The ghrelin receptor agonist HM01 mimics the neuronal effects of ghrelin in the arcuate nucleus and attenuates anorexia-cachexia syndrome in tumor-bearing rats

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The ghrelin receptor agonist HM01 mimics the neuronal effects of ghrelin in the arcuate nucleus and attenuates anorexia-cachexia syndrome in tumor-bearing rats. Am J Physiol Regul Integr Comp Physiol 311: R89–R96, 2016. First published May 4, 2016; doi:10.1152/ajpregu.00044.2016.—The gastric hormone ghrelin positively affects energy balance by increasing food intake and reducing energy expenditure. Ghrelin mimetics are a possible treatment option against cancer anorexia-cachexia syndrome (CACS). This study aimed to characterize the action of the nonpeptidergic ghrelin receptor agonist HM01 on neuronal function, energy homeostasis and muscle mass in healthy rats and to evaluate its possible usefulness for the treatment of CACS in a rat tumor model. Using extracellular single-unit recordings, we tested whether HM01 mimics the effects of ghrelin on neuronal activity in the arcuate nucleus (Arc). Furthermore, we assessed the effect of chronic HM01 treatment on food intake (FI), body weight (BW), lean and fat volumes, and muscle mass in healthy rats. Using a hepatoma model, we investigated the possible beneficial effects of HM01 on tumor-induced anorexia, BW loss, muscle wasting, and metabolic rate. HM01 (10−7–10−6 M) mimicked the effect of ghrelin (10−8 M) by increasing the firing rate in 76% of Arc neurons. HM01 delivered chronically for 12 days via osmotic minipumps (50 µg/h) increased FI in healthy rats by 24%, paralleled by increased BW, higher fat and lean volumes, and higher muscle mass. Tumor-bearing rats treated with HM01 had 30% higher FI than tumor-bearing controls and were protected against BW loss. HM01 treatment resulted in higher muscle mass and fat mass. Moreover, tumor-bearing rats reduced their metabolic rate following HM01 treatment. Our studies substantiate the possible therapeutic usefulness of ghrelin receptor agonists like HM01 for the treatment of CACS and possibly other forms of disease-related anorexia and cachexia.

The cancer anorexia-cachexia syndrome (CACS) is characterized by reduced eating, increased catabolism, and body weight loss. CACS is a major cause for the decline in the clinical status of affected patients and is associated with increased mortality. CACS is present in up to 80% of cancer patients at death and represents the most significant negative predictor of treatment outcome (12, 23, 41). Affected cancer patients show a poor responsiveness to anticancer therapies, irrespective of the type of malignancy (1, 9). The effectiveness of nutritional care as a therapeutic option against CACS is limited, and insufficient progress has been made in the development of specific pharmacological approaches (21).

Because of positive effects on energy balance, the gastrointestinal hormone ghrelin or ghrelin analogs are considered as a possible treatment option for CACS. Ghrelin is mainly secreted from the stomach, and it has been identified as a high-affinity ligand for the growth hormone secretagogue receptor (GHS-R) (20). Plasma levels of ghrelin rise during fasting and shortly before meals. Pharmacological doses of ghrelin stimulate food intake and promote body weight gain in rodents (36, 42). These effects are thought to be mediated via the hypothalamic arcuate nucleus (Arc), which is of high importance for the control of food intake and energy homeostasis (32). The GHS-R is highly expressed in the Arc and ghrelin’s effects on neuronal activity of Arc neurons have been characterized in various electrophysiological and immunohistological studies (13, 16, 17). Ghrelin activates neuropeptide Y-expressing neurons in the Arc, which is widely considered as the neuronal correlate for ghrelin’s actions on food intake and body weight (5, 34, 45). Moreover, ghrelin also promotes muscle cell differentiation in vitro and ameliorates skeletal muscle atrophy in mice, suggesting that ghrelin may also exert direct effects on muscle that are independent of its central actions (10, 31).

