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A cyclic peptide significantly **improves** thyroid function, thyrotropin-receptor antibodies and orbital mucine /collagen content in a long-term Graves' disease mouse model

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ABSTRACT

Background: BALB/c mice which received long-term immunizations of adenovirus (Ad) expressing thyrotropin receptor A-subunits (TSHR) developed stable Graves' disease (GD). TSHR-derived cyclic peptide 19 (P19) was identified as effective therapy in this model.
Methods: In Ad-TSHR mice, we investigated shorter disease intervals up to 4 months for histological alterations of the orbits, fine tuning of *anti*-TSHR antibodies (Ab) and free thyroxine (fT4) hormone levels by using novel detection methods in an independent laboratory. Therapy (0.3 mg/kg P19 or vehicle) was given intravenously after the fourth Ad-TSHR immunization (week 11) and continued until week 19.
Results: Thyrotropin binding inhibitory immunoglobulins (TBII, bridge immunoassay), blocking (TBAb) and stimulating (TSAb) TSHR-Ab (both cell-based bioassays) and serum levels of fT4 were significantly elevated at week 11 in Ad-TSHR-immunized mice versus none in control mice. For the first time, TSAb, TBAb, and thyroperoxidase-Ab were detected in 17 of 19, 12/19 and 6/19 Ad-TSHR immunized mice, respectively at week 21. Also, for the first time, this study showed that P19 treatment markedly reduced serum TBII ($p < 0.0001$), serum fT4 ($p = 0.02$), and acidic mucins and collagen content in the orbital tissue of Ad-TSHR-immunized mice.
Conclusion: P19 significantly improved thyroid function, confirming previous results in an independent second laboratory. A relevant shift of *anti*-TSHR antibody subpopulations in response to P19 therapy may help explain its immunological effects. Moreover, P19 exerted a beneficial effect on mucine and collagen content of orbital tissue. Hence, P19 offers a potential novel therapeutic approach for GD and associated orbitopathy.

Author statement

Tanja Diana: Investigation, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Martin Ungerer: Conceptualization, Supervision, Resources, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Christian Wüster: Resources, Writing – review & editing, Julia Faßbender: Investigation, Zhongmin Li: Investigation, Formal analysis. Andreas Reimann: Investigation. Hans-Peter Holthoff Investigation. Michael Kanitz: Investigation. George J Kahaly: Initiation, Lab-Infrastructure, Methodology, Conceptualization, Supervision, Resources, Visualization, Writing – original draft, Writing – review & editing, Project administration. All co-authors approved the content of the finalized manuscript subsequent to critical review.

1. Introduction

Graves' disease (GD) can be replicated in a long-term mouse model utilizing continuing immunizations for nine months of the recombinant

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Abbreviations

Ab	Antibodies
Ad-GFP	Adenovirus containing only the reporter gene GFP
Ad-TSHR	Adenovirus expressing the extracellular A-subunit of the human TSHR
AITD	Autoimmune thyroid disease
CHO	Chinese hamster ovary
FT3	Free triiodothyronine
FT4	Free thyroxine
GD	Graves' disease
GFP	Green fluorescent protein
LRRD	Leucine rich repeat domain
P19	Peptide 19
TSHR	Thyrotropin receptor
TSAb	TSHR stimulating antibodies
TBAb	TSHR blocking antibodies
TBII	TSHR-binding inhibitory immunoglobulins

adenovirus expressing the first 289 amino acids of the human thyrotropin receptor (Ad-TSHR) [1–3]. Interestingly, TSHR-stimulating antibodies (TSAb) and TSHR-blocking antibodies (TBAb) were highly prevalent and persistent in Ad-TSHR immunized mice over seven immunizations (27 weeks after the first immunization) [3], hence confirming the successful establishment of a long-term murine model for GD [3]. *Anti*-TSHR-Ab may mimic or block the action of TSH or be functionally neutral [4–7]. TSAb are responsible for several clinical manifestations of GD and are specific biomarkers of this autoimmune disease [8–11]. All TBAb and TSAb-positive Ad-TSHR immunized mice were TSHR-binding inhibiting autoantibodies (TBII) positive, showing that binding immunoassays are not able to discriminate TSHR-Ab functionality [3,9,10,12].

Graves' orbitopathy (GO) is the most common extrathyroidal manifestation of GD [13–17]. Histological serial orbital sections revealed that orbital fibrosis reflected GO severity [18]. Previous studies showed that cyclic peptides are able to simulate the tertiary structure of single cylindrical loops of the leucine rich repeat domain (LRRD) of the TSHR [19,20]. Novel peptides markedly reduced thyroid size and tachycardia in Ad-TSHR-immunized mice [20]. Especially, the cyclic peptide (P) 19 was derived from the first loop of the TSHR LRRD. This 11-meric peptide is a shortened version of peptide 829 [20]. Further, it encompasses amino acid residues, which play a crucial role for TSHR interaction and the purely stimulating human monoclonal antibody M22 at positions 34–36 [20].

In the present work, the effect of this novel P19 on thyroid function, thyroid-related antibodies and orbital morphology and tissue remodeling was evaluated in our established long-term GD mouse model.

2. Material and methods

2.1. Compliance with ethical standards and ethical approval

All applicable international, national and institutional guidelines for the care and use of animals were followed. All animal experiments were approved by the local animal welfare authority and Ethics committee at the "Regierung von Oberbayern" (Government Upper Bavaria) in Munich, Germany (no. 55.2-1-54-2531-25-12), and carried out in accordance to the European Commission guidelines. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

