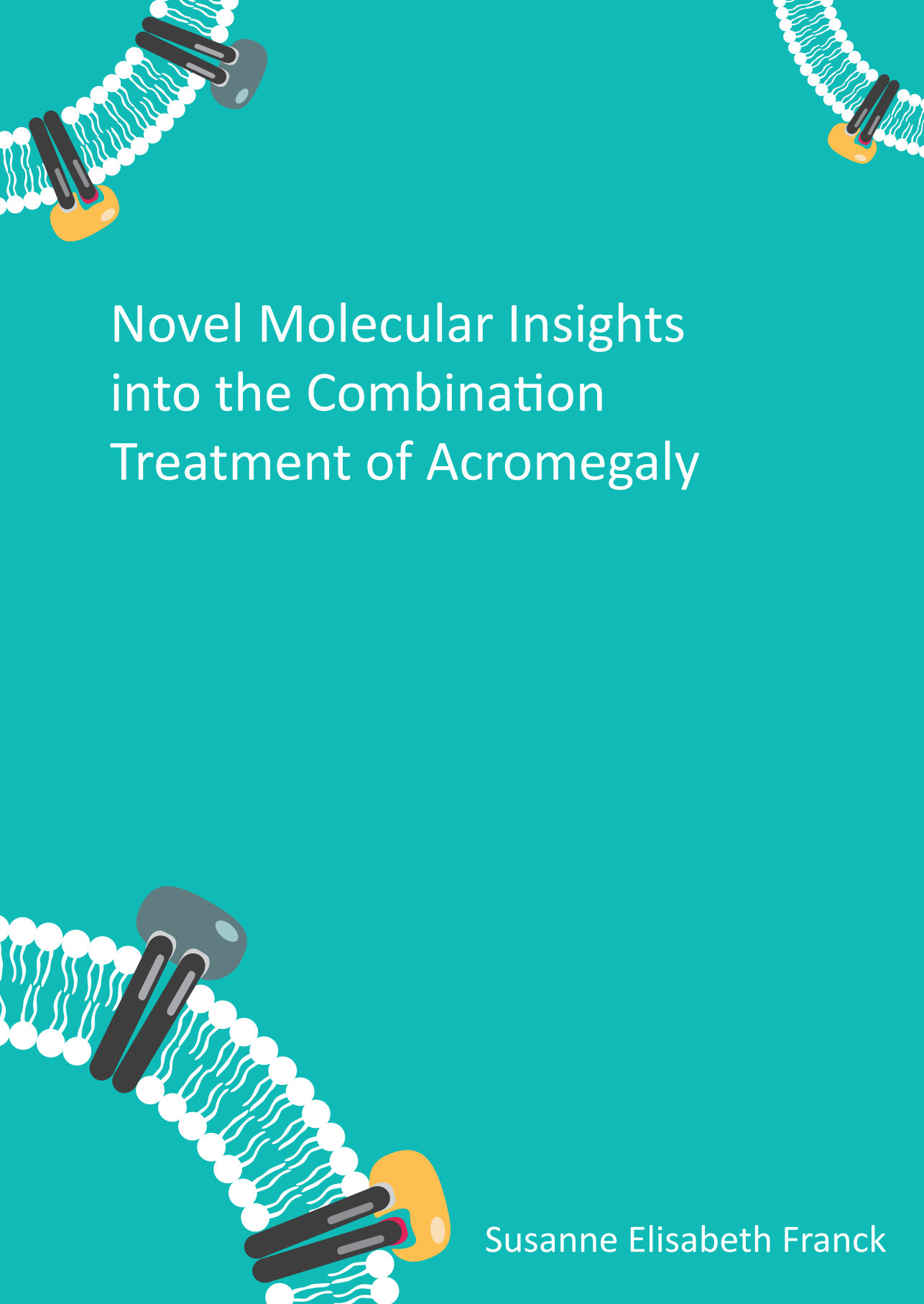

Novel Molecular Insights into the Combination Treatment of Acromegaly

Susanne Elisabeth Franck

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NOVEL MOLECULAR INSIGHTS INTO THE COMBINATION TREATMENT OF ACROMEGALY

