation with SST_2 mRNA expression (unpublished data of LJ Hofland et al). **Chapter 9** discussed the role of these epigenetic modifications in small intestinal (SI) NET samples. The analyses revealed that SI-NETs have lower SST_2 promoter methylation levels and lower H3K27me3 methlyation levels compared to normal SI-tissue. However, while negative correlations between SST_2 mRNA expression level and mean level of DNA methylation within the SST_2 promoter region was found, no correlation was found between histone modification marks and SST_2 expression. While our data confirm the importance of epigenetics in SI-NET tumorigenesis, the regulation of SST_2 expression appears to be mainly through DNA methylation. One possible explanation for this divergent finding compared with pancreatic NETs could be that NETs of different origins have different epigenetic control mechanisms of the SST_2 promoter. That DNA methylation is an important factor in tumorigenesis of NETs is consistent with recent data showing tissue- and tumor-specific distribution of DNA methylation across the genome. (25). The collection of genome-wide DNA methylation profiles enabled a precise entity-specific diagnostic classification within a broad spectrum of tumors (25-27). Such an entity-specific classification is currently missing for NENs. That this could be a promising approach was shown by our preliminary data on global promoter methylation patterns of SI-NET samples compared with the matching normal SI tissue samples (*Figure 1*).

Although it could be argued that a true comparison would require enteroendocrine cells, this initial evaluation already demonstrated the future possibilities.

With the help of a machine learning approach, using an algorithm that can improve automatically through experience and the use of data (26, 28), DNA methylation could be used to define a specific profile for any NENs, including NENs of unknown origin. We are currently testing this approach in the ongoing NEMESIS trial (NCT05013957), in which DNA methylation analyses are performed in gastroenteropancreatic and lung NETs, matched to outcome data, and added to the open-access online epigenomic diagnostics portal at www.EpiDiP.org, which uses an unsupervised machine learning algorithm. Since the algorithm needs a certain amount of samples to classify them correctly, first results are expected in 2024.

Several *in vitro* and *in vivo* studies have demonstrated an increase in SST_2 expression levels by reducing DNA methylation and increasing histone acetylation levels of the SST_2 gene promoter region in NET cell lines (29-32). This provided the basis for a clinical trial in patients (**Chapter 10**). Using the anti-epileptic drug valproic acid, a histone deacetylase (HDAC) inhibitor, and the DNA methyltransferase (DNMT) inhibitor hydralazine, 9 patients with advanced well-differentiated NETs of the lung, pancreas, intestinal, rectum und thymus and low baseline SST_2 expression were treated for 14 days (33). Unfortunately, epigenetic treatment did not result in an increase of SST_2 expression as measured by in vivo

