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ORIGINAL SCIENTIFIC REPORT (INCLUDING PAPERS PRESENTED AT SURGICAL CONFERENCES)

Chemoprevention with Somatuline© Delays the Progression of Pancreatic Neuroendocrine Neoplasms in a Mouse Model of Multiple Endocrine Neoplasia Type 1 (MEN1)

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Abstract

Objective Long-acting synthetic somatostatin analogues (SSA) are an essential part of the treatment of neuroendocrine neoplasms. We evaluated the chemopreventive effects of a long-acting somatostatin analogue on the development of pancreatic neuroendocrine neoplasms (pNENs) in a genetically engineered MEN1 knockout mouse model.

Materials and methods Heterozygote MEN1 knockout mice were injected every 28 days subcutaneously with the somatostatin analogue lanreotide (Somatuline Autogel©; Ipsen Pharma) or a placebo starting at day 35 after birth. Mice were euthanized after 6, 9, 12, 15 and 18 months, and the size and number of pNENs were measured due histological analysis and compared to the placebo group.

Results The median tumor size of pNENs was statistically significantly smaller after 9 (control group vs. SSA group; 706.476 μm^2 vs. 195.271 μm^2; $p = 0.0012$), 12 (placebo group vs. SSA group 822.022 vs. 255.482; $p \leq 0.001$), 15 (placebo group vs. SSA group 1192.568 vs. 273.533; $p \leq 0.001$) and after 18 months (placebo group vs. SSA group 1328.299 vs. 864.587; $p \leq 0.001$) in the SSA group. Comparing the amount of tumors in both groups, a significant reduction was achieved in treated *Men1*$^{(+/-)}$ mice (41%, $p = 0.002$). Immunostaining showed, however, no significant difference in the expression of the apoptosis marker caspase-3, but a significant difference in Ki67 index as a marker for tumor cell proliferation ($p \leq 0.005$).

Conclusion Long-acting somatostatin analogues may be an effective chemopreventive approach to delay the progression of MEN1-associated pNENs. After our preclinical results, we would recommend to evaluate the effects of long-acting SSA in a prospective clinical trial.

Introduction

Multiple endocrine neoplasia type 1 (MEN1) is a hereditary tumor syndrome affecting all endocrine glands. The combination of primary hyperparathyroidism, neuroendocrine pancreatic and duodenal neoplasms as well as the anterior pituitary gland is characteristic for this autosomal dominant inherited disorder. In addition, the adrenal glands and—in less cases—the thymus and the bronchi can also be affected [1–4]. Germline mutations in a tumor suppressor gene (*Menin)* on chromosome 11q13 cause the syndrome [5]. Since the identification of the underlying mutation in

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1997, over 1000 different germline mutations have been detected [2–4]. Due to a result of standardized screening programs with improved imaging studies (especially endoscopic ultrasonography), the prevalence of pancreaticoduodenal neoplasms (pNEN) in MEN1 patients is, however, steadily increasing [6–9]. Thymic carcinoids as well as pNENs are the most common causes of death in patients with MEN1 [10]. The MEN1-associated pNENs are an inhomogeneous group with functioning or non-functioning and benign or malignant neoplasms. Most of the patients develop not only one but multiple pNENs. Surgery is recommended in MEN1-associated non-functional-pNENs when they reach a size >2 cm in current practice guidelines as well as in insulinomas, glucagonomas and vipomas [11–13]. There is a high risk of a recurrence or a progression in all MEN1 patients due to genetic predisposition of the disease [14]. Therefore, in most of the patients, more than one surgical procedure is necessary—a fact that might both affect the quality of life of these mostly young patients and can lead to long-term problems like adhesions, incisional hernias and/or diabetes mellitus.

Considering all these facts, a chemopreventive approach may avoid early pancreatic surgery as long as possible.