Nieuwe moleculaire inzichten
in de combinatietherapie van acromegalie

Proefschrift

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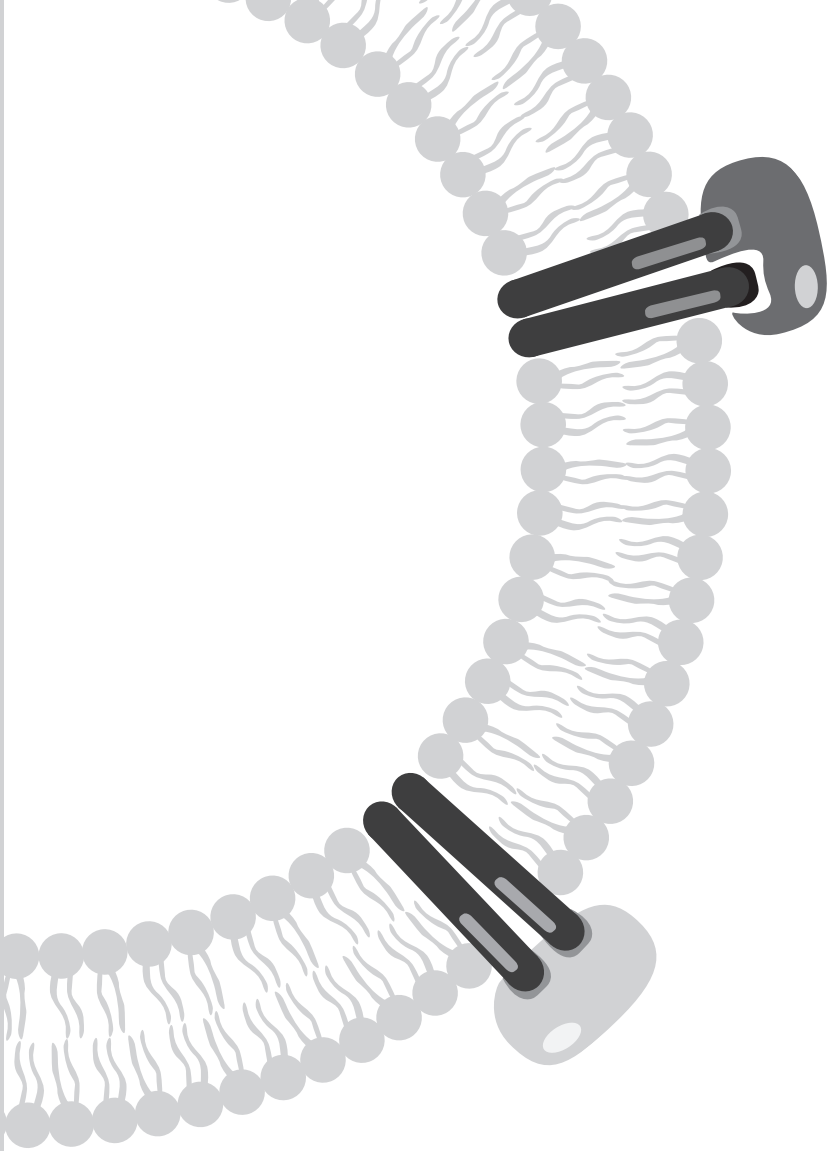

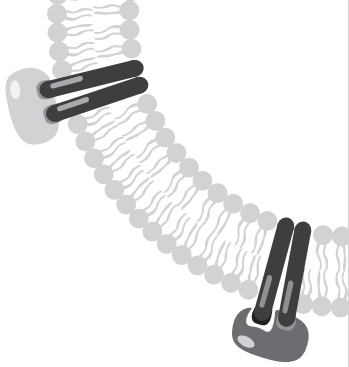

Chapter 1

General introduction and outline of this thesis

PARTLY BASED ON:
Combined Treatment of Somatostatin Analogues
with Pegvisomant in Acromegaly
S.E. Franck, A. Muhammad, A.J. van der Lely, S.J.C.M.M. Neggers
Endocrine 2016; 52(2): 206-213

Extra-Hepatic Acromegaly
S.E. Franck, A.J. van der Lely, S.J.C.M.M. Neggers
European Endocrinology 2013; 9(1): 54-58
US Endocrinology 2013; 9(1): 66-70

1. ACROMEGALY

Acromegaly is a rare disease caused by a growth hormone (GH) secreting pituitary adenoma and resulting in associated chronic elevated insulin-like growth factor I (IGF-I) levels, the mediator of most GH-activity (1). The disease presents either because of signs and symptoms due to GH hypersecretion, or mechanical compression by local space occupation or symptoms due to panhypopituitarism. Population based studies have reported that 9-17% of pituitary adenomas arose from somatotroph cells and cause excessive GH excretion (2-6), in 25% GH secreting pituitary adenomas co-secrete prolactin (7). Prevalence of acromegaly is between 40-70 cases per million inhabitants with an incidence rate of 3-4 new cases per million inhabitants a year (8-10). However, more recent studies report higher prevalence of the disease between 70-120 cases per million inhabitants and incidence rates between 7.7-11 new diagnosed acromegaly patients per million per year (2,3,11,12), indicating that acromegaly is likely of being underdiagnosed. Signs and symptoms develop slowly over time, causing a median delay in diagnosing the disease by 6 years (13). Acromegaly can be diagnosed at every age, however the majority is diagnosed between the age of 40-50 years (13).