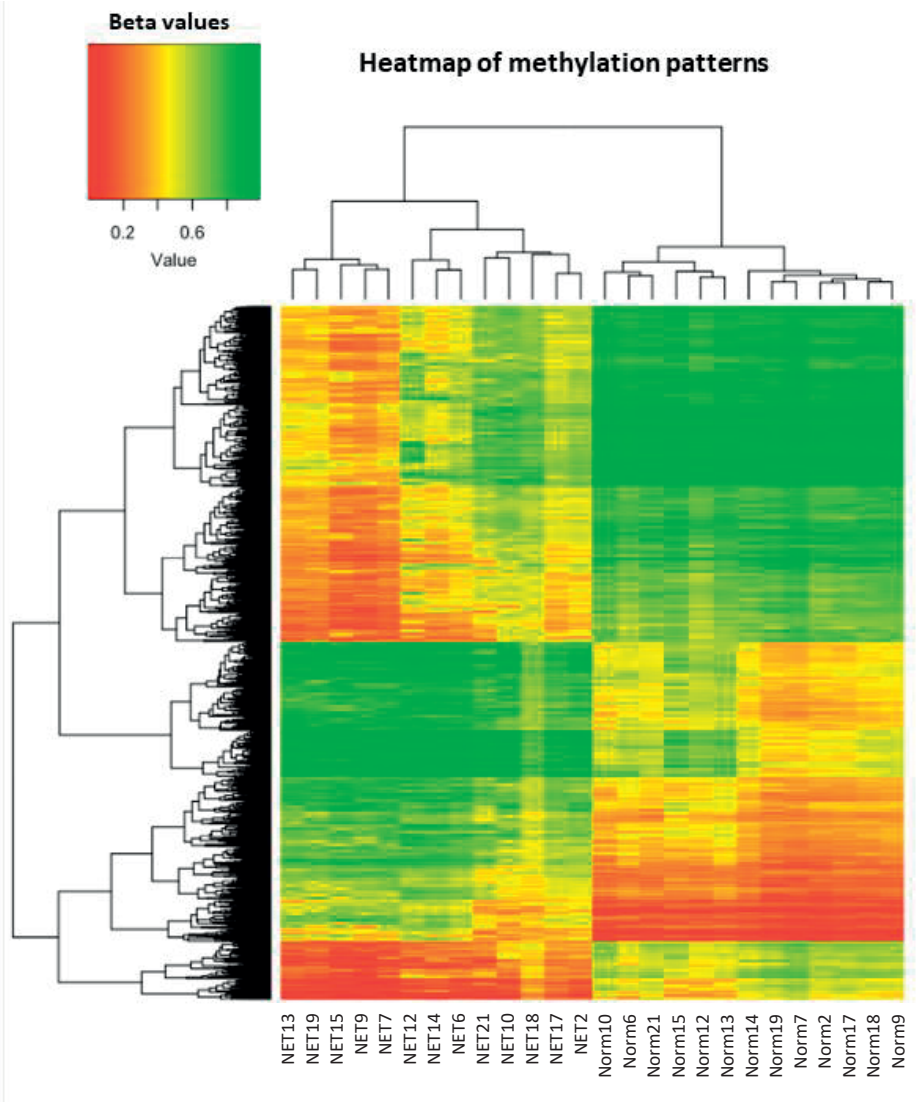


Figure 1: DNA methylation distribution of small intestinal neuroendocrine tumor (NET) samples with their corresponding normal (Norm) small intestinal tissue according to unsupervised clustering. The color range reaches from sites with low methylation (red) to highly methylated sites (green). NET = small intestinal neuroendocrine tumor, Norm = normal small intestinal tissue

^{68}Ga-DOTATATE uptake in tumor lesions, contradicting the in vitro data. However, a 27% increase in kidney uptake was observed. Simultaneously, another preclinical study showed that in contrary to the *in vitro* results, the observed increase in tumoral ^{177}Lu-DOTATATE uptake *in vivo* after treatment with valproic acid was not caused by SST_2 upregulation, but by increased ^{177}Lu-DOTATATE circulation time and renal toxicity (34). While the only

other clinical trial involving five well-differentiated SI-NET patients with sufficient ^{68}Ga-DOTATOC uptake on baseline PET scan showed an 11% uptake after a 4-days treatment with the HDAC inhibitor vorinostat (35), the average change in SUVmax across all patients was only 1.3, calling into question its clinical significance. Of note, when the analysis was repeated on liver lesions with lower uptake, no effect was seen. Accordingly, there are three possibilities regarding the effect of epigenetic treatment to upregulate SST_2 expression: first, it is only efficacious in tumors that already express SST_2. This hypothesis is contradicted by the in vitro data, which showed the least upregulation in the cell line with the highest baseline expression. Second, although the same therapeutic dosages were used in the clinical trial as in the cell line experiments, the intertumoral dose may have been too low to induce epigenetic changes. Since the dosages cannot be further increased due to dose limiting toxicity, another agent with better cell uptake would need to be evaluated. Third, the regulation of SST_2 expression is more complex than an on/off switch, and SST-negative tumors seem to have a different tumor biology. This would then require a new diagnostic stratification of these tumors and a separate evaluation of therapeutic options. Accordingly, new epigenetic agents should still be studied *in vitro* but should be translated to the clinic more quickly, as there seems to be a discrepancy between preclinical and clinical effects. In addition, NENs with low SST expression should be further investigated for differences in tumor biology.

Additional expected future developments in the diagnosis and treatment of neuroendocrine disorders

Regarding disorders of salt-water homeostasis, many developments in diagnostic and therapeutic procedures can be expected in the coming years. For example, the results of our diagnostic multicenter study (CARGOx, NCT03572166) will determine which stimulation test will be used to diagnose AVP deficiency in the future. In addition, several studies are currently investigating stimulatory tests as well as the use of oxytocin substitution in patients with AVP deficiency (NCT04648137; NCT04789148; NCT04897802). Oxytocin, also known as the bonding hormone, promotes feelings of intimacy and closeness and is synthesized and secreted through the same anatomical pathways as AVP. Recent data (36) indicated decreased oxytocin levels in AVP-deficient patients. Because many patients with AVP deficiency suffer from impaired quality of life despite balanced salt water homeostasis (37), such treatment could alter the well-being of many of them.