Mouse models can serve as effective experimental surrogates in case of inherited disorders, such as the MEN1 syndrome. Therefore, they can give remarkable insights in the genetic and/or molecular interrelations leading to the onset of neoplasms. Chu et al. [15] and others have shown that heterozygous *Men1+/−* mice develop pancreatic neoplasms and other endocrine manifestations extremely comparable to that in MEN1-patients. Furthermore, not only the manifestations but also timing of developing such endocrine neoplasms is very alike to those in humans [15–17].

Somatostatin (somatotroph release-inhibiting factor) was initially described as a growth hormone secretion inhibitor. It acts through five somatostatin receptors (SSTR1–SSTR5), which are variably expressed not only in normal but also in tumor cells causing—among many other functions—an anti-proliferative effect [18]. In addition, somatostatin inhibits the secretion of gastrointestinal hormones and is today part of a treatment of particularly metastatic endocrine neoplasm of gastroenteropancreatic origin [19]. Because of a short half-life of the natural somatostatin peptides (∼ 1 min), somatostatin analogues (SSAs) have been synthesized. Among them, octreotide (Sandostatin, Novartis, East Hanover, NJ, USA) and lanreotide (Somatuline Autogel©; Ipsen Pharma, Paris, France) have been intensively investigated and are used today in the treatment of acromegaly as well as metastatic neuroendocrine neoplasms. Both octreotide and lanreotide act mainly via the somatostatin receptor type 2 (SSTR2). In addition, recent studies proved [20, 21] an effect of pasireotide. Pasireotide is a multiple receptor targeted somatostatin analogue for the chemoprevention of pNENs, acting via SSTR1, 2, 3 and 5.

In this study, we aimed to evaluate a chemopreventive effect of the somatostatin analogue lanreotide in a MEN1 knockout mouse model.

Materials and methods

Animals

For this study, we used conventional heterozygous knockout mice ($Men1^{+/-}$) generated through a Cre-mediated deletion of the second exon of the *MEN1 gene*—kindly provided by Dr. Chang-Xian Zhang, International Agency for Research on Cancer, Lyon, France.

Not only the maintenance of the animals but also all experiments were performed in accordance with the guidelines of the committee for animal care of the Philipp's University Marburg, Germany. In particular, the principles of "The Three R's" (reduction, refinement and replacement) in animal testing were followed precisely. All animals used in this study were placed in microisolator cages on a determined dark/light cycle (12 h) and were alimented with standard diet ad libitum.

Genotyping

For breeding, heterozygous Men1 mutant mice were used. After obtaining a large cohort of animals, a polymerase chain reaction (PCR) was initiated to differ between wild-type and $Men1^{+/-}$ mice. For the PCR, genomic DNA extracted from tail cutting was used. Primer sequences were (a) 3f1, 5′-GGATTCTGCCCCAGGC and (b) 3r1, 5′-CACCTCCATCTTACGGTCG.

Treatment

The composition of the particular groups as well as the algorithm of treatment is described in Fig. 1. Starting at day 35, the SSA groups (five mice) were treated with monthly subcutaneous injections of lanreotide (Somatuline Autogel©; Ipsen Pharma; SSA group) or a placebo (five mice). In the placebo group, sterile sodium chloride solution was injected.

Necropsy

After 6, 9, 12, 15 and 18 months, mice were sacrificed by CO_2 inhalation. Pancreata from all mice were collected,