Ghrelin has been tested as anti-CACS treatment in clinical human trials and rodent cancer models (4, 8, 15, 25–27, 39, 46). Most of these studies support a beneficial effect of ghrelin on food intake and body weight. However, because of its peptidergic nature and its short half-life time of 15–20 min, the usefulness of native ghrelin as a therapeutic agent is limited (44). HM01 is a synthetic small-molecule compound that acts as a GHS-R agonist. It has high receptor binding affinity, high brain permeability, and a higher plasma half-life compared with ghrelin (19). Besides other possible therapeutic indications, it might, therefore, be used as an anti-CACS treatment. It was the overall aim of our studies to evaluate the possible usefulness of HM01 against tumor-dependent anorexia and body weight loss. First, we sought to confirm the ghrelin-like excitatory action of HM01 in electrophysiological recordings of the Arc of rats. Second, we investigated the effect of chronic HM01 treatment on food intake, body weight, muscle mass, and body composition in healthy non-tumor-bearing rats. Third, we used a rat Morris-7777 hepatoma model to test whether HM01 ameliorates tumor anorexia, body weight loss, and muscle wasting, and whether HM01 affects nutrient utili-
zation and metabolic rate. The Morris-7777 hepatoma model is well established and characterized by a clear anorectic response and body weight loss (35). Since an attenuation of anorexia is one important mode of action for ghrelin-based approaches, we chose a tumor model in which anorexia is a major contributor to body weight loss. Another advantage of this tumor model is that anorexia and body weight loss are less severe than in other tumor models. A very rapid deterioration of the health status caused by aggressive tumors often limits the usefulness of such tumor models for chronic treatment paradigms.

METHODS

Animals. Adult male Wistar rats (Elevage Janvier, France), weighing between 230 and 270 g, were used for all electrophysiological and behavioral experiments involving non-tumor-bearing animals. Animals were kept in a temperature-controlled room (21 ± 1°C) on a 12:12-h light-dark cycle with ad libitum access to standard chow (890 kcal/kg; Altromin). For the in vivo testing of the GHS-R agonist HM01, rats were single-housed in wire-mesh cages. Before the experiments, rats were handled daily and kept in the cages for an adaptation period of at least 1 wk.

For the behavioral and metabolic studies involving tumor-bearing animals, adult male Buffalo rats (Charles River) weighing around 280 g were used. The animals were single-housed in metabolic cages (TSE Phenomaster, TSE Systems) and were adapted to the housing conditions for 7 days before the start of the experiment. To evaluate the effects of tumor growth on muscle mass, Buffalo rats were single-housed in wire-mesh cages. All animal procedures were approved by the Veterinary Office of the Canton of Zurich, Switzerland.

Electrophysiology. The electrophysiological recording technique was the same as described previously (3). The rats were decapitated using a guillotine at the same time point in the middle of the light phase to minimize differences in the circadian and prandial state of the animals. After decapitation, the brain was quickly removed and superfused with ice-cold artificial cerebrospinal fluid (aCSF) that was oxygenated and pH-equilibrated with oxycarbon (pH 7.4; 290 mosmol/kg H2O). Coronal brain slices (700 μm) were cut at the mid-rostral-caudal level of the Arc using a vibratome (Leica VT1000S, Leica Microsystems). A rectangular 3 x 3-mm slice preparation containing the Arc was manually dissected under a dissection microscope and transferred to a temperature-controlled (37°C) incubation chamber filled with constantly oxygenated aCSF. For recordings, the Arc preparations were transferred to a temperature-controlled (37°C) recording chamber that was constantly perfused with oxygenated and prewarmed aCSF at a rate of 1.6 ml/min. Extracellular single-unit recordings were obtained using home-made glass-coated platinum-iridium electrodes. Recordings were conducted in the medial arcuate nucleus, in which the majority of neurons are excited by ghrelin (34).

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HM01 (provided by Helsinn Healthcare, Lugano, Switzerland) was superfused at a concentration of 10⁻⁶ or 10⁻⁷ M. The cosensitivity of the recorded neurons to ghrelin was tested by stimulation with rat ghrelin (10⁻⁸ M; Bachem).

Effects of HM01 in non-tumor-bearing rats. Osmotic minipumps (model 2ML2, ALZET osmotic pumps; DURECT) were implanted subcutaneously into the left flank at the lower level of the abdomen between the chest and the hind limbs. Minipumps were filled with HM01 (10 μg/μl) or saline for controls. The minipumps released a constant amount of HM01 (50 μg/h at a pump rate of 5 μl/h). The capacity of the minipumps (2 ml) allowed a constant compound release for 14 days. Body weight and food intake were measured daily. At the end of the experiment, animals were euthanized for the measurement of body composition by computed tomography (CT) scanning and muscle mass.