In the field of hyponatremia the development of a urea product with better palatability led to a re-evaluation of this therapeutic approach. Three recent studies confirmed the efficacy of urea in SIAD-induced hyponatremia and were effective even in the difficult-to-treat cases with fluid-restriction-refractory hyponatremia and tumor-induced SIAD (38-40). While long-term safety data are still missing, this offers a further cost-effective extension of the therapeutic measures. In addition, another prospective study (NCT04987385) is currently

investigating whether SIAD patients might benefit from higher protein intake instead of urea administration. Based on early animal data (41), a high-protein diet could increase serum urea concentrations, leading to osmotic diuresis with corresponding hyponatremia amelioration. With regard to the elderly polymorbid SIAD-patients (10, 22), this study will further pursue the holistic approach that is urgently needed in this population. Finally, several trials are aiming at prevention of hyponatremia instead of its correction. This includes the currently evaluated recommendation to limit fluid intake after pituitary surgery to avoid delayed hyponatremia (NCT03636568) - a phenomenon observed after trauma to the pituitary stalk or posterior pituitary (42). Another study aims to prevent thiazide-induced hyponatremia by measuring urinary prostaglandins, which could indicate patients at risk for this common cause of hyponatremia (NCT05542056).

In conclusion, although widespread recognition of the importance as well as proper management of salt-water balance disorders are still unsatisfactory, current research is moving in a much-needed patient-oriented direction. The pending studies will impact and likely positively influence future diagnostic and treatment guidelines.

Several improvements are also emerging in the management of NENs. Since molecular imaging and PRRT are the cornerstones of NEN diagnosis and treatment, this outlook will focus on them.

The search for the optimal imaging technique has triggered rapid development and improvement of this field. While ^{68}Ga-DOTA-PETs use SST agonists to visualize NENs (43), an alternative approach is the use of SST antagonists. The advantage of SST antagonists is that, unlike SST agonists, they are not restricted to the active SST receptors, resulting in multiple binding sites and consequently increased tumor uptake (44). The so developed new PET-tracer ^{68}Ga-OPS202 showed a higher lesion based overall sensitivity and higher detection rate of liver metastases than ^{68}Ga-DOTATOC (45, 46). Another alternative imaging technique lies in the use of copper radioisotopes in combination with SST agonists or antagonists. The long half-life of around 13 hours of ^{64}Cu is convenient in clinical practice as it opens the scanning window to up to 3 hours (47). In addition, its ability to build stable complexes to different chelators and its potential for higher spatial resolution due to its decay mode are additional advantages. Accordingly, ^{64}Cu-SST-PET might become a diagnostic alternative for NEN centres without access to ^{68}Ga or which require their supply on long distance. ^{18}F-Fluorodeoxyglucose (^{18}F-FDG) has emerged as a diagnostic option for patients with negative lesions on SST-PET, particularly in poorly differentiated NENs whose high glucose turnover rate can be visualized with ^{18}F-FDG PET (48). The combined use of SST

PET and FDG PET imaging has been proposed for the thorough evaluation of patients with NENs (49, 50), but is currently rarely used because of financial and radiation limitations. The fibroblast activation protein (FAP) is a serine proteinase which is overexpressed on the cell surface of cancer-associated fibroblasts in tumor stroma (51). FAP-specific inhibitors (FAPI) have recently been developed as radioligands for PET imaging with excellent image contrast (52, 53). Because SI-NENs are characterized by extensive fibrosis surrounding the primary tumor and mesenteric metastases leading to bowel obstruction and ischemic complications (51), FAPI-based PET imaging may be of particular interest in these patients.

Because NENs can be diagnosed, localized, and treated with the same peptide alternatively labeled with a diagnostic or therapeutic radioisotope (theranostic approach), therapeutic options with PRRT are also evolving. Concerning the therapeutic counterpart for the SST antagonist, the radiotracers ^{177}Lu-DOTA-JR11 and ^{177}Lu-DOTA-LM3 are being evaluated (54-57). Since SST antagonists show no internalization, their effect is focused on the tumor cell membrane. A phase I study in 20 patients with advanced well-differentiated NEN using ^{177}Lu-DOTA-JR11 showed promising results with a complete response in 1 (5%), a partial response in 8 (40%), and stable disease in 8 (40%) patients. However, the protocol had to be modified during the study to limit cumulative bone marrow uptake because grade 4 hematologic toxicity occurred in 57% (4 of 7) patients after the second cycle (58).