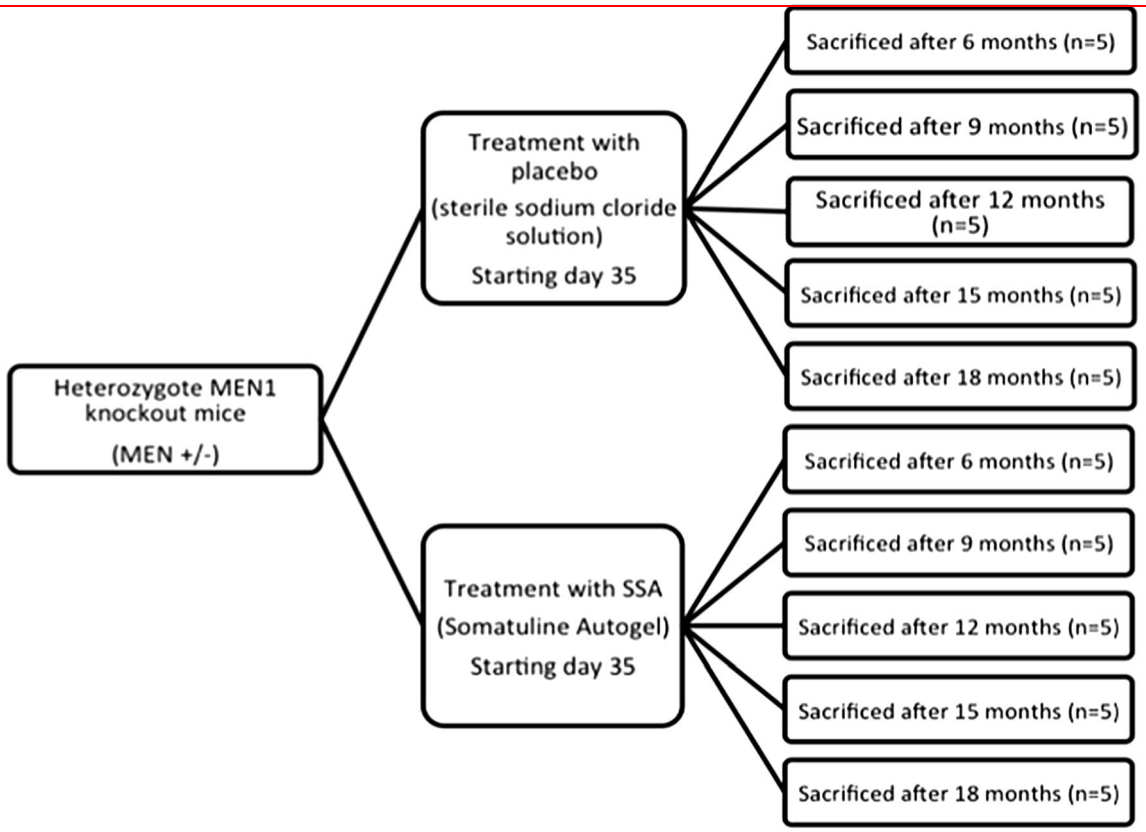

Fig. 1 Oversight study. Heterozygote MEN mice were treated either with Somatuline© or placebo, starting at day 35 of age and sacrificed after a determinant time

weighed, inspected for visible tumors and at last prepared for microscopical analysis.

The pancreas was preserved in a 10% formalin solution (Sigma-Aldrich). In addition, the number of pNENs was counted. To compare and evaluate the size and number of islet cell tumors, five H&E-stained sections from each mouse were analyzed (1 µm, 10 µm, 20 µm and 40 µm from the surface of the pancreas). For morphometric analysis, 100 µm sections were digitalized and the surface of the tumors was measured using a computerized pixel counting.

Immunostaining

As described previously [22], the samples were fixed in formalin, embedded in paraffin and stained. Rabbit anti-synaptophysin (*Invitrogen*), rabbit anti-cleaved caspase-3 (*Cell Signaling*), rabbit anti-Ki67 (*Abcam)* and anti-SSTR2 (*Abcam*) were used as primary antibodies. (Concentrations are available upon request.) First, the slides were heated (60 °C/1 h) and deparaffinized with xylene. Using a graded series of ethanol, the slides were afterward hydrated. For antigen retrieval, microwave heating (in sodium citrate buffer, pH 6.0, for 10 min) was used followed by quenching the endogenous peroxidase activity through 10 min incubation (in 3% H_2O_2). A 10% serum was used to block any non-specific binding. Afterward, the slides were treated overnight (at 4 °C) with the primary antibodies. Antibody bounding was visualized by the avidin–biotin-complex (ABC) peroxidase method (ABC Elite Kit, Vector Labs, Burlingame, CA). Finally, using the Sigma FAST DAB peroxidase substrate kit (Sigma, Deisenhofen, Germany), staining was performed.