2.2. Synthesis of cyclic peptide 19

P19 was designed in analogy to the first loop of the TSHR LRD, and includes amino acid residues EED, which are considered crucial for the interaction of the TSHR and the prototypical patient M22 at positions 34–36. Fig. 1 shows the structure of the peptide, which was fully soluble in 0.9% NaCl. The peptide was synthesized by Biosyntan Berlin according to described protocols of fluorenylmethoxycarbonyl (FMOC) resin-based amino acid chain elongation, and subsequent head-to-tail cyclization. Fmoc amino acid or Fmoc dipeptide was attached to the 2-Chlorotrityl chloride resin (RAPP Polymere GmbH, Germany) yielding a loading of 0.30 mmol/g resin. Peptide synthesis was done by a standard cycle of deblocking with 30% piperidine/N,N-dimethylformamide (DMF, 5 + 12 min) and coupling with 3 eq. Fmoc-amino acid/O-(7-Azabenzotriazol-1-yl)-N,N,N′,N'-tetramethyluronium-hexafluorophosphate (HATU)/6 eq. N-methylmorpholine (NMM) in DMF (double coupling, 2 × 30 min). After cleavage from the resin by 20% hexafluoroisopropanol (HFIP)/DCM (2 × 20 min) the isolated crude peptide was cyclized by 1,5 eq. 7-azabenzotriazol-1-yloxy-tripyrrolidinophosphonium hexafluorophosphate (PyAOP)/3 eq. diisopropylethylamine (DIEA) in DMF overnight, the solvent evaporated and the crude peptide deblocked by trifluoroacetic acid (TFA)/water/thioanisol (TIS) (95:5: 3) during 2 h. Then, the peptide was purified up to 95% by means of HPLC and analyzed by MALDI-TOF mass spectrometry. This quality control reconfirmed that amino acids had been correctly included into the peptide, and the cyclization was evident from the experimental determination versus theoretical prediction of molecular weights: the value of observed versus predicted molecular weight of P19 was 1364.6 vs. 1363.5 Da.

2.3. Animal studies

Mice immunized with the Ad-TSHR either with 0.3 mg/kg body weight P19 dissolved in 0.9% NaCl or with 0.9% NaCl vehicle control were compared to mice immunized with the Ad-TSHR or recombinant adenovirus containing only the reporter gene GFP (Ad-GFP) and to native non-immunized mice over six immunizations (21 weeks after the first immunization). P19 was intravenously (i.v.) administered at weeks 11 and 21. Female BALB/c mice were handled as described previously [3]. Twenty-nine (ten Ad-TSHR + P19, nine Ad-TSHR + NaCl, five Ad-GFP immunized and five native) mice were investigated. Therapy (P19 dissolved in 0.9% NaCl without further additives, or 0.9% NaCl vehicle control) was given one week after the fourth immunization (week 11) by i.v. Injection into the tail vein and then continued at

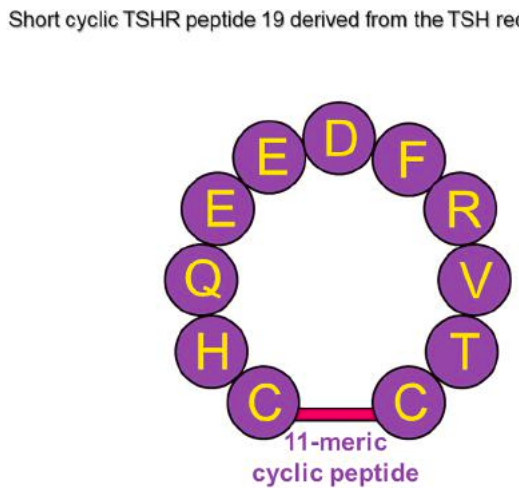

Fig. 1. Structure of the cyclic peptide P19. Amino acid residues are shown. Cyclisation occurs during synthesis by disulphide bridge formation between cysteine residues.

four-weekly intervals until week 19 [20]. Blood was drawn at same intervals and final blooding and sacrifice took place at week 21. All investigators including veterinarians, technicians and histologists were blinded to the treatment. Serum levels of free thyroxine (fT4) and thyroid-related antibodies and thyroid/orbital morphology were investigated.

2.4. Thyroid-related hormones and antibodies

Serum fT4 and TBII levels were measured in a blinded manner with a bridge TSHR-Ab binding immunoassay Immulite 2000 XPi (Siemens, Erlangen, Germany) according to the manufacturer's instructions. The reference ranges for fT4 and TBII are 0.9–1.8 ng/ml and 0.1–40.0 IU/L, respectively while the cut-off for the TBII immunoassay is at 0.55 IU/L. Serum levels of anti-thyroglobulin (Tg)-Ab, and anti-thyroid peroxidase (TPO)-Ab concentrations were measured using the Cobas e411 analyzer, Elecsys (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The reference ranges for *anti*-Tg-Ab, and *anti*-TPO-Ab were 115 IU/ml, and 34 IU/ml, respectively.

2.5. Cell-based bioassays for functional TSH-receptor antibodies

Serum TSAb were measured with an FDA-cleared cell-based bioassay (Thyretain®, Quidel, San Diego, USA) according to the manufacturer's instructions [21–23]. Briefly, Chinese Hamster Ovary (CHO)-MC4 cells were seeded and grown to confluent cell monolayers in 96-well plates for 15–18 h. Serum samples, as well as positive, reference and normal controls were diluted 1:11 in reaction buffer, added to the cell monolayers and each plate was incubated for 3 h at 37 °C, 5% CO2. Subsequently, the CHO-MC4 cells were lysed and the relative light unit values were quantified in a luminometer (Infinite M200; Tecan, Crailsheim, Germany). The assay cut-off is at a percentage specimen-to-reference-ratio (SRR) of 140%.

Serum TBAb levels were measured according to the manufacturer's (Quidel, San Diego, USA) instructions of the CE-marked cell-based bioassay [24,25]. The cut-off is at 34% inhibition. In both bioassays, all samples were measured in duplicates in a blinded fashion.

2.6. Histological analysis

For orbital preparations, complete dissection of the orbital and periorbital areas was performed, all orbital tissues, eyelids, and adjacent tissues were collected [19,20]. As previously reported [26], quantification of fibrosis using digitized Adobe Photoshop analysis of Masson trichrome stain was validated.