1.1. Anatomy

The pituitary is located in the sella turcica below the hypothalamus, both structures are connected via the pituitary stalk. The optic chiasm (N. II) is situated between the hypothalamus and the pituitary. The nervus oculomotorius (III), trochlearis (IV) and abducens (VI) are also closely situated around the pituitary gland. Anatomical structures around the pituitary are depicted in Figure 1. The pituitary is the central endocrine gland, about the size of a pea, which coordinates (together with the hypothalamus and its releasing hormones) the peripheral endocrine glands via production of several hormones. The anterior lobe of the pituitary, connected to the hypothalamus via a capillary system, provides all hormones (GH, LH, FSH, TSH, ACTH and prolactin (PRL)) needed to control multiple processes in the human body such as growth, metabolism, control of the sex organs, the thyroid gland, the adrenals, physiology during pregnancy and nursing. The posterior lobe, connected to the hypothalamus via axons of hypothalamic neurons, is responsible for the secretion of oxytocin (important during childbirth) and vasopressin, which regulates blood pressure by reabsorption of water by the kidneys. The pituitary is highly vascularized by a portal system, which releases the hormones in the circulatory system. The central nervous system is separated from the circulatory system by the blood-brain barrier (BBB). However, the pituitary and many areas of the hypothalamus lack a BBB (14).

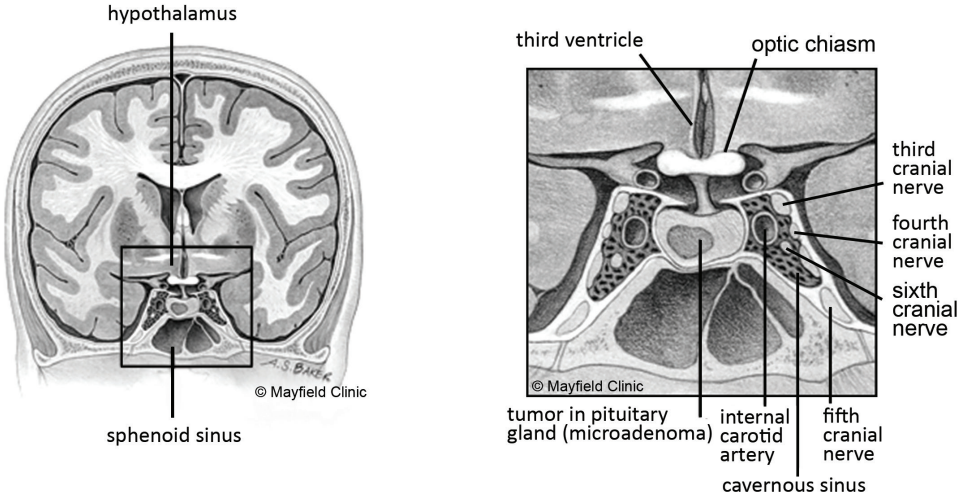

Figure 1. A microadenoma in the pituitary
Coronal cross-section of the head at the level of the pituitary gland depicting its relationship to the unharmed surrounded anatomy such as the optic chiasm above, the sphenoid sinus below, and the cavernous sinuses.
This Figure is printed with permission from Mayfield Clinic.

1.2. Growth hormone

GH promotes (bone/muscle) growth and influences metabolism which includes processes such as promoting protein synthesis, cell proliferation, gluconeogenesis, lipolysis and inhibition of glycogen storage and apoptosis. GH stimulates synthesis of IGF-I in peripheral tissues, predominantly in the liver. Many (but not all) effects of GH on peripheral tissues are physiologically mediated by IGF-I (15-17). Several studies have shown that the GH-IGF-I axis plays an important role during processes as longevity and ageing (18). It is hard to address the individual effects of GH and IGF-I in psychological conditions at the tissue level. What we know is that IGF-I and GH are both strong growth promoters as described previously. However, GH possesses anti-insulin or diabetogenic activity (19). On the other hand, insulin and IGF-I have similar actions, this clearly demonstrates that GH and IGF-I also exhibit different physiological actions.