The results of the immunohistochemistry staining were analyzed as described previously [22]. In a defined 10 × 10 fields of view (n = 3/section; 3 sections/animal were analyzed). The caspase-3 positive cells were counted by manual assessment. Sections were viewed under light microscopy, and images were recorded using a digital camera (both Leica, Wetzlar, Germany). Proliferation rates were calculated as mean ± SEM of max. twenty pancreatic neoplasms of five different mice. Analyses of the particular sections were performed with the established Leica QGo Algorithm using the established BrdU staining (Bromodeoxyuridine).

In every tumor, the percentage of positive stained cells was calculated and a boxplot was generated.

Statistical analysis

For descriptive and explorative statistics, we used median and range; for univariate analyses, *t* test, Mann–Whitney *U* test and ANOVA were carried out. For the comparison of more than two groups, we used one-way ANOVA with post hoc Holm–Sidak analysis for pairwise comparisons. A p value <0.05 was considered statistically significant. For all statistics, the SPSS software version 16 was used (SPSS, Inc.); statistical analysis and graphic visualization within the immunostaining were performed with GraphPad Prism 7.

Results

Treatment with Somatuline© inhibits tumor growth and tumor number

The results of the microscopical analysis revealed a highly statistical significant difference in tumor size between the two groups 8 (placebo vs. Somatuline Autogel©). The median tumor size of pNENs differed statistically significant after 9 months (control group vs. SSA group; 706.48 µm^2 vs. 195.27 µm^2; $p = 0.0012$), 12 months (placebo group vs. SSA group 822.02 vs. 255.48; $p \leq 0.001$), 15 month (placebo group vs. SSA group 1192.57 vs. 273.53; $p \leq 0.001$) and 18 months (placebo group vs. SSA group 1328.23 vs. 864.59; $p \leq 0.001$) (s. Fig. 2), respectively. At the age of 6 months, the tumor size of the placebo group tended to be higher, but there was yet no statistically significance detectable. Because of the high statistical significance in tumor size, the specimens from the 12- and 15-month group (SSA and placebo) were used for further analysis.

As mentioned above, the tumor amount was counted using the light microscope in H&E-stained probes. In untreated Men1$^{+/-}$ mice, a median number of 12 pNENs could be detected. After treatment with Somatuline Autogel©, a significant tumor amount reduction was achieved with a median pancreatic tumor amount of 7—resulting in a reduction of 41% (12 vs. 7 pNENs, $p = 0.002$).

Expression of synaptophysin and cleaved caspase-3

Immunostaining against synaptophysin confirmed the neuroendocrine origin of the pancreatic tumors (Fig. 3). As mentioned above Somatuline© has a high affinity for SSTR2; therefore, the expression of SSTR2 in the murine pancreatic neoplasms was investigated using chemical

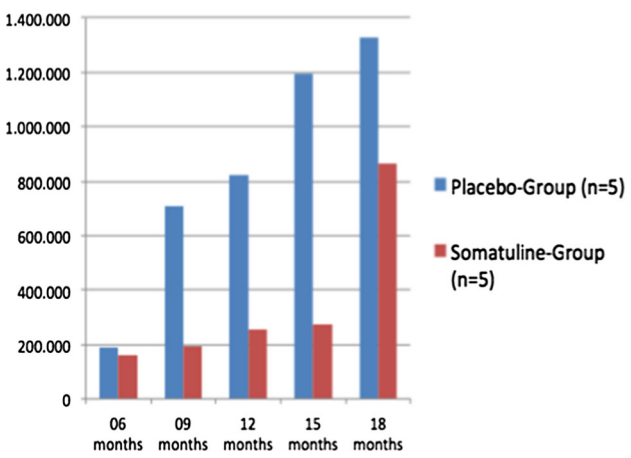

Fig. 2 Comparison tumor size (µm^2). To evaluate the change in islet cell tumor growth, five sections from each mouse were analyzed. These sections were taken 1 µm, 10 µm, 20 µm and 40 µm from the surface of the pancreas and H&E stained according to standard procedures. For morphometric analysis, 100-µm sections were digitalized and the surface of the tumors was measured using a computerized pixel counting