2.7. Alcian blue staining in orbit samples

For orbital preparations, the animals were euthanized, and complete dissection of the orbital and periorbital areas was carried out as described previously [19]. A serial of seven micron coronary cryosections were cut in a Leica cryostat (CM1850 cryostat; Leica Biosystems, Buffalo Grove, IL), as done before [19]. Mucin-stained areas in the extra-orbital adipose tissue and extra-orbital muscle regions were identified by their blue color. Digitized image analysis of blue color pixels was carried out using the luminescence tool of Adobe Photoshop vCS5, as shown previously [20].

2.8. Fibrosis in orbital sections

The orbital sections were viewed at 4× objective lens (Axioscope, Zeiss) captured with an Axiovision digital cam system and recorded with 2560× 1920 pixel resolution [19].

2.9. Statistical analyses

Statistical differences between the groups were analyzed by analysis of variance (ANOVA) for comparison between groups using GraphPad Prism Software, Inc., version 9.0.0, GraphPad, San Diego, CA, USA, followed by least significant difference post hoc testing or Student's t-test where appropriate. For comparison of values at various times within one group, repeated-measures ANOVA was used where appropriate.

3. Results

3.1. Free thyroxine levels

Serum fT4 concentrations are shown at week 11 (before start of treatment with P19 or vehicle) and week 21 in all individual investigated mice groups (Fig. 2). At week 11, fT4 levels were significantly higher in Ad-TSHR immunized mice versus control (Ad-GFP and native) mice, but did not differ between the treatment groups, which were defined by subsequent randomization. Subsequent to administration of P19 at weeks 11, 15 and 19, fT4 levels were markedly lower ($p = 0.02$) in individual Ad-TSHR-immunized mice treated with P19 vs. Ad-TSHR-immunized, vehicle-treated mice at week 21. Due to low serum volumes obtained at weeks 15 and 19, samples were pooled in the Ad-TSHR + P19 (ten mice) and in the Ad-TSHR + NaCl (nine mice) groups, respectively. In pooled serum samples at weeks 15 and 19, median values of serum fT4 in Ad-TSHR + P19 immunized mice were 10 ng/ml and 5 ng/ml vs. 20.2 ng/ml and 10.7 ng/ml in Ad-TSHR + NaCl immunized mice, respectively.

3.2. Thyrotropin receptor antibodies

Serum levels of TBII (Fig. 3), TBAb (Fig. 4, panel A) and TSAb (figure four, panel B) are represented at weeks 11 and 21 in all individual investigated mice groups. All Ad-GFP-immunized and native non-immunized mice were negative for TBII, TBAb and TSAB at all-time points. At week 11, serum levels of TBII were strongly elevated in Ad-TSHR immunized mice in contrast to control mice ($p < 0.0001$). At week 21, ten weeks after the first administration of P19, serum TBII levels were markedly reduced ($p < 0.0001$) in individual Ad-TSHR + P19 vs. Ad-TSHR + NaCl vs. immunized mice. TSAb and TBAb were positive in 17 of 19 (89.5%) and 12/19 (63%) individual Ad-TSHR immunized mice respectively at week 21.

Median TBII levels in pooled serum samples at weeks 15 and 19, were 200 IU/L and 40 IU/L in Ad-TSHR + P19 immunized mice vs. 200 IU/L and 200IU/L in Ad-TSHR + NaCl immunized mice, respectively. In line with this, in Ad-TSHR + P19 immunized mice, median, 25 and 75 quartiles of TSAb levels in pooled serum samples at weeks 15 and 19 were 289 (222, 326) and 273 (211, 297) vs. 349 (151, 428) and 280 (122, 373) SRR% in Ad-TSHR + NaCl immunized mice, respectively. TBAb in pooled Ad-TSHR + P19 immunized mice serum samples at weeks 15 and 19 were 27 (19, 49); 27 (25, 47) vs. 13 (−8, 68); eight (−8, 72) percent inhibition in Ad-TSHR + NaCl immunized mice, respectively.

3.3. Thyroperoxidase and thyroglobulin antibodies

Anti-thyroperoxidase-Ab were present in six of 19 samples (32%) of Ad-TSHR immunized mice versus none in control mice (Ad-GFP and native animals). Serum levels of *anti*-Tg-Ab were negative in all investigated animal groups at all measured time points.

3.4. Orbital and thyroid morphology

Acidic mucin and glycosaminoglycan (GAG) deposition (Alcian blue staining) was significantly increased in the orbital tissues of Ad-TSHR-immunized mice versus control mice (Fig. 5, panel A). The mucin

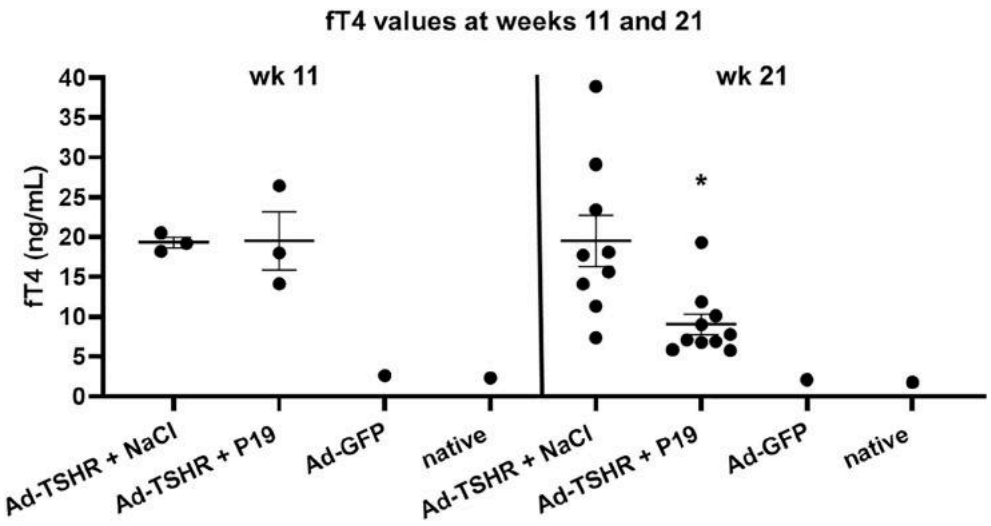

Fig. 2. Free T4 serum concentration values at weeks 11 and 21. *p < 0.05, comparing vehicle control (NaCl) versus 0.3 mg/kg body weight peptide P19 in Ad-TSHR-immunized mice.