Once secreted and excreted by somatotroph cells in the pituitary, GH is entering the circulatory system by pulsatile-release. GH is released, particular, during stress, exercise and sleep. GH and IGF-I levels are high during adolescence, elevated during pregnancy and decline trough adult life. The IGF-I upper limit of normal, therefore, is age and sex dependent, which has to be taken into account during interpreting IGF-I values (20). GH-release is controlled by growth hormone releasing hormone (GHrH) which stimulates GH secretion/release, while endogenous somatotropin releasing-

inhibiting factor (SRIF, also known as somatostatin) suppresses its release, both hormones are derived from the hypothalamus (21). Ghrelin, a hormone produced by the stomach, also promotes secretion of GH by the pituitary, and is influenced by food-intake (22). Additionally, negative feedback is also promoted by GH itself at the level of the hypothalamus. IGF-I has a negative feedback loop at the level of the hypothalamus and the pituitary, depicted in Figure 2.

The binding of GH to its receptor stimulates Janus kinase 2 (JAK2) in signaling signal transducers and activators of transcription (STATs) to dimerize. The dimerized STATs translocate to the nucleus to regulate transcription of GH-target genes (23). A GH receptor (GHR) polymorphism common in the general population, which lacks exon 3 (d3-GHR), lead to greater stimulation of the intracellular JAK2-STAT5 pathway in response to GH, and therefore results in increased transcription of these target genes. This enhanced signal transduction via the d3-GHR was first shown in vitro by transfection experiments (24). Thereafter, clinical studies were performed in GH deficient children and acromegaly patients. More about this topic is written in the outlines of this thesis, chapter 4 and 5.

1.3 Pathophysiology of acromegaly

Acromegaly develops when somatotroph cells proliferate and together form a benign monoclonal adenoma, which hypersecrete GH that is released to the peripheral circulation. Based on the largest diameter of the adenoma on a magnetic resonance imaging (MRI), adenomas are subdivided in microadenomas (<1 cm) and macroadenomas (≥1 cm). The majority (78%) of the GH-secreting pituitary adenomas are macroadenomas (13). In chapter 2 of this thesis (Figure 2), the MRIs depict macroadenomas. Acromegaly is almost exclusively caused by a GH-secreting pituitary adenoma, however very rare acromegaly cases are described caused by extra-pituitary hypersecretion of GH in an ectopic pancreatic islet-cell tumor or non-Hodgkin's lymphoma (25,26). Excessive production of GHrH could also induce acromegaly, via a central hypothalamic tumor or a peripheral neuroendocrine tumor mainly from pancreatic or bronchial origin (27). The vast majority of the GH-secreting pituitary adenomas occur sporadically, although reports are increasingly published about hereditary acromegaly cases including Multiple Endocrine Neoplasia 1, Carney complex, and McCune-Albright syndrome (28). Familial acromegaly without other syndromic features have also been described in literature. The majority (one fifth) of these familial cases show a mutation in the Aryl Hydrocarbon Receptor Interacting Protein (AIP) gene (29). Recently, a rare syndrome called X-linked acrogigantism (X-LAG) is discovered, which causes acromegaly in young children (30).