analysis with anti-SSTR2. The results showed that there was a high expression of SSTR2 in both groups (Somatuline© vs. placebo). Further, both in the SSA group and the placebo group, a SSTR2 expression was detected after 12 and 15 months, but could not reach statistical significance (Fig. 3). Immunostaining for caspase-3 as a marker for apoptosis showed no significant difference between mice treated with Somatuline© and the placebo group (Fig. 4).

Treatment with Somatuline© inhibits tumor proliferation

When staining with anti-Ki67 as a marker for cell proliferation, we found that within the Somatuline© group the Ki67 expression was significantly lower compared to the placebo group (SSA group 35.08% vs. placebo group 56.88%, $p \leq 0.001$), proving the ability of the drug to decrease tumor cell proliferation (Fig. 5).

Discussion

Over the past years, several reports, preclinical and at last clinical studies confirmed an anti-proliferative effect of synthetic, long-lasting somatostatin analogues [23–25]. These reports revealed that the effects of the somatostatin analogues are partly caused by the regulation of the PI3K/Akt signaling (MAP kinase) pathway (via SSTR mediation). This pathway leads not only to an increased expression of tumor suppressor genes and an increased induction of cell cycle arrest but also to apoptosis. [25].

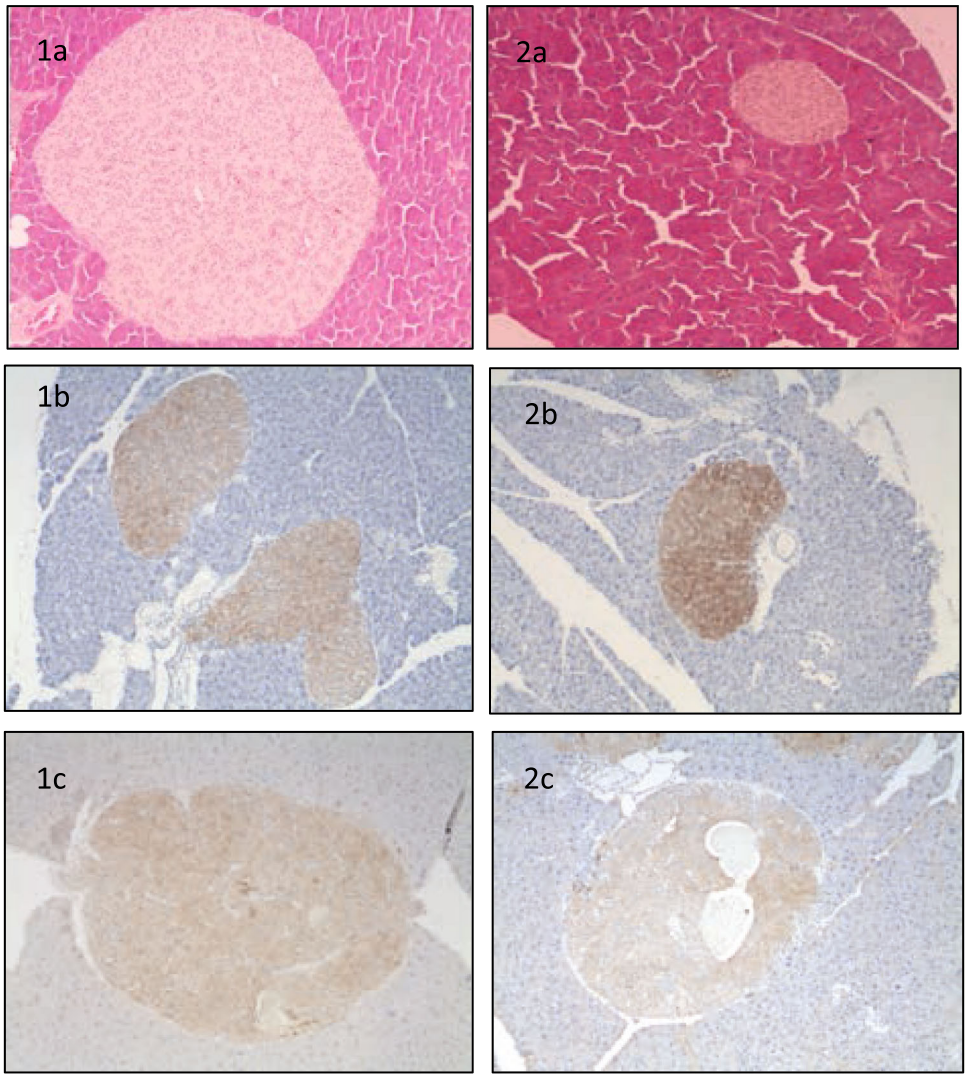

Fig. 3 H&E staining and immunohistochemistry of murine neuroendocrine neoplasms. Murine tumor samples from the placebo group (**1a–c**) were stained with H&E (**1a**) or underwent immunohistochemistry using the primary antibodies anti-synaptophysin (**1b**) and anti-SSTR2 (=somatostatin receptor type 2) (**1c**). Samples from the Somatuline© group (**2a–c**) were treated analogous; H&E staining (**a**), synaptophysin (**b**), SSTR2 (**c**)