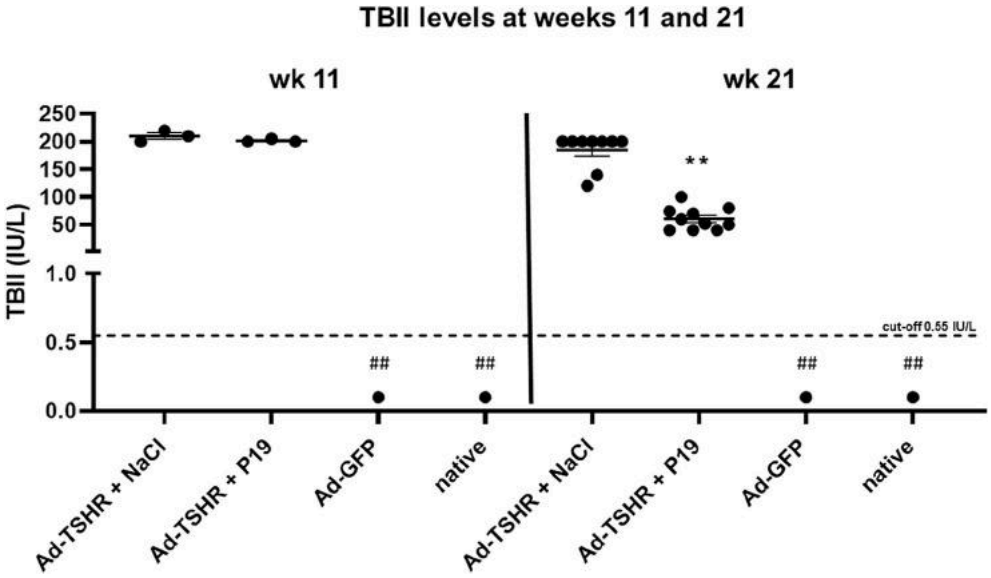

Fig. 3. Binding TSHR-Ab (TBII) serum levels in all individual investigated mice groups at weeks 11, and 21. **p < 0.0001, comparing vehicle control (NaCl) versus 0.3 mg/kg body weight P19 in Ad-TSHR-immunized mice at week 21. ##p < 0.0001, comparing vehicle control (NaCl) versus Ad-GFP-immunized and native mice at weeks 11 and 21.

amount significantly decreased when comparing Ad-TSHR-immunized mice treated with 0.3 mg/kg body weight P19 vs. vehicle control (NaCl) (figure five, panel B). The same hold true for the collagen content (mm^3) which was significantly higher in Ad-TSHR-immunized mice versus control mice (figure six, panel A). The collagen deposition significantly declined in the P19 treated animals ($p < 0.05$) vs. vehicle control mice. Collagen contents in the orbits (mm^3) were markedly different comparing 0.3 mg/kg body weight P19 (Fig. 6, panel B). Immune cell infiltration was estimated from hematoxylin eosin stains, and was qualitatively reduced with P19 therapy. In addition, we also included thyroid sizes as outcome parameters into the study protocol. However, as multiple tissues were harvested at the end of the study with a focus on orbital mucins (GAG), which were measured for the first time in our hands, the quality of thyroid assessment suffered unfortunately, probably due to longer than usual processing time. Therefore, these tissues could only partially be analyzed. As previously reported [20], the size of the thyroid glands were increased in Ad-TSHR + NaCl immunized mice. As far as could be judged, P19 administration led to a trend towards decreased thyroid sizes (data not shown). The findings were less pronounced than in the previous study, since these measurements can only be done once at the end of the experiments, i.e. 10 weeks after initiation of therapy in this study vs. 24 weeks after start of therapy in our previous study [27].

4. Discussion

To the best of our knowledge and for the first time, this study shows that further to the serological antibody positivity, elevated serum levels of thyroid-related hormones were already noted after four immunizations with the TSHR. Also new, six mice immunized with the TSHR developed *anti*-thyroperoxidase antibodies versus none of the control mice. Additionally, typical morphological changes of the orbital tissue with enhanced mucopolysaccharide and collagen deposition were observed. For the first time, subsequent to P19 administration, serum