Additional data to further evaluate optimal dosing and treatment schedule are currently pending. At the University Hospital Basel, Switzerland, ^{177}Lu-DOTA-JR11 is used as add-on treatment in NET-patients with early progression after standard PRRT. Dosimetry and treatment outcome data for the first fifteen patients are expected in mid-2023.

Because the efficacy of PRRT needs to be improved to go beyond disease stabilization to cure, other radiopharmaceuticals that may be more effective are being investigated. Such an alternative could be alpha particles since they have a high particle energy and short range, thereby ensuring target damage (59). First clinical data using Bismuth-213 or Actinium-225 on patient's refractory to standard PRRT showed promising results leading to partial remission or stable disease (60, 61). However, further studies are needed as the use of these radionuclides may be challenging due to their decay mode, which carries the risk of side effects, and complicated production. Another way to improve the efficacy of PRRT may be to combine the beta-emitting radioisotope terbium-16 with SST antagonists. Terbium-161 has the advantage to co-emit conversion and Auger electrons (62, 63). First preclinical data showed promising results of this combined use (64) and a randomized, cross-over, open label phase 0 study, comparing the dosimetry of ^{177}Lu-DOTATOC and ^{161}Tb-DOTA-LM3 in the same patients is planned to start in march 2023 (NCT05359146). Lastly, the effect of PRRT could be improved by the concomitant rather than sequential administration of targeted

therapy or chemotherapy. However, these treatments carry the risk of hematologic toxicities, which limits their use (65, 66).

In summary, several diagnostic and therapeutic improvements for NEN patients can be expected in the coming years. However, it is important to emphasize that although the results of these smaller studies are promising, large randomized clinical trials are needed to make real progress. Unfortunately, such trials are still too infrequently conducted due to the larger patient numbers required, the organizational and the financial burden. The financial issue is of particular importance because outcomes such as progression-free survival or mortality are only available with a large time latency in these comparatively slow-growing tumors. Therefore, international collaborations and funding are needed. Such efforts are currently being encouraged by the professional societies and will hopefully pave the way for future collaborations.

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12 Summary

SUMMARY

Neuroendocrine disorders are complex and multifaceted, affecting not only their local environment but also other organ systems and hormone regulatory pathways. This has a major impact on patient relevant outcomes. Their holistic view is accordingly crucial when evaluating new diagnostic and therapeutic measures.

Outcome assessment and diagnostic evaluations of neuroendocrine disorders

Dysregulation of the neuroendocrine hypothalamic pituitary renal axis leads to an imbalance in the water sodium homeostasis. The most common electrolyte disorder resulting from this disturbance is hyponatremia. Despite its prevalence, it is considered by many physicians to be a non-relevant disorder, requiring diagnostic and therapeutic consideration only in its acute and severe form. However, as shown in **Chapter 2**, chronic hyponatremia – even the mild form – is symptomatic, leading to impaired neurocognitive and neuromuscular function. In this study comparing 19 hyponatremic patients to 10 healthy volunteers, patients scored lower in the neurocognitive and – muscular performance tests. Interestingly, 14-day treatment with fluid restriction and salt tablets lead to amelioration of symptoms in all patients but only improved neurocognitive function in sodium-normalized patients. This underlines the treatment indication in these patients.

That neuroendocrine disorders can have different impacts on involved organ systems was shown in **Chapter 3**. Here, the influence of hypo- or hypernatremia was evaluated on outcome of neuroendocrine neoplasm (NEN) patients undergoing treatment with peptide receptor targeted therapy (PRRT). While the median overall survival of the 57 hyponatremic patients was significantly shorter at 25 months compared to the 512 normonatremic patients at 55 months, the 80 hypernatremic patients had a longer median overall survival of 94 months. No additional associated factors were found to explain this difference, so further studies are needed to evaluate this opposite result.

That the outcome of NENs depends on several factors, was shown in **Chapter 4**. Because NENs are usually slow-growing tumors, they are prone to develop liver metastases. Although these metastases can be quite extensive, reports of liver failure are rare. However, hyperammonemia may occur in NEN patients with liver metastases due to inadequate hepatic clearance, but this NEN-specific complication might be under recognized in clinical practice. A systematic evaluation of 44 NEN patients with documented hyperammonemia showed that the presence of hyperammonemia was a high-risk factor for death, with a median survival of only 1.7 months after diagnosis. Further risk factors were hyperbilirubinemia, hypoalbuminemia, elevated international normalized ratio (INR), presence of liver insufficiency,

encephalopathy and ascites. The Child-Pugh score – known as a prognostic tool for patients with liver disease – summarizes these parameters and could be helpful to identify patients at risk of this underdiagnosed condition.