Because of these data, over the last 20 years, somatostatin analogues became a relevant treatment option in metastatic neuroendocrine tumors. The control of clinical symptoms and stabilization of tumor growth lead both to an increase in quality of life and an improved overall survival in these patients.

At present, the long-acting somatostatin analogues octreotide (Sandostatin, Novartis, East Hanover, NJ, USA) and lanreotide (Somatuline Autogel©, Ipsen Pharma, Paris, France) are most established. In addition, the somatostatin analogue pasireotide (Signifor LAR®; Novartis, East Hanover, NJ, USA) may play an important role in the future.

Recently published data of the randomized, placebo-controlled phase II study (PROMID) confirmed that in patients with well-differentiated advanced midgut NENs octreotide can inhibit tumor progression [26]. In a corresponding study regarding the effects of Somatuline© (double blind, randomized, placebo controlled), the authors showed that the progression-free survival was significantly better in the treatment group than in the placebo group (not reached vs. 18 months in the placebo group, $p < 0.001$). In addition, in the treatment arm, the 2-year progression-free survival was also better (65.1% vs. placebo 33%). To note, the overall survival was not significantly different between both arms [27].

Recent studies proved [20, 21] an effect of pasireotide. In a study of Walls et al. [20], the authors showed that in pasireotide-treated Men1(+/−) mice survival increased (pasireotide, 80.9% vs. PBS, 65.2%; $p < 0.05$) and pancreatic NENs were fewer (pasireotide, 86.9% vs. PBS, 96.9%; $p < 0.05$). Thus, treatment with pasireotide can act as an anti-proliferative and pro-apoptotic therapy.

There are, however, until today no data regarding a chemopreventive effect of somatostatin analogues in patients with MEN1. The MEN1 knockout mouse model,