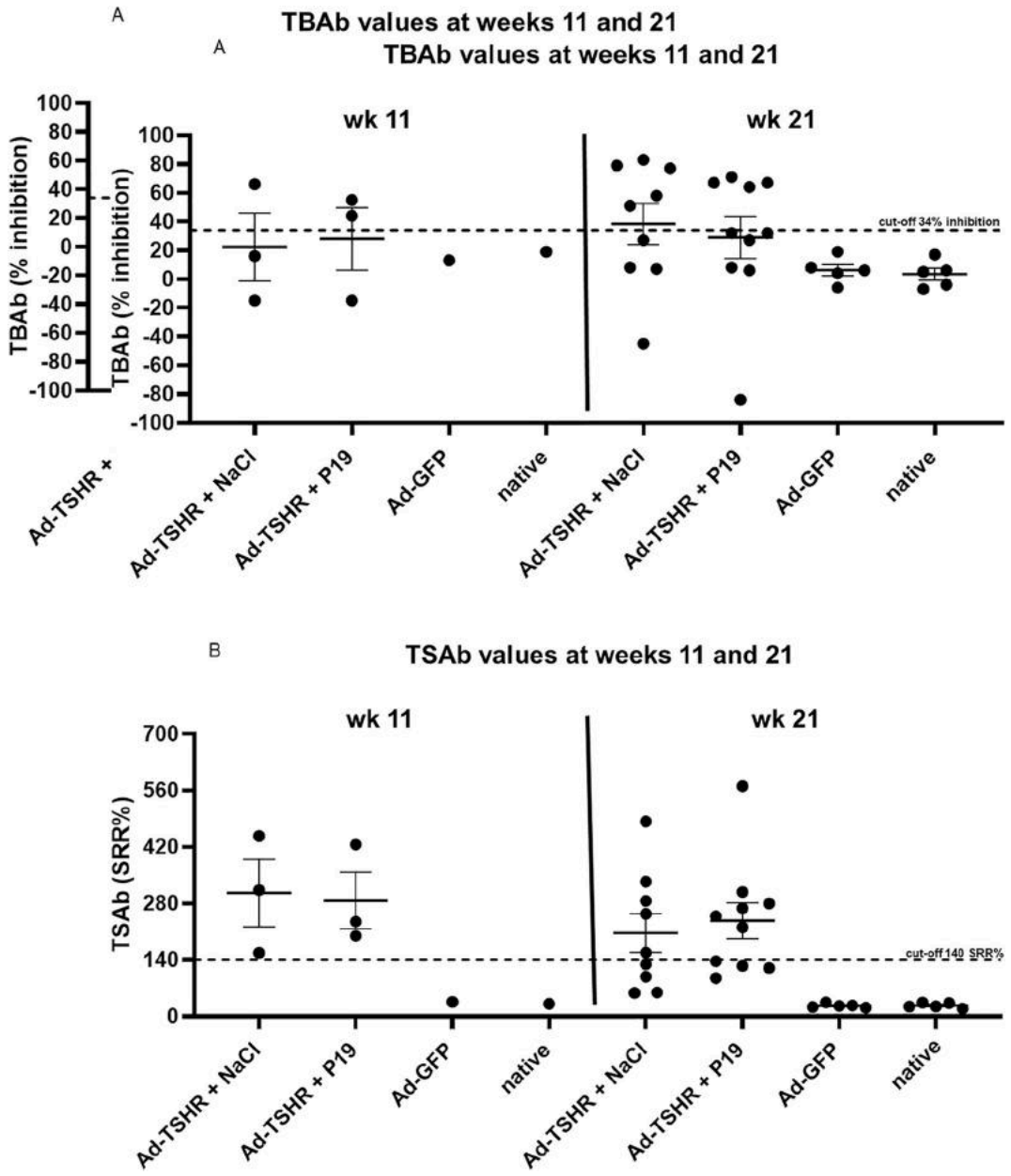

Fig. 4. Panel A, TBAb bioassay Blocking TSHR-Ab (TBAb) serum levels in all individual investigated mice groups at weeks 11, and 21. Panel B, TSAb bioassay Stimulatory TSHR-Ab (TSAb) serum levels in all individual investigated mice groups at weeks 11, and 21.

TBII and free T4 levels were markedly decreased, whereas other measured *anti*-TSHR titers were unchanged. This interesting finding indicates that fine-tuning of specific *anti*-TSHR antibody subpopulations may be involved in the therapeutic effects of antigen-derived cyclic peptides such as P19. Hence, as previously shown in humans [3,9,10,12] differentiating TSHR-Ab functionality in mice immunized with the adenovirus is exclusively feasible via cell-based bioassays, only.

This manuscript also demonstrates that a long-term GD mouse model as previously reported by our group [3,9,10,12] was verified, reconfirmed, and expanded by the detection of specific functional TSHR-Ab in all mice immunized with the TSHR in an independent laboratory. In contrast, *anti*-TSHR-Ab were not detected in any Ad-GFP immunized and native non-immunized mice at any investigated time points throughout the study. In this animal model, administration of P19 led to both a significant reduction of thyroid-related hormones and antibodies as well as to orbital tissue remodeling, retro bulbar fibrosis and collagen accumulation, hence demonstrating the relevant therapeutic effect of this novel peptide. These findings corroborate those of a previous long-time study on P19 [27] by measurements in a second independent laboratory.

The applied P19 imitates the first cylindrical loop of the leucine rich repeat domain of the TSHR. During synthesis, cyclization of P19 occurred spontaneously by building disulfide bonds [20]. Various novel peptides were designed to diminish antibody secreting immune cells in TSHR-Ab mediated GD [19,20]. In an earlier attempt, mice were immunized with murine fibroblasts transfected with the TSHR and a murine major histocompatibility complex class II molecule [28]. Intranasal administration of the TSHR peptide caused a decreased proliferative response of lymph node cells to the peptide. Instead of reducing both severity and frequency of GD, the application of this peptide led to a boost [28]. Another approach showed that a single injection of glycosylated TSHR A-subunit prior to TSHR immunization drifted the immune response, thereby preventing mice from development of GD [27]. It is not feasible to reverse generated hyperthyroidism in mice and therefore it is not suitable to treat these immunized mice [27]. Titers of