Effects of HM01 in tumor-bearing rats. The hepatoma tumor model was used in our experiments, as previously described (35). Morris hepatoma 7777 cells (McA-RH7777, cat. no. CRL-1601, American Type Culture Collection) were cultured under standard conditions in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Semiconfluent McA-RH7777 Petri dishes were washed with DMEM repeatedly to detach the cells from the surface. After confirming the viability of the cells with Trypan blue, 10⁷ cells were inoculated subcutaneously in 250 μl PBS under short isoflurane anesthesia between the scapulas.

HM01 or saline treatment was applied as described above. Minipumps were implanted on day 11 after tumor inoculation, shortly after the onset of the anorectic response. Treatments continued for 6 days until the animals had to be euthanized for ethical reasons, according to our criteria for the termination of experiments. Food intake and respiratory gas exchange (O2 and CO2) were recorded automatically at 17-min intervals throughout the entire experiment. Body weight was measured daily. After euthanizing the rats, tumors and limb muscles were resected and weighed, and body composition was determined by CT scan (see Computed tomography scanning and muscle weight measurements).

To examine the effects of tumor growth on muscle mass, tumor-bearing and non-tumor-bearing rats were used. After adaptation, tumor growth was induced as described above; control animals received the same volume of PBS without tumor cells. Rats were euthanized 16 days after tumor induction, and limb muscles were dissected and weighed.

Computed tomography scanning and muscle weight measurements. Total carcass lean and fat volumes were measured by quantitative microcomputed tomography (La Theta LCT-100A scanner, Hitachi-Aloka Medical). Sequential 2-mm slice images with a pixel size of

Fig. 1. Recording of a ghrelin-excited Arc neuron, which was also reversibly stimulated by superfusion of HM01. Horizontal bars indicate time of superfusion.

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Data were expressed as means ± SE. These values were corrected for the weight of minipump implantation from the body weight at the end of the treatment period by subtracting the body weight at the time of minipump implantation from the body weight. Body weight change following treatment was calculated for body weight relative to controls, which became significant from day 2 after minipump implantation. Group means were analyzed with one-way ANOVA followed by the Tukey post hoc test. After tumor resection, tumor weights were compared between control animals and HM01-treated rats by Student’s t-test (two-sided). For all statistical analyses, P < 0.05 was considered significant.

**RESULTS**

*Electrophysiology.* Seventeen Arc neurons were recorded. Similar to previous studies, the majority (13/17; 76%) of neurons recorded in the medial Arc was reversibly excited by ghrelin (10⁻⁸ M) (34). All ghrelin-excited cells were also activated by superfusion of HM01 at a concentration of 10⁻⁶ M (see example in Fig. 1). Interestingly, neurons that were insensitive (3/17; 18%) or inhibited (1/17; 6%) by ghrelin showed the same type of responses to HM01, resulting in 100% concordant responses. The average response latency was significantly longer for the lower concentration of HM01 (10⁻⁶ M) compared with ghrelin or HM01 at 10⁻⁸ M. The absolute changes in firing rate and the peak responses were not significantly different between the stimuli. However, HM01 tended to induce longer-lasting responses compared with ghrelin, which reached statistical significance for the higher concentration of HM01 (Table 1).

Effects of HM01 in non-tumor-bearing rats. HM01 significantly stimulated food intake starting on the first day after minipump implantation. This effect remained stable during the 12 days of measurements. On average HM01-treated rats consumed 6.0 ± 0.3 g (24 ± 1%) more food per day than controls (Fig. 2A). Over the 12-day treatment period, this resulted in a significantly higher cumulative food intake (HM01, 405 ± 6 g; saline, 329 ± 7 g; P < 0.001). This orexigenic effect of HM01 was paralleled by an increase in body weight relative to controls, which became significant on day 5 after minipump implantation (Fig. 2B). At the end of the experiments, the difference in body weight was 35 g.