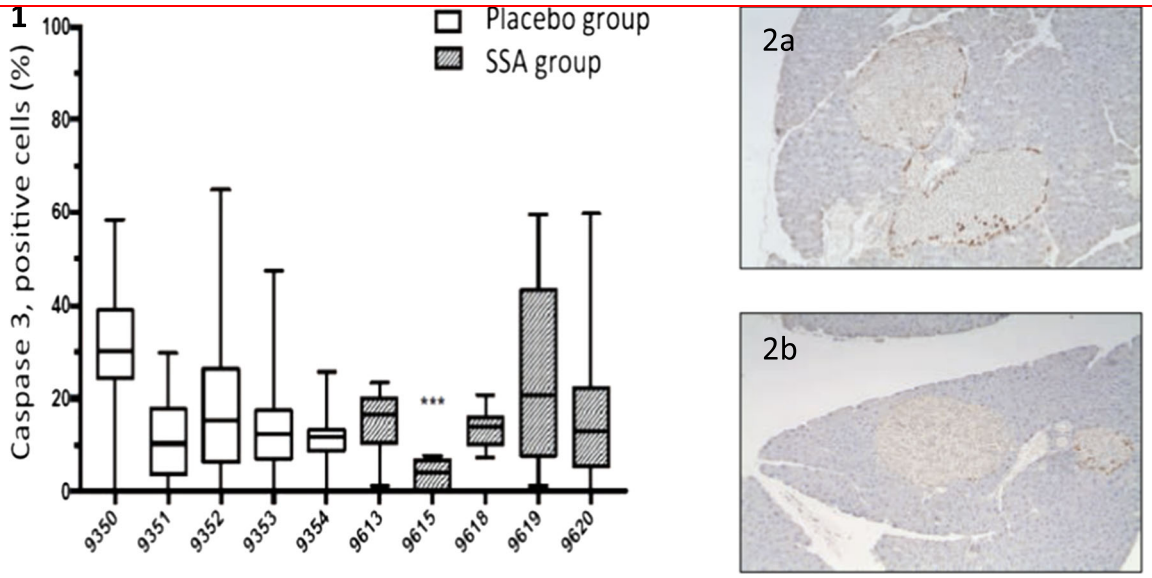

Fig. 4 Apoptosis index of 15-month-old MEN knockout mice (Men+/−). Immunostaining for caspase-3 as a marker for apoptosis was performed. In every tumor, the percentage of positive stained cells was calculated and a boxplot was generated; cleaved caspase-3 index (**1**); $*p < 0.05$; $**p \leq 0.01$; $***p \leq 0.001$. 10 × magnification of caspase positive cells (**2**), an exemplary sample of the placebo group (**2a**) and the Somatuline© group (**2b**)