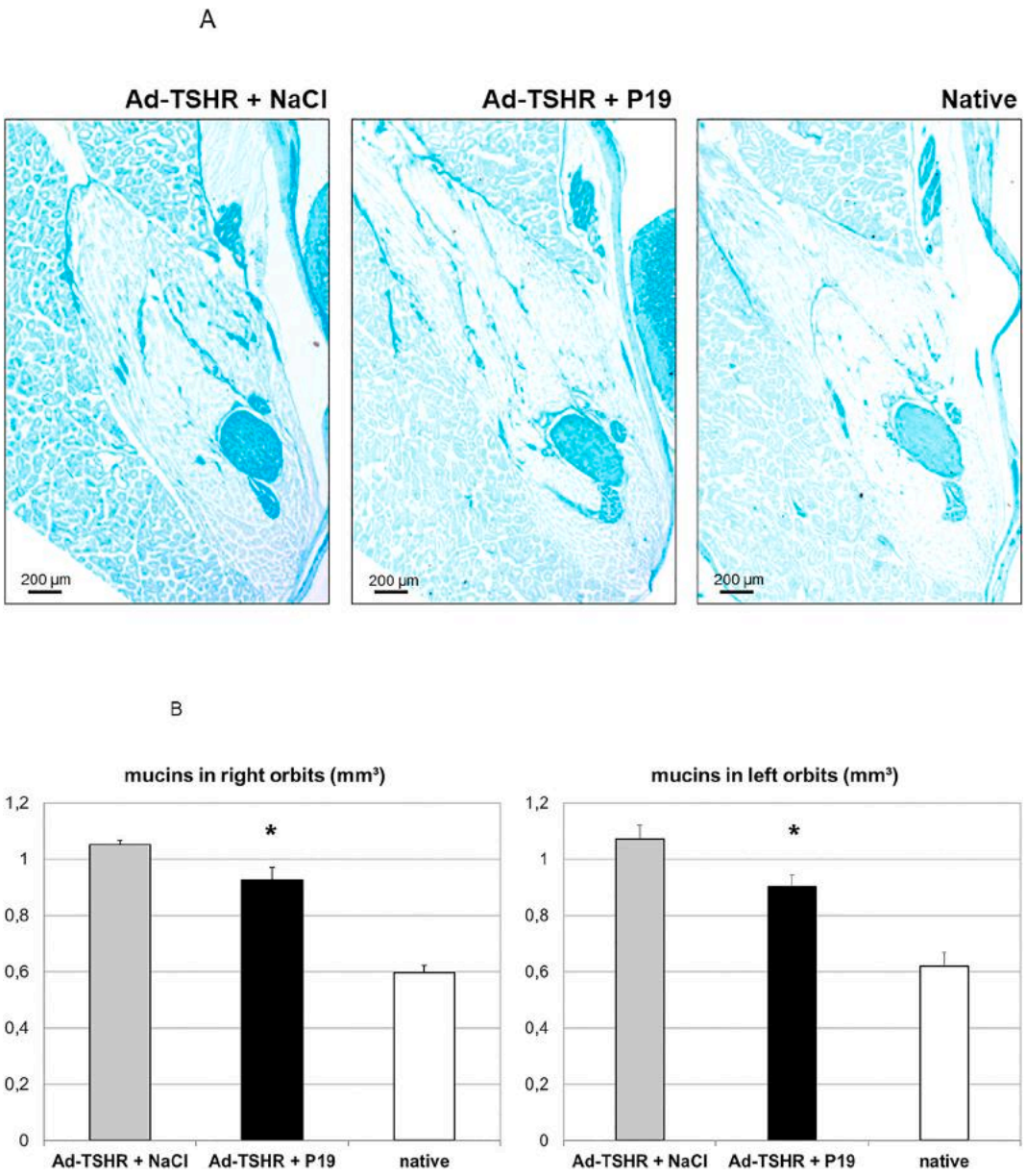

Fig. 5. Panel A, immunohistochemically staining with Alcian blue. Acidic mucins and glycosaminoglycans were detected in Ad-TSHR + NaCl, Ad-TSHR + P19 and native mice. Panel B, Mucins in the right and left orbits (mm^3). *$p < 0.05$, comparing vehicle control (NaCl) versus 0.3 mg/kg body weight P19 in Ad-TSHR-immunized mice.

anti-TSHR-Ab did not decline and T4 levels did not change.

Differences to the reported protocol and the present is that we are applying a long-term instead of a short-term model. In comparison to a short-term mouse model, a long-term model using regular adenoviral induced TSHR A-domain immunizations revealed a steadier phenotype of GD. In line with our results is a recently reported long-term adenovirus mouse model expressing the human TSHR A-subunit [29]. Orbital changes were noted in 7/10 of Ad-TSHR A-subunit mice after the ninth injection. Retro-orbital fibrosis and adipogenesis were observed in seven and four mice, respectively. Orbital immunohistochemically staining demonstrated the shift in $CD4^+$ T cell subsets. RNA sequencing revealed in mice with orbitopathy that various genes associated in T cell receptor signaling, proliferation, adhesion, inflammation, and cytotoxicity were upregulated.

Both in the above paper as well as in the present work, long-term immunization with the TSHR seems to produce better and more reproducible results as a short-term model, hence offering a reliable tool to study GD and associated orbitopathy. More importantly, with this established in-vivo tool, the inhibiting effect of both synthesized peptides as well as small molecules or monoclonal antibodies binding to the TSHR transmembrane region or extracellular ectodomain can be extensively tested [30,31]. Following up on the results described here, further mechanistic studies on how P19 reverses the immune response by phenotyping will be carried out, e. g. by investigating effects of P19 on blocking T-cell activation, B-cells or plasma cells, which will also include further control groups such as unrelated or sequence-scrambled peptides. To this end, an extensive investigation is being prepared in Ad-TSHR-immunized mice in which splenic as well as peripheral blood cells will be isolated and studied according to established methods [32]. These studies will importantly complement existing results on the effects of P19 on physiological, histological and hormonal changes in the model. Future steps to evaluate the efficacy and safety of P19 as well as potential application in humans are ongoing.