![Fig. 2. Daily food intake and body weight of HM01-treated (50 μg/h) and vehicle-treated animals. A: HM01-stimulated food intake starting from the day after minipump implantation. This effect remained stable during the 12-day treatment period. B: HM01-induced increase in food intake was paralleled by an increase in body weight relative to controls, which became significant from day 5 after minipump implantation. Data analyzed using the Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001).](http://ajpregu.physiology.org/doi/10.1152/ajpregu.00044.2016)
HM01 significantly increased lean and fat body mass after the 12-day treatment period (229.7 ± 4.3 vs. 209.5 ± 4.3; \( P < 0.05 \), Fig. 3A). Moreover, rats treated with HM01 had a higher gastrocnemius weight compared with saline controls (HM01: 1.795 ± 0.052 g vs. control: 1.508 ± 0.076 g; \( P < 0.05 \), Fig. 3B).

**Effects of HM01 in tumor-bearing rats.** In tumor-bearing animals food intake started to decline around day 7 after tumor induction. After implantation of minipumps on day 11, HM01 prevented a further decrease in eating, and HM01-treated rats consumed significantly more food than control animals (Fig. 4A). On average, daily food intake during the treatment period (i.e., from day 12 to day 17 after tumor induction) was 18.5 ± 0.6 g vs. 14.3 ± 0.5 g (saline controls), which corresponds to a 30% higher food intake of HM01-treated rats compared with controls (Fig. 4B). While tumor-bearing controls showed a loss of body weight during the treatment period, rats receiving HM01 stabilized their body weight. The difference in absolute body weight did not reach statistical significance; however, the change in body weight was highly significant between the treatment groups (HM01: 1.1 ± 2.0 g vs. control: 10.4 ± 1.7 g; \( P < 0.001 \); Fig. 4C). This difference in body weight change appeared to be mainly due to lower fat mass in tumor-bearing control rats because HM01-treated rats showed significantly higher fat mass but similar lean mass compared with control animals (Fig. 5A). Tumor-bearing rats had lower gastrocnemius, tibialis, and soleus muscle mass compared with controls (1.077 ± 0.018 vs. 1.208 ± 0.038, 0.342 ± 0.012 vs. 0.397 ± 0.022, 0.059 ± 0.002 g vs. 0.067 ± 0.001 g; \( P < 0.05 \); in all cases, Fig. 5B). HM01 led to a higher gastrocnemius and soleus mass compared with tumor-bearing controls (1.422 ± 0.050 vs. 1.253 ± 0.054, 0.091 ± 0.005 g vs. 0.072 ± 0.005 g; \( P < 0.05 \); respectively, Fig. 5C).

Relative to baseline conditions before the onset of anorexia (days 1 and 2), control rats had significantly lower RER values on days 14 and 15 reflecting a shift toward lipid metabolism. This tumor-dependent reduction in RER was partly prevented by HM01 treatment (Fig. 6A). Interestingly, energy expenditure was significantly lower in HM01-treated compared with control rats during the treatment period (days 14 and 15) and also lower relative to baseline conditions on days 1 and 2 (Fig. 6B). Chronic HM01 treatment did not affect tumor growth.

![Fig. 3](http://ajpregu.physiology.org/)

**Fig. 3. Computed tomography-based assessment of lean and total fat volumes, and weights of gastrocnemius, tibialis, and soleus muscle.** A: rats treated with HM01 (50 \( \mu \)g/h) displayed higher lean and fat volumes than vehicle-treated animals. B: HM01 treatment also resulted in higher gastrocnemius and total muscle mass (sum of gastrocnemius, tibialis, and soleus mass) compared with controls. Data were analyzed using the Student’s t-test (*\( P < 0.05 \); ns, not significant).

![Fig. 4](http://ajpregu.physiology.org/)

**Fig. 4. Daily food intake, mean daily food intake during treatment, and body weight development of HM01-treated (50 \( \mu \)g/h) and vehicle-treated tumor-bearing (TB) rats following minipump implantation.** A: HM01 significantly attenuated the anorectic response induced by tumor growth. B: on average, HM01-treated TB rats had a 30% higher daily food intake compared with TB controls. C: chronic HM01 administration prevented tumor-induced body weight loss. Data analyzed using the Student’s t-test (*\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \)).
because average tumor weight was not significantly different between treatment groups (8.9 ± 1.0 g vs. 8.2 ± 0.7 g, Fig. 7).