Another important factor for the diagnosis and treatment of NENs are somatostatin receptors (SST). Their sufficient expression on the surface of NENs is crucial for treatment with somatostatin analogues (SSA) and PRRT, and a correspondingly worse prognosis has been described for SST-negative patients. **Chapter 5** further analyses this issue by comparing the outcomes of 77 SST-negative and 248 SST-positive advance stage NEN patients. SST-negative patients had a significantly lower median overall survival of 53 months compared to 131 months in the SST-positive patients. To adjust for potential confounders, the two populations were matched using a propensity score approach taking into account age, gender, tumor grade and site of origin. Despite this adjustment, the worse outcome of the SST-negative patients persisted. Further analysis revealed differences in tumor biology as a likely explanation for the results.

Improving diagnostic and therapeutic options for neuroendocrine disorders

Physiologic studies in patients with SIAD-induced hyponatremia are difficult to perform because they are usually elderly polymorbid patients taking multiple medications, which may affect interpretation of the data. To tackle this problem, **chapter 6** discussed the opportunities of inducing an artificial SIAD-model in 14 healthy volunteers. Through the administration of desmopressin and fluid, mild hyponatremia was successfully induced in all participants and the changes of different biomarkers involved in salt water balance could be studied. This SIAD-model was then used to test a possible new therapeutic option for SIAD-induced hyponatremia. As described in **chapter 7**, the 14 healthy participants received the sodium glucose cotransporter 2 (SGLT2) inhibitor empagliflozin 25mg or placebo in a randomized cross-over trial, two hours after induction of the artificial SIAD. Empagliflozin induces glucosuria, which was hypothesized to induce osmotic diuresis. While no changes in serum sodium levels were observed after the 6-hour monitoring period, the hypothesis was confirmed with significantly higher total urinary excretion (579 ml vs. 367 ml) with empagliflozin. These results were the basis for the study of empagliflozin in patients with SIAD as conducted in the randomized, placebo-controlled crossover study of 14 patients with chronic SIAD-induced hyponatremia described in **chapter 8**. Patients received a 4-week treatment with empagliflozin 25mg/day or placebo in a randomized order with a wash-out period of at least two weeks between the treatments. At baseline and after each treatment cycle, different tests assessing neurocognitive and –muscular function were performed. Treatment with empagliflozin led to a significant increase in serum sodium of 4.1 mmol/L compared to placebo. In addition, improvement in neurocognitive testing was observed under empagliflozin in the

exploratory analyses. With the treatment being well-tolerated, empagliflozin might become a new treatment option for patients with SIAD.

Taking the focus back to NENs, **chapter 9** shed new light on the regulation of SST receptor expression in NETs. Using fresh frozen small intestinal NET samples, it was shown that DNA methylation and histone acetylation surrounding the *SST* gene are associated with SST_2 expression, however this correlation was less clear than expected. This could signify, that NETs are more diverse than previously thought, calling for an individual diagnostic and treatment approach.

Lastly, **chapter 10** brought the preclinical work to the clinic. In a proof of concept trial, nine advanced NET patients with low SST expression were treated for 14 days with the histone deacetylase (HDAC) inhibitor valproic acid and the DNA methyltransferase (DNMT) inhibitor hydralazine. Unfortunately, this epigenetic treatment did not lead to an increase in tumor-uptake of ^{68}Ga-DOTATATE, contradicting the *in vitro* data. In addition, an increase in renal uptake was observed. While other potential epidrugs are on the horizon that will hopefully have a more specific effect on NENs, further studies on the differences in tumor biology of NENs are needed to better understand this complex disease.

12 Samenvatting

SAMENVATTING

Neuro-endocriene aandoeningen zijn complex en veelzijdig. Zij beïnvloeden niet alleen de lokale omgeving, maar ook andere organen en hormonale systemen. Dit heeft grote gevolgen voor de uitkomsten van patiënten. Een holistische visie is dan ook van belang bij de evaluatie van nieuwe diagnostische en therapeutische maatregelen.