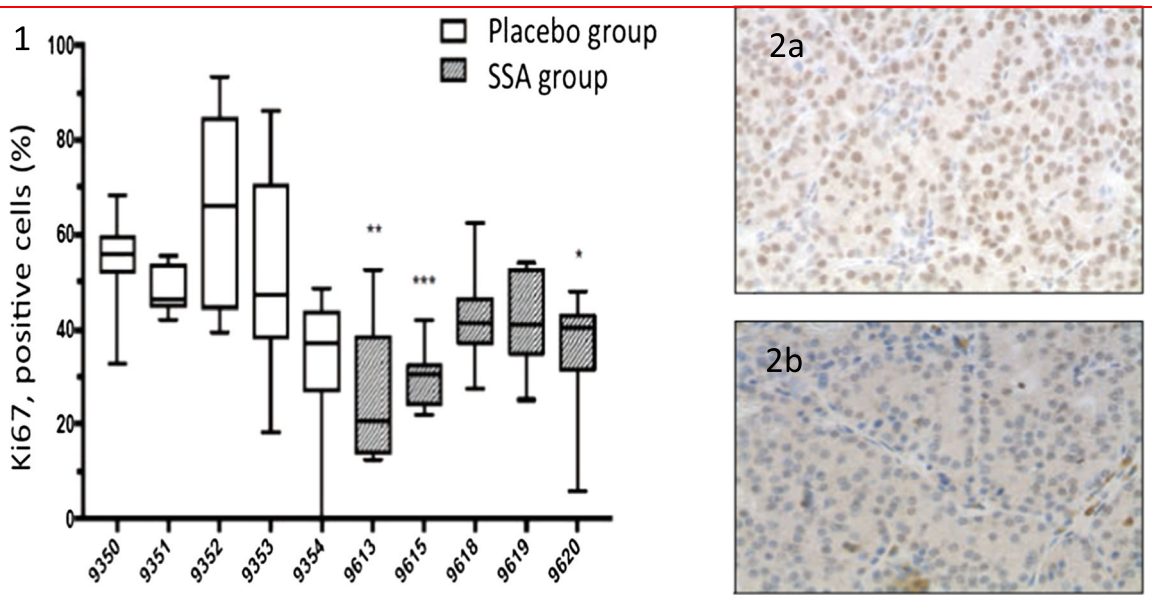

Fig. 5 Proliferation index of 15-month-old MEN knockout mice (Men+/−). The immunostaining with anti-Ki67 as a marker for cell proliferation showed a significant difference; in every tumor, the percentage of positive stained cells was calculated and a boxplot was generated; 1: Ki67 index (**1**); $*p < 0.05$; $**p \leq 0.01$; $***p \leq 0.001$. 10× magnification of Ki67 positive cells (**2**), an exemplary sample of the placebo group (**2a**) and an exemplary sample of the Somatuline© group (**2b**)

however, qualifies to evaluate a possible chemopreventive effect due the genetic background of the syndrome. As shown in various reports [15–17], the heterozygous MEN1 knockout mice suffer from pancreatic neoplasms and other endocrine lesions which are comparable to MEN1 patients. Furthermore, both manifestations and timing of developing such endocrine neoplasms are very alike to those in humans [15–17].

Knowing that the majority of MEN1 patients develop pNENs at a very young age with a need for major pancreatic surgery in a lot of those patients, a chemopreventive

therapy may delay the time of surgical procedures and thus improve the quality of life.

In 2012, Quinn et al. [28] showed that the somatostatin analogue pasireotide (SOM 230; Signifor, Novartis) caused an anti-secretory, anti-proliferative and pro-apoptotic activity in a MEN mouse model of insulinoma. In this study, eight MEN1 knockout mice with an insulinoma received—similar to the present study—monthly subcutaneous injections of the somatostatin analogue ($n = 4$) or a placebo ($n = 4$). The results confirmed both an anti-secretory effect (measured by insulin and glucose serum levels) in favor of the treatment group and a reduction in tumor activity on PET/CT scan (measured by SUV comparison). Furthermore, survival was improved in mice of the treatment group (four mice in the treatment group vs. one mouse in the placebo group; $p = 0.012$). As mentioned above, current guidelines recommend surgery in MEN1-associated functional tumors as well as non-functional-pNENs >1–2 cm in size [11–13]. A chemopreventive effect may, however, delay the progression of pNENs and may therefore prolong the time until pancreatic surgery is necessary.

Recently reported data from our group regarding the surgical approach in MEN1 patients with pNENs showed that the median age of patients undergoing pancreatic surgery was 35 years (range 9–58 years) [29]. It is, however, to notice that in this retrospective study we initially pursued a more aggressive surgical approach (until 2011, we recommended a surgical approach if the tumor size of non-functioning pNEN was >1 cm), but revised our indication for surgery in 2012 in accordance with current ENETs guidelines. Since 2012, we did recommend a surgical approach, if the tumor size reached 2 cm. However, if suspicious endosonographic features or a rapid progression during follow-up was observed, we also scheduled patients with pNENs with a size between 1 and 2 cm for surgery. Having this in mind, the median age of MEN1 patients in need of surgery may be higher in the future. To delay the progression of pNENs may, however, be an enormous benefit for these patients.

The strength of this study is the long treatment period; it has, however, some limitations: First, we treated only a small number of animals, and second, only the development of pancreatic neuroendocrine tumors was monitored and analyzed. The presence of other MEN1-associated neoplasms and a possible effect on those due the treatment with Somatuline© were not evaluated and might be explored in further studies.

In conclusion, the present data suggest that a long-acting somatostatin analogue might be an effective chemopreventive approach resulting in a delay of the progression of MEN1-associated pNENs. On the basis of our preclinical results, we would recommend to evaluate the effects of somatostatin analogues in a prospective clinical trial.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research/data reported. The somatostatin analogue Somatuline Autogel© used in this trail was, however, provided gratis from Ipsen Pharma, Paris, France.

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