**DISCUSSION**

We provide in vitro and in vivo characterization of the synthetic GHS-R agonist HM01 under nonpathological conditions and in the context of cancer anorexia and body weight loss. In particular, we showed that HM01 acts as a ghrelin analog in the Arc, increases food intake and body weight in healthy rats, and prevents the tumor-induced body weight loss by attenuating anorexia and by reducing energy expenditure. In the electrophysiological experiments, HM01 and ghrelin showed a concordant response profile in all tested neurons. Hence, HM01 mimicked the effects of ghrelin on neuronal activity of Arc neurons.

We performed recordings using two concentrations of HM01 (10⁻⁷ M and 10⁻⁶ M), and we observed a significantly longer response duration after superfusion of the higher concentration of HM01 (10⁻⁶ M) compared with ghrelin. However, no difference was seen in the absolute response and the peak response. It was not the aim of this study to establish a complete dose-response characteristic for the excitatory effects of HM01, but only to provide proof of concept that HM01 and ghrelin exert concordant actions on Arc neurons. To use effective stimuli, we used concentrations that are clearly above the EC₅₀ of 1–2 nM for HM01 (19). Therefore, a possible ceiling effect might be the reason why the absolute and peak responses caused by the two concentrations were similar. We cannot exclude differences in the dynamics of receptor activation by ghrelin and HM01, respectively, which might be reflected by the longer latency of HM01 (10⁻⁷ M) compared with ghrelin (10⁻⁸ M). In addition to the direct excitatory effect of ghrelin on NPY neurons, ghrelin indirectly inhibits POMC neurons in the Arc (5). It was not our aim to characterize the effect of HM01 on second-order neurons. Nevertheless, an indirect inhibition of POMC neurons is a likely downstream effect of HM01.

The ghrelin-like action of HM01 on neuronal activity in the Arc is in line with the orexigenic effect of HM01 observed in the current study. HM01 treatment in healthy rats was associated with increased body weight gain and increased lean and fat mass. These effects were similar to the action induced by chronic treatment with the ghrelin analog BIM-28131, which has been considered as one of the most potent GHS-R agonists (28, 29, 38). Interestingly, we observed an increase in both absolute fat and lean volumes and in gastrocnemius muscle mass in HM01-treated rats. While most studies using ghrelin or GHS-R agonists demonstrate increased adiposity in healthy rodents, treatment effects on lean and muscle mass appear to be less robust (22, 24, 38, 42). Ghrelin or BIM-28131-induced positive effects on lean body mass in healthy rats, although most of these effects only occurred after longer treatment periods compared with the current study (4, 28, 29, 38).

On the basis of previous studies, a possible feeding-independent adipogenic action of HM01 could be due to an up-regulation of lipogenic enzymes and a reduction of lipid export in adipose tissue (7, 30). Another factor promoting adiposity may be a reduction of metabolic rate, which is a well-documented effect of ghrelin. This effect is mediated via reduced sympathetic outflow to brown adipose tissue that leads to decreased uncoupling protein-1 expression (43, 48). It was beyond the scope of our study to reconfirm the aforementioned mechanisms. On the basis of the similarity between the action of ghrelin and HM01, it appears plausible that similar adipogenic and metabolic processes might also be engaged by HM01.

Similar to its orexigenic action, ghrelin’s effect on GH release depends on the GHS-R, although both effects occur...
of physical activity in hepatoma tumor-bearing rats in previous studies. We did not detect a tumor-dependent alteration in tumor-induced muscle loss in our hepatoma tumor model. Whether similar mechanisms contribute to the HM01-mediated attenuation of muscle atrophy is currently unknown. Moreover, HM01 reduced metabolic rate, lipid metabolism, and muscle wasting.

The relative increase in food intake of HM01-treated animals was similar in tumor-bearing and in non-tumor-bearing rats (30% and 24%, respectively). The magnitude of the positive effects of HM01 on food intake and body weight gain was also similar to the actions of ghrelin analogs used in previous studies (8). However, direct comparisons between the effectiveness of HM01 and ghrelin analogs used in other studies are limited due to different tumor models and treatment paradigms, as well as potential differences in dose-response relationships and pharmacokinetics of the substances used.