Beoordeling van uitkomsten en diagnostische evaluaties van neuro-endocriene aandoeningen

Verstoring van de neuro-endocriene hypothalamus-hypofyse-nieras leidt tot een problemen met de water-natriumhomeostase. De meest voorkomende elektrolytenstoornis als gevolg van deze verstoring is hyponatriëmie. Ondanks de prevalentie ervan wordt het door veel artsen beschouwd als een niet-relevante stoornis, die alleen in acute en ernstige vorm diagnostische en therapeutische aandacht behoeft. Zoals aangetoond in **hoofdstuk 2**, is chronische hyponatriëmie - zelfs de milde vorm - echter symptomatisch en leidt het tot een verminderde neurocognitieve en neuromusculaire functie. In deze studie, waarin 19 hyponatremische patiënten werden vergeleken met 10 gezonde vrijwilligers, scoorden de patiënten lager op de neurocognitieve en neuromusculaire testen. Interessant is dat een 14-daagse behandeling met vochtbeperking en zouttabletten leidde tot verbetering van de symptomen bij alle patiënten, maar alleen de neurocognitieve functie verbeterde bij de natriumgenormaliseerde patiënten. Dit onderstreept het belang van behandeling bij deze patiënten.

Dat neuro-endocriene aandoeningen verschillende effecten kunnen hebben op betrokken orgaansystemen werd aangetoond in **hoofdstuk 3**. Hier werd de invloed van hypo- of hypernatriëmie geëvalueerd op de uitkomst van neuro-endocriene neoplasma (NEN) patiënten die een behandeling ondergingen met peptidereceptor radionucleide therapie (PRRT). Terwijl de mediane overleving van de 57 hyponatremische patiënten met 25 maanden significant korter was dan die van de 512 normonatremische patiënten met 55 maanden, hadden de 80 hypernatremische patiënten een langere mediane overleving van 94 maanden. Er werden geen bijkomende factoren gevonden om dit verschil te verklaren, zodat verdere studies nodig zijn om dit tegengestelde resultaat te evalueren.

Dat de uitkomst van NENs afhankelijk is van verschillende factoren, bleek in **hoofdstuk 4.** Omdat NENs meestal langzaam groeiende tumoren zijn, zijn ze vaak geassocieerd met de aanwezigheid van levermetastasen. Alhoewel deze uitzaaiingen vrij uitgebreid kunnen zijn, zijn meldingen van leverfalen zeldzaam. Bij NEN patiënten met levermetastasen kan echter hyperammonemie optreden als gevolg van onvoldoende leverklaring, maar deze NEN-specifieke complicatie wordt in de klinische praktijk mogelijk onvoldoende onderkend. Een systematische evaluatie van 44 NEN patiënten met gedocumenteerde hyperammonemie

liet zien dat de aanwezigheid van hyperammonemie een risicofactor was voor vroegtijdig overlijden, met een mediane overleving van slechts 1,7 maanden na diagnose. Andere risicofactoren voor overlijden waren hyperbilirubinemie, hypoalbuminemie, verhoogde internationale genormaliseerde ratio (INR), aanwezigheid van leverinsufficiëntie, encefalopathie en ascites. De Child-Pugh score - bekend als prognostisch instrument voor patiënten met een leverziekte - vat deze parameters samen en zou nuttig kunnen zijn om patiënten te identificeren die risico lopen op deze ondergediagnosticeerde aandoening.

Een andere belangrijke factor voor de diagnose en behandeling van NENs zijn somatostatinereceptoren (SST). Hun voldoende expressie op het oppervlak van NENs is belangrijk voor behandeling met somatostatine-analogen (SSA) en PRRT, en voor SST-negatieve patiënten is een slechtere prognose beschreven. In **hoofdstuk 5** wordt deze kwestie verder geanalyseerd door de uitkomsten van 77 SST-negatieve en 248 SST-positieve uitgezaaide NEN patiënten te vergelijken. SST-negatieve patiënten hadden een significant lagere totale overlevingstijd van 53 maanden vergeleken met 131 maanden bij de SST-positieve patiënten. Om te corrigeren voor potentiële verstorende factoren werden de twee populaties gekoppeld met behulp van een propensity score benadering, rekening houdend met leeftijd, geslacht, tumorgraad en plaats van herkomst. Ondanks deze aanpassing bleef de slechtere uitkomst van de SST-negatieve patiënten bestaan.Verdere analyse wees uit dat verschillen in tumorbiologie een waarschijnlijke verklaring vormen voor de resultaten.