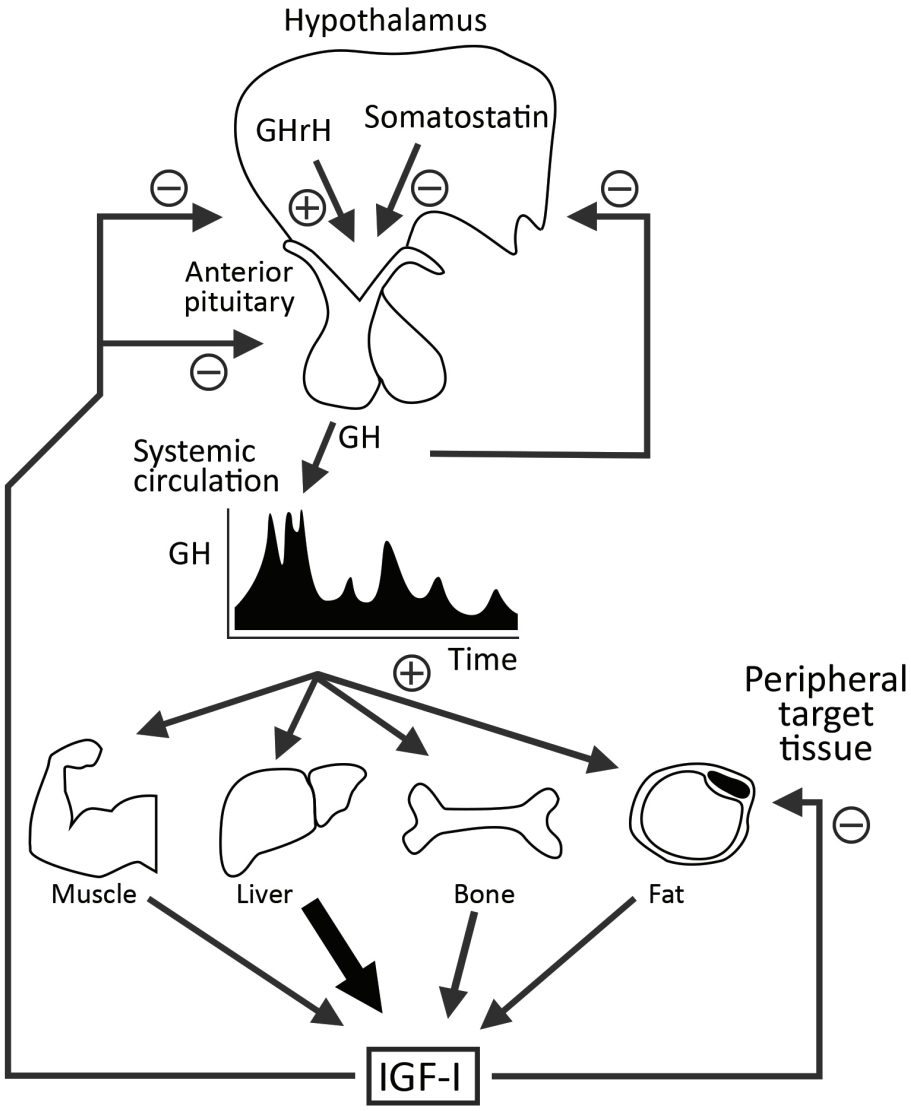

Figure 2. Feedback system of the GH-IGF-I axis

Physiology of the GH-IGF-I axis by the hypothalamus, pituitary and the peripheral tissues, such as muscle, liver, bone and white adipose tissue. Somatostatin in this Figure is the same as endogenous somatotropin releasing-inhibiting factor (SRIF).

GHrH: growth hormone releasing hormone, GH: growth hormone, IGF-I: insulin-like growth factor I.

1.4 Symptoms, co-morbidities and mortality

Hypersecretion of GH and the associated elevated IGF-I levels result in increased morbidity and mortality (1). Symptoms associated with acromegaly are either caused by the GH/IGF-I hypersecretion itself and/or due to panhypopituitarism and/or local tumor mass effects. Prolonged exposure to increased GH and IGF-I levels can cause an acromegalic phenotype. The disease is characterized by excessive skeletal growth, soft tissue enlargement and multiple other comorbidities, listed in Table 1 (1). Some GH-secreting pituitary adenomas cause serious symptoms, while others slowly cause non-specific complains or even remain symptomless and are diagnosed incidentally. Gigantism only arises in young acromegaly patients before fusion of epiphyseal growth plates of the bone. Mortality rates in acromegaly patients are approximately two to three times higher compared with age- and sex-matched controls (31), and are depending on how well GH/IGF-I levels are controlled. Cardiovascular, cerebrovascular and respiratory diseases are the major causes of death in acromegaly patients (31).

1.5. Diagnosis

The cornerstone of diagnosing acromegaly consists of IGF-I levels above the age-adjusted normal value, and an insufficient GH suppression during oral glucose loading (32), both measurements depend on the upper limit of normal for the locally used assay. These biochemical measurements should be done in response to clinical parameters such as the patient's (acromegalic) phenotype and/or coexisting comorbidities, described in Table 1. Following biochemical diagnosis of the disease, pituitary-imaging via an MRI scan is recommended in order to determine and visualize a GH-secreting adenoma and its size as well as its parasellar extent (32).