Hepatoma tumor-bearing rats had lower muscle mass compared with non-tumor-bearing controls, indicating tumor-induced muscle degradation. HM01 not only stimulated muscle growth in healthy animals, but it also increased muscle mass in tumor-bearing rats. Muscle atrophy during cancer is associated with decreased expression of different markers for muscle formation such as myoD, myogenin, and mTOR, and increased proteolytic markers like myostatin, activin A, FOXO, MURF-1, and atrogin-1/MAFbx (two muscle-specific ubiquitin ligases) (2). Chronic ghrelin treatment prevented the cancer-induced increase of myostatin, MURF-1, and atrogin-1/MAFbx (two muscle-specific ubiquitin ligases) (2). Chronic ghrelin treatment prevented the cancer-induced increase of myostatin, MURF-1, and atrogin-1/MAFbx (two muscle-specific ubiquitin ligases) (2).

Although we did not measure locomotor activity in the present study, we did not detect a tumor-dependent alteration in the expression of mTOR. We did not observe any significant differences in the expression of mTOR in tumor-bearing rats treated with HM01 compared with non-tumor-bearing controls. The latter effect is consistent with the preservation of fat depots by HM01 treatment.
observation that HM01 does not promote tumor growth is consistent with these findings. The lack of difference in the tumor weight between the HM01-treated animals and the control group is an important finding that is relevant for future long-term clinical applications.

In the context of CACS, the role of endogenous ghrelin is controversial. Ghrelin has been found increased, unchanged, or decreased in different rodent tumor models and among patients with different types of cancers (6, 11, 14, 18). We did not detect any changes in total or active ghrelin circulating level in our animals at the end of experiment (data not shown). Hence, a reduction in endogenous ghrelin levels does not appear to mediate the observed reduction in food intake in this rat tumor model.

Some studies postulated that CACS could lead to partial ghrelin resistance, which might eventually limit the effectiveness of ghrelin-based therapy (11, 47). This might be the reason why some ghrelin-based clinical trials have failed to attenuate CACS (39). However, under our experimental conditions, the anorectic action of HM01 and its positive effect on body weight were still significant at the end of the treatment period. Moreover, the relative difference in food intake between HM01-treated rats and controls was similar in tumor-bearing and non-tumor-bearing rats, suggesting the absence of profound ghrelin resistance. In our studies, the effect of HM01 on food intake became more variable toward the end of the experiment, that is, just before our criteria for the termination of the experiment were reached. It is a general and expected phenomenon that the increasing tumor burden overrides anti-CACS treatment effects at late disease stages in experimental cancer models (15). Notably, an important purpose of anti-CACS treatments is to positively influence the health status and the anti-cancer treatment success before the patients undergo end-stage disease. For these reasons, we did not attempt to characterize the effectiveness of HM01 to counteract CACS at later time points.

In addition to the direct effect of HM01 on energy homeostasis, there might be beneficial effects resulting from anti-inflammatory actions. Ghrelin has been shown to attenuate the inflammatory cytokine response in tumor-bearing mice (4). In contrast to other tumor models, the possible inflammatory or cytokine-dependent mechanisms that contribute to CACS in our hepatoma tumor model have not yet been identified. Systemic levels of IL-1β, IFN-γ, IL-6, and TNF-α have been reported to be unaltered in this tumor model (35). Although this does not exclude that other cytokines contribute to CACS in hepatoma tumor-bearing rats, other tumor models appear to be more appropriate to investigate anti-inflammatory effects of HM01 (4). An attenuation of the inflammatory response might not only indirectly attenuate anorexia, but also cytokine-mediated effects on muscle wasting.

**Perspectives and Significance**

We demonstrated that HM01 mimicked the actions of ghrelin on Arc neurons in vitro and stimulated food intake and body weight gain in healthy rats. More importantly, HM01 attenuated cancer anorexia in a rat hepatoma model, and it positively affected metabolism, body weight development, and muscle wasting. Thus, ghrelin agonists, such as HM01, may be a useful therapeutic approach for the treatment of CACS and possibly other forms of pathological anorexia, malnutrition, and muscle wasting.

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**DISCLOSURES**

Claudio Pietra and Claudio Giuliano are employees of Helsinn Healthcare, Lugano, Switzerland.

**AUTHOR CONTRIBUTIONS**

T.B. and T.R. conception and design of research; T.B. and L.L. performed experiments; T.B. and L.L. analyzed data; T.B., L.L., and T.R. interpreted results of experiments; T.B. prepared figures; T.B. and L.L. drafted manuscript; T.B., C.P., C.G., T.A.L., and T.R. edited and revised manuscript; T.B., L.L., C.P., C.G., T.A.L., and T.R. approved final version of manuscript.

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