Verbetering van de diagnostische en therapeutische opties voor neuro-endocriene aandoeningen

Fysiologische studies bij patiënten met SIAD-geïnduceerde hyponatriëmie zijn moeilijk uit te voeren omdat het meestal oudere polymorbide patiënten zijn die meerdere medicijnen gebruiken, wat de interpretatie van de gegevens kan beïnvloeden. Om dit probleem aan te pakken werden in **hoofdstuk 6** de mogelijkheden besproken om een kunstmatig SIAD-model te induceren bij 14 gezonde vrijwilligers. Door toediening van desmopressine en vocht werd bij alle deelnemers met succes een milde hyponatriëmie opgewekt en konden de veranderingen van verschillende biomarkers die betrokken zijn bij de zoutwaterbalans worden bestudeerd. Dit SIAD-model werd vervolgens gebruikt om een mogelijke nieuwe therapeutische optie voor SIAD-geïnduceerde hyponatriëmie te testen.

Zoals beschreven in **hoofdstuk 7**, kregen de 14 gezonde deelnemers de natrium-glucose cotransporter 2 (SGLT2) remmer empagliflozin 25 mg of placebo in een gerandomiseerde cross-over proef, twee uur na start van de kunstmatige SIAD. Empagliflozin induceert glucosurie, waarvan werd aangenomen dat het osmotische diurese zou bevorderen. Alhoewel na de controleperiode van 6 uur geen veranderingen in het serumnatriumgehalte werden waar-

genomen, werd de hypothese bevestigd met een significant hogere totale urine-uitscheiding (579 ml vs. 367 ml) met empagliflozin.

Deze resultaten vormden de basis voor de studie van empagliflozin bij patiënten met SIAD, zoals uitgevoerd in de gerandomiseerde, placebogecontroleerde cross-overstudie van 14 patienten met chronische, door SIAD veroorzaakte hyponatriëmie, beschreven in **hoofdstuk 8**. De patiënten kregen een 4-weekse behandeling met empagliflozin 25mg/dag of placebo in een gerandomiseerde volgorde met een wash-out periode van ten minste twee weken tussen de behandelingen. Op de basislijn en na elke behandelingscyclus werden verschillende testen ter beoordeling van de neurocognitieve en neuromusculaire functie uitgevoerd. Behandeling met empagliflozin leidde tot een significante stijging van het serumnatrium met 4,1 mmol/L in vergelijking met placebo. Bovendien werd onder empagliflozin in de verkennende analyses een verbetering van de neurocognitieve tests waargenomen. Aangezien de behandeling goed wordt verdragen, zou empagliflozin een nieuwe behandelingsoptie kunnen worden voor patiënten met SIAD.

Terugkerend naar NENs, wierp **hoofdstuk 9** nieuw licht op de regulatie van SST receptor expressie in NEN's. Met behulp van vers ingevroren dunne darm NEN monsters werd aangetoond dat DNA methylering en histon acetylering rond het SST gen geassocieerd zijn met SST_2 expressie, maar deze correlatie was minder duidelijk dan verwacht. Dit zou erop kunnen wijzen dat NEN's diverser zijn dan eerder werd gedacht, waardoor een individuele diagnose en behandeling nodig is.

Hoofdstuk 10 ten slotte bracht het preklinische werk naar de kliniek. In een proof-of-concept studie werden negen gevorderde NEN patiënten met lage SST expressie gedurende 14 dagen behandeld met de histon deacetylase (HDAC) remmer valproïnezuur en de DNA methyltransferase (DNMT) remmer hydralazine. Helaas leidde deze epigenetische behandeling niet tot een toename van de tumoropname van ^{68}Ga-DOTATATE, hetgeen in tegenspraak is met de in vitro gegevens. Bovendien werd een toename van de renale opname ^{68}Ga-DOTATATE waargenomen. Hoewel andere potentiële epigenetische medicijnen in het verschiet liggen die hopelijk een meer specifiek effect hebben op NENs, zijn verdere studies naar de verschillen in tumorbiologie van NENs nodig om deze complexe ziekte beter te begrijpen.