In conclusion, the novel P19 seems to be a promising therapeutic

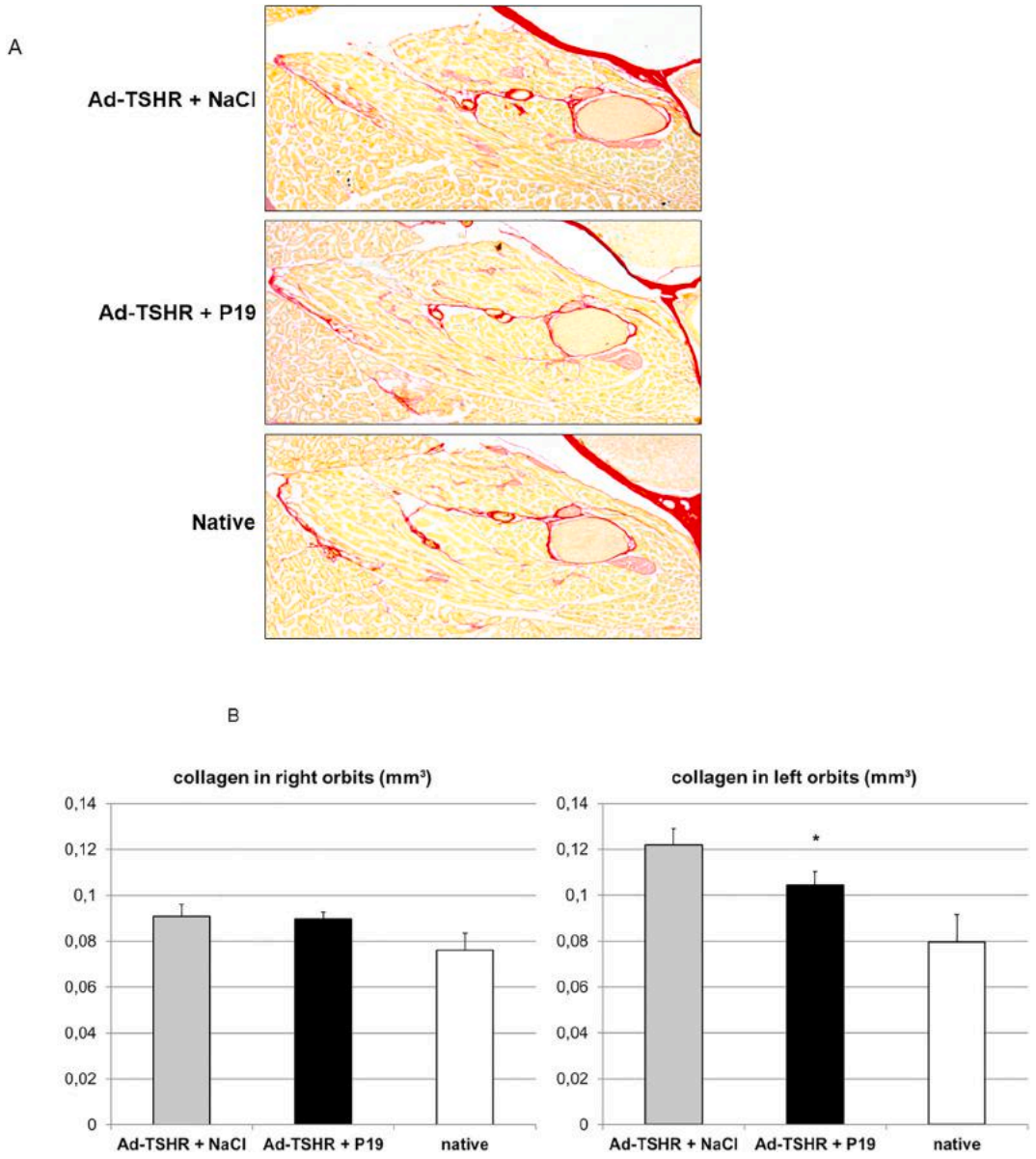

Fig. 6. Panel A Collagen content in orbits of Ad-TSHR + NaCl, Ad-TSHR + P19 and native mice. Panel B Collagen volume in right and left orbits (mm^3). *$p < 0.05$, comparing vehicle control in Ad-TSHR-immunized mice versus 0.3 mg/kg body weight P19.

alternative to treat GD and associated orbitopathy.

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Disclosure statement

TD, CW, and MK have nothing to disclose. MU and HPH are employees of ISAR Bioscience GmbH, and JF, ZL and AR are employees of Advancecor GmbH, a biotech company, which has filed patents on TSHR P19. GJK consults for Quidel.

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References

[1] H.P. Holthoff, S. Goebel, Z. Li, J. Fassbender, A. Reimann, S. Zeibig, et al., Prolonged TSH receptor A subunit immunization of female mice leads to a long-term model of Graves' disease, tachycardia, and cardiac hypertrophy, Endocrinology 156 (4) (2015) 1577–1589.
[2] M. Ungerer, J. Fassbender, H.P. Holthoff, Antigen-specific therapy of Graves disease and orbitopathy by induction of tolerance, Front. Biosci. 23 (2018) 2044–2052.
[3] T. Diana, H.P. Holthoff, J. Fassbender, C. Wuster, M. Kanitz, G.J. Kahaly, et al., A novel long-term graves' disease animal model confirmed by functional thyrotropin receptor antibodies, Eur Thyroid J 9 (Suppl 1) (2020) 51–58.
[4] G.J. Kahaly, T. Diana, TSH receptor antibody functionality and nomenclature, Front. Endocrinol. 8 (2017) 28.
[5] T. Diana, G.J. Kahaly, Thyroid stimulating hormone receptor antibodies in thyroid eye disease-methodology and clinical applications, Ophthalmic Plast. Reconstr. Surg. 34 (4S Suppl 1) (2018) S13–S19.
[6] T. Diana, P.D. Olivo, G.J. Kahaly, Thyrotropin receptor blocking antibodies, Horm. Metab. Res. 50 (12) (2018) 853–862.
[7] G.J. Kahaly, T. Diana, P.D. Olivo, Tsh receptor antibodies: relevance & utility, Endocr. Pract. 26 (1) (2020) 97–106.
[8] T. Diana, R.S. Brown, A. Bossowski, M. Segni, M. Niedziela, J. Konig, et al., Clinical relevance of thyroid-stimulating autoantibodies in pediatric graves' disease-a multicenter study, J. Clin. Endocrinol. Metab. 99 (5) (2014) 1648–1655.