2. TREATMENT MODALITIES

Treatment of acromegaly is aimed to reduce signs and symptoms, improve quality of life, and decrease morbidity and mortality. Depending on patient characteristics, pituitary adenoma size and localization, a treatment modality should be chosen. Available modalities are surgery, medical therapy, radiotherapy or a combination of these. Treatment mainly focuses on suppressing GH hypersecretion, normalization of IGF-I serum levels and tumor shrinkage or at least tumor size control (33). Treatment efficacy is monitored by GH/IGF-I serum levels and by an MRI to assess changes in tumor volume. Biochemical target goals are a random GH level of <1.0 g/l and an age-normalized serum IGF-I value, resulting in relief of signs and symptoms (32). Life expectancy in successfully treated acromegaly patients is approaching that of the general population (34,35).

Table 1. Symptoms and comorbidities of acromegaly

Symptoms:	**Comorbidities:**
Fatigue	Hypertension
Headache	Hypertriglyceridemia
Visual field defects	Cardiomyopathy
Dysfunction cerebral nerves (sporadically)	Diabetes mellitus type II
Snoring	Sleeping apnea
Deepening of the voice	Carpal tunnel syndrome
Excessive sweating	Osteoarthrosis
Oily skin	Colon polyps
Skin tags	Organomegaly *i.e.*:
Thickness of soft tissue hands/feet	Hepatomegaly
Arthropathy	Splenomegaly
Facial changes *i.e.*:	Dolichocolon
Frontal skull bossing	Panhypopituitarism *i.e.*:
Enlargement of facial features	Erectile dysfunction in men
Prognathism	Menstrual cycle dysfunction in women
Macrognathia	
Diastema	Secondary thyroid deficiency
Macroglossia	Secondary adrenal deficiency

2.1 Transsphenoidal adenectomy

Surgery is in general the first treatment modality and the only possibility to cure the disease, but is not always successful, as the majority of the patients have a macroadenoma (1). An urgent call for transsphenoidal adenectomy is visual field deficit, which needs direct pressure-relief of the tumor mass on the optic chiasm. The reported cure rates of transsphenoidal surgery vary widely, mainly depending on tumor size, tumor invasiveness and the experience of the neurosurgeon (36). Surgery of microadenomas (<1 cm in diameter) has an average cure rate of 78%, whereas with macroadenomas (≥1 cm in diameter) the average cure rate is ≤50% (36). More recent data from daily practice was shown in a study with data gathered from the UK National Acromegaly Registry. This study reported cure rates between 20 and 40% (37). Complications of transsphenoidal surgery are meningitis, bleeding, spinal fluid leakage, diabetes insipidus and (partial) panhypopituitarism.

Major events as visual field loss and carotid artery injury occur, albeit rarely (38,39). Curation is determined by an oral glucose tolerance test with a GH level <1 g/l (32). The recurrence rate of patients who initially were cured after transsphenoidal surgery is 19%, in which the vast majority presents disease recurrence within the first 5 years of follow-up (40).

2.2 Radiotherapy

First-line treatment in acromegaly remains surgery and/or medical treatment, but when biochemical control is not possible and the patient has an aggressive adenoma radiotherapy could be proposed. Conventional radiotherapy is administered in 20-30 fractions, eventually resulting in a total dose of 45-50 Gray (41). The GH-decline during the first two years after radiotherapy are ranging between 50-70% of the initial value, thereafter characterized by a prolonged slow GH decrease over 10-20 years (41). Disadvantages of radiotherapy are the delayed time to control the disease compared to surgery and medical treatment, apart from the unneglectable risk of adverse events. Hypopituitarism occurs in the vast majority over time (42-44). Other severe side effects are rare such as visual field deficits, radiation-induced cerebral tumors and vascular injuries (41), but have to be taken into account when considering this treatment modality. A relatively new irradiation technique is the gamma-knife, in which a more precise stereotactically mapped region could be administered by one single high dose of radiotherapy, also called stereotactic radiosurgery. This results in a lower dose on the healthy surrounding tissue and probably a lower incidence of adverse events. Stereotactical radiotherapy can be used in the same cases as conventional radiotherapy, although this technique requires a well-defined target adenoma, restraining its applicability. In addition, it must be taken into account that especially long-term safety data are lacking, and final conclusions cannot be drawn about these new irradiation techniques.