13

- List of publications
- PhD portfolio
- Curriculum vitae
- Acknowledgements

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*equally contributing first authors

Co-authorships

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PHD PORTFOLIO

Erasmus MC Rotterdam	Internal Medicine, Section Endocrinology
University Hospital Basel	Internal Medicine, Department Endocrinology
Research Period	2017-2021
Promotors	Prof. W.W. de Herder / Prof. Mirjam Christ-Crain
Co-promotor	Dr. Johannes Hofland

Erasmus General Academic Courses:	**Year**	**ECTS**
Introduction in GraphPad Prism Version 7	2019	0.3
CC02A Biostatistical Methods I	2019	2
Basistraining OpenClinica	2019	0.5
Basiscursus Stralingsbescherming (Introduction, open / closed radioactive sources (1.5 day)	2019	1
Basiscursus Survial Analysis	2020	0.6
Basis Course on R	2021	2
Gene expression data analysis using R	2021	2
Scientific Integrity	2021	0.3
Clinical Courses and meetings		
European Congress of Endocrinology (ECE) 2017	2017	1
Annual Swiss Endocrine Society Meeting (SGED) 2017	2017	1
Post-graduate course 15th annual ENETS conference	2018	1
ENETS 15th annual conference	2018	1
Annual Endocrine Society Meeting (ENDO) 2018	2018	1
Annual Swiss Endocrine Society Meeting (SGED) 2018	2018	1
European Endocrine Society Meeting (ECE) 2019	2019	1
Post-graduate course 16th annual ENETS conference	2019	1
ENETS 16th annual conference	2019	1
Annual Endocrine Society Meeting (eENDO)	2020	0.5
European Endocrine Society Meeting (eECE) 2020	2020	0.5
Annual Swiss Endocrine Society Meeting (SGED) 2020	2017	0.5
Erasmus MC neuroendocrine tumor board	2019-20	2
Erasmus MC neuroendocrine tumor meeting	2019-20	2
Conferences: oral presentations		
Annual Swiss Endocrine Society Meeting (SGED) 2017: Copeptin based Approach for Diabetes insipidus	2017	0.5
Annual Endocrine Society Meeting (ENDO) 2018: Copeptin based Approach for Diabetes insipidus	2018	0.5

Annual Swiss Endocrine Society Meeting (SGED) 2018:		
Arginine stimulated Copeptin	2018	0.5
European Endocrine Society Meeting (ECE) 2019:		
Empagliflozin treatment for SIAD	2019	0.5
Conferences: Poster presentations		
European Congress of Endocrinology (ECE) 2017:		
Effect empagliflozin in artificial SIADH	2017	0.5
Annual Swiss Endocrine Society Meeting (SGED) 2018:		
Differences in Plasma and Whole Blood Na Measurements	2018	0.5
Annual Swiss Endocrine Society Meeting (SGED) 2018:		
FGF21-levels in Polyuria-Polydipsia Syndrome	2018	0.5
eENETS 2020: Survival outcome SSTR-negative NET patients	2020	0.5
eENETS 2020: Epidrug induced upregulation SST2 in NET	2020	0.5
Science Days Internal Medicine Erasmus MC 2020:		
Survival outcome SSTR-negative NET patients	2020	0.5
ENETS 2021: Dysnatremia effect on NET treatment outcome	2021	0.5
Teaching activities		
Lectures for medical students / resident physicians 2017-2018	2017-18	1
Lectures for patients with diabetes mellitus type 1	2017-18	1
Lectures for internists Basel internist conventions 2017-2019	2017-19	1
Lecture for endocrinologists 24th ESE post-graduate course	2019	0.5
Lecture for endocrinologists 45th Erasmus Endocrinology Cursus	2019	0.5
Lecture for laboratory technician Uppsala	2019	0.5
Lecture endocrinologists Finnish Endocrine Meeting	2020	0.5
Total points:		**33.7**

ABOUT THE AUTHOR

Julie Refardt was born in Geneva, Switzerland, on July 28th 1982. After graduating from High School in St. Gallen, Switzerland, in 2001, she started medical school at the University of Basel, Switzerland. She graduated from medical school in 2008 with a thesis on white coat hypertension in the outpatient setting.

After completing her residency in several hospitals in the the region of Basel, Switzerland, she finished her specialization in internal medicine in 2015, followed by her specialization in endocrinology in 2017 at the University Hospital Basel, Switzerland.

In 2013 she started her career as a clinical researcher in the research team of Prof. Mirjam Christ-Crain, conducting investigator-initiated clinical trials in the field of water sodium disturbances. In 2019 she decided to broaden her research horizon by completing her PhD project with 2 years in one of the top European Centers for Neuroendocrine Tumors, the Erasmus Medical Center in Rotterdam, the Netherlands, under the guidance of Dr. Hans Hofland, Prof. Wouter W. de Herder and Prof. Leo Hofland.

Returning in 2021 to the University Hospital Basel, Switzerland, as a senior physician, she became a research group leader and continued her research on neuroendocrine disorders.

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