[9] T. Diana, C. Wuster, M. Kanitz, G.J. Kahaly, Highly variable sensitivity of five binding and two bio-assays for TSH-receptor antibodies, J. Endocrinol. Invest. 39 (10) (2016) 1159–1165.
[10] T. Diana, C. Wuster, P.D. Olivo, A. Unterrainer, J. Konig, M. Kanitz, et al., Performance and specificity of 6 immunoassays for TSH receptor antibodies: a multicenter study, Eur Thyroid J 6 (5) (2017) 243–249.
[11] T. Diana, A. Daiber, M. Oelze, S. Neumann, P.D. Olivo, M. Kanitz, et al., Stimulatory TSH-receptor antibodies and oxidative stress in graves disease, J. Clin. Endocrinol. Metab. 103 (10) (2018) 3668–3677.
[12] S. Allelein, T. Diana, M. Ehlers, M. Kanitz, D. Hermsen, M. Schott, et al., Comparison of a bridge immunoassay with two bioassays for thyrotropin receptor antibody detection and differentiation, Horm. Metab. Res. 51 (6) (2019) 341–346.
[13] S.D. Lytton, K.A. Ponto, M. Kanitz, N. Matheis, L.D. Kohn, G.J. Kahaly, A novel thyroid stimulating immunoglobulin bioassay is a functional indicator of activity and severity of Graves' orbitopathy, J. Clin. Endocrinol. Metab. 95 (5) (2010) 2123–2131.
[14] G.J. Kahaly, M. Riedl, J. Konig, S. Pitz, K. Ponto, T. Diana, et al., Mycophenolate plus methylprednisolone versus methylprednisolone alone in active, moderate-to-severe Graves' orbitopathy (MINGO): a randomised, observer-masked, multicentre trial, Lancet Diabetes Endocrinol 6 (4) (2018) 287–298.
[15] Y.P. Hai, A.C.H. Lee, L. Frommer, T. Diana, G.J. Kahaly, Immunohistochemical analysis of human orbital tissue in Graves' orbitopathy, J. Endocrinol. Invest. 43 (2) (2020) 123–137.
[16] T. Diana, K.A. Ponto, G.J. Kahaly, Thyrotropin receptor antibodies and Graves' orbitopathy, J. Endocrinol. Invest. 44 (4) (2021) 703–712.
[17] A.C.H. Lee, M. Riedl, L. Frommer, T. Diana, G.J. Kahaly, Systemic safety analysis of mycophenolate in Graves' orbitopathy, J. Endocrinol. Invest. 43 (6) (2020) 767–777.
[18] M. Ungerer, J. Fassbender, Z. Li, G. Munch, H.P. Holthoff, Review of mouse models of graves' disease and orbitopathy-novel treatment by induction of tolerance, Clin. Rev. Allergy Immunol. 52 (2) (2017) 182–193.
[19] H.P. Holthoff, Z. Li, J. Fassbender, A. Reimann, K. Adler, G. Munch, et al., Cyclic peptides for effective treatment in a long-term model of graves disease and orbitopathy in female mice, Endocrinology 158 (7) (2017) 2376–2390.
[20] J. Fassbender, H.P. Holthoff, Z. Li, M. Ungerer, Therapeutic effects of short cyclic and combined epitope peptides in a long-term model of graves' disease and orbitopathy, Thyroid 29 (2) (2019) 258–267.
[21] S.D. Lytton, Y. Li, P.D. Olivo, L.D. Kohn, G.J. Kahaly, Novel chimeric thyroid-stimulating hormone-receptor bioassay for thyroid-stimulating immunoglobulins, Clin. Exp. Immunol. 162 (3) (2010) 438–446.
[22] J.J. Leschik, T. Diana, P.D. Olivo, J. Konig, U. Krahn, Y. Li, et al., Analytical performance and clinical utility of a bioassay for thyroid-stimulating immunoglobulins, Am. J. Clin. Pathol. 139 (2) (2013) 192–200.
[23] T. Diana, M. Kanitz, M. Lehmann, Y. Li, P.D. Olivo, G.J. Kahaly, Standardization of a bioassay for thyrotropin receptor stimulating autoantibodies, Thyroid 25 (2) (2015) 169–175.
[24] Y. Li, J. Kim, T. Diana, R. Klasen, P.D. Olivo, G.J. Kahaly, A novel bioassay for anti-thyrotrophin receptor autoantibodies detects both thyroid-blocking and stimulating activity, Clin. Exp. Immunol. 173 (3) (2013) 390–397.
[25] T. Diana, Y. Li, P.D. Olivo, K.J. Lackner, H. Kim, M. Kanitz, et al., Analytical performance and validation of a bioassay for thyroid-blocking antibodies, Thyroid 26 (5) (2016) 734–740.
[26] G.M. Dahab, M.M. Kheriza, H.M. El-Beltagi, A.M. Fouda, O.A. El-Din, Digital quantification of fibrosis in liver biopsy sections: description of a new method by Photoshop software, J. Gastroenterol. Hepatol. 19 (1) (2004) 78–85.
[27] A.V. Misharin, Y. Nagayama, H.A. Aliesky, Y. Mizutori, B. Rapoport, S. M. McLachlan, Attenuation of induced hyperthyroidism in mice by pretreatment with thyrotropin receptor protein: deviation of thyroid-stimulating to nonfunctional antibodies, Endocrinology 150 (8) (2009) 3944–3952.
[28] T. Arima, N. Shimojo, K. Yamaguchi, M. Tomiita, L.D. Kohn, Y. Kohno, Enhancement of experimental Graves' disease by intranasal administration of a T cell epitope of the thyrotropin receptor, Clin. Immunol. 127 (1) (2008) 7–13.
[29] M. Zhang, X. Ding, L. P. Wu, M. Q. He, Z. Y. Chen, B. Y. Shi et al. A promising mouse model of graves' orbitopathy induced by adenovirus expressing thyrotropin receptor A subunit. Thyroid (2021 Jan 5. doi: 10.1089/thy.2020.0088. Epub ahead of print. PMID: 33076782.).
[30] A.C.H. Lee, G.J. Kahaly, Novel approaches for immunosuppression in graves' hyperthyroidism and associated orbitopathy, Eur Thyroid J 9 (Suppl 1) (2020) 17–30.
[31] S. Neumann, C.C. Krieger, M.C. Gershengorn, Targeting TSH and IGF-1 receptors to treat thyroid eye disease, Eur Thyroid J 9 (Suppl 1) (2020) 59–65.
[32] H. Degen, I. Gavvovidis, T. Blankenstein, K. Uhland, M. Ungerer. Thyrotropin receptor-specific lymphocytes in adenovirus-TSHR-immunized native and human leukocyte antigen-DR3-transgenic mice and in graves' disease patient blood. Thyroid (2021 Feb 8. doi: 10.1089/thy.2020.0338. PMID: 33208049).