2.3 Medical treatment

2.3.1 Somatostatin analogues

GH-secreting pituitary adenomas express different subtypes of somatostatin receptors (SSTRs), predominantly SSTR subtypes 2 and 5 (45), these receptors play a major role in reducing GH and IGF-I levels. Long-acting somatostatin analogues (LA-SSAs) have high-binding affinity for SSTR2 and a moderate affinity for SSTR5. According to current guidelines, LA-SSAs are considered to be the first line medical treatment modality after unsuccessful surgery as well as primary treatment option in selected cases (32,33). Meta-analyses of clinical trials showed that LA-SSAs alone normalize GH and IGF-I levels in about half of the patients (46). However, due to selection bias this efficacy rate is probably an overestimation. In unselected

treatment-naive patients an LA-SSA efficacy rate of 40% seems to be more common (47). An additional advantage of LA-SSA treatment is its capacity to reduce tumor volume. Recent prospective multicenter clinical trial observed tumor shrinkage in 63% of primary treated patients with 120 mg Lanreotide Autogel administered every 28 days (48). Tumor shrinkage was defined as clinically significant when ≥20% tumor volume reduction was observed after 48 weeks of Lanreotide Autogel administration. No differences between Lanreotide SR and Octreotide LAR were observed regarding tumor shrinkage and normalization of IGF-I levels (49). Most side effects of LA-SSA-treatment are transient and of mild-to-moderate intensity, most commonly are injection-site discomfort/erythema and gastrointestinal symptoms such as nausea, vomiting, abdominal pain, biliary sludge or gallstones and diarrhea (50).

2.3.2 Dopamine agonists

Dopamine agonists (DAs) suppress GH hypersecretion in patients with acromegaly, not only in mixed PRL-GH-secreting pituitary adenomas but also on pure GH-secreting pituitary adenomas (51-54). Cabergoline (CAB) is more effective and tolerated than bromocriptine, however less effective than LA-SSA, but cheaper and can be taken orally (55). A meta-analysis concluded that CAB as monotherapy normalizes IGF-I levels in one third of patients with acromegaly (55). When CAB was added to LA-SSA, which fails to control acromegaly as monotherapy, IGF-I levels normalized in about half of the patients (55). An advantage of DAs is that the drug is given orally in 1-3 weekly doses. Side effects are headache, dizziness and nausea.

2.3.3 Pegvisomant

Pegvisomant (PEGV) is a PEGylated recombinant analogue of GH and thereby functions as a GH antagonist. The molecule contains, similar like human GH, 191 amino acids and harbors several amino acid changes with respect to human GH. Most significantly is the replacement of glycine by lysine at the position 120 corresponding with binding site 2 of the drug, which leads to a lack of function of GH signal transduction (56,57), depicted by Figure 3. Additional mutations corresponding with binding site 1 increase the affinity of PEGV with the GHR. Besides these changes, the drug is modified by the addition of five polyethylene-glycol polymer chains, which is performed to prolong the half-life of the molecule, and improves its clinical applicability (58). PEGV is a competitive blocker of the GHR, pharmacology dictates that in principle it should be possible to control IGF-I levels in virtually every patient with acromegaly, provided that the appropriate PEGV dose is used (59). Monotherapy of PEGV requires a high cumulative weekly dose of around 130-140 mg to achieve a 89-97% normalization rate (59,60).

PEGV-treatment is generally well tolerated and is increasingly considered to be safe (61,62). More data is available where it comes to medical treatment of LA-SSA and DA, as PEGV has only been commercially available since 2003. Lipohypertrophy (3-14%) and hepatotoxicity (4-14%) are the most frequently reported adverse events during long-term PEGV monotherapy (63-66). Lipohypertrophy is an increased subcutaneous fat deposition around the injection sites of PEGV and appeared to be reversible by a more frequent rotation of the injection-site in the majority of the patients (64). It is suggested that the blockade of GHRs causes unopposed insulin effects and, therefore, promotes lipogenesis (67). The PEGV-induced hepatocellular elevated enzymes are usually mild and self-limiting (63,64). These transient elevated transaminases (TET) are defined as alanine transaminase (ALT) and/or aspartate transaminase (AST) levels of more than three times the upper limit of normal.

As PEGV systemically blocks GHRs, and does not prevent tumor growth of the pituitary adenoma, concerns were previously raised whether PEGV might induce growth of the pituitary adenoma. Despite the fact that in a few cases an increase in tumor size during PEGV therapy was reported, data indicates that its incidence is extremely rare and these concerns are conceived as being unfounded (61,68). However, more long-term data is needed to confirm these findings.

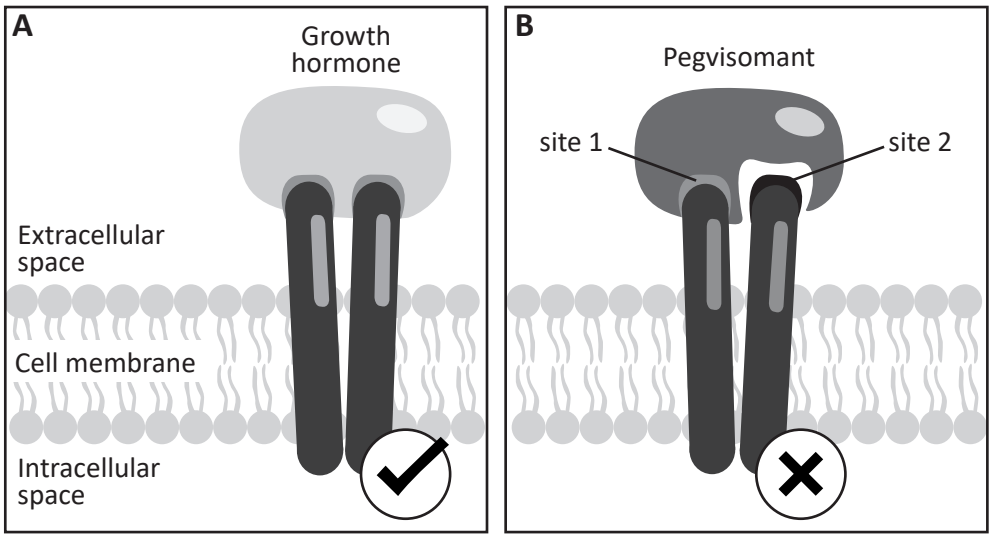

Figure 3. Binding site 1 and 2 of growth hormone and pegvisomant
A) Illustrative picture of growth hormone binding to its dimerized receptor resulting in activation of a downstream signal transduction, which eventually will lead to transcription of GH-target genes.
B) Illustrative picture of pegvisomant binding to the dimerized GH receptor. Binding site 1 of pegvisomant improve binding to the GH receptor. Binding site 2 contains the replacement of glycine by lysine at the position 120 substitution that blocks the conformational change of the GH receptor and thereby inhibits downstream signal transduction.

3. COMBINATION TREATMENT OF LA-SSA AND PEGV

An attractive way to biochemically control disease activity in acromegaly patients who are uncontrolled by LA-SSA treatment alone, is to add PEGV because of the two different modes of action. LA-SSA reduces hypersecretion of GH by binding to SSTRs on the cell surface of the pituitary adenoma and has the capacity to reduce tumor volume. While, PEGV acts by reducing excessive GH actions in peripheral tissues and blocks the increased production of IGF-I by the liver. LA-SSA combined with PEGV is suitable for adjuvant treatment and primary medical treatment. However, in the current guidelines combination treatment is only recommended for acromegaly patients who are not controlled by LA-SSA monotherapy (33). LA-SSA in combination with PEGV as pre-surgical treatment to reduce morbidity is very questionable, as there is no supporting data yet.

3.1 Efficacy and dose reduction

As stated in the paragraph about PEGV monotherapy, PEGV in principle should be able to biochemically control disease activity, provided that the appropriate PEGV dose is used. Several studies have reported similar normalization rates of LA-SSA combined with PEGV, ranging between 67-100% and cumulative weekly PEGV doses range between 60-140 mg depending on disease activity, depicted in Table 2 (63,66,69-71). Co-administration of the highest dose of LA-SSA on top of PEGV monotherapy appears to reduce the necessary mean PEGV dose by 50% (70), although with a high individual variability (72). Analysis of eight patients whose mean IGF-I levels were similar during PEGV monotherapy and on at least one moment during the co-administration of LA-SSA, showed that these patients were able to reduce their PEGV dose from 131.3 $^{+}$/$_{-}$36.2 to 62.5 $^{+}$/$_{-}$16.7 mg/week (70). Besides the LA-SSA-induced decrease in GH secretion of the adenoma, LA-SSAs also have direct and indirect effects that result in a GH-independent decrease of the IGF-I production (73,74), which will be further explained in the next paragraph. Moreover, a Danish study reported that PEGV serum levels increase by 20% when combined with LA-SSA (75). PEGV dose reduction during combination treatment might improve cost-effectiveness of medical treatment in acromegaly and may reduce injection frequency for patients. However, there are no direct studies comparing cost-effectiveness of the median required weekly PEGV dose during mono- and combination treatment.

3.2 Quality of life

Physicians tend to be mainly focused on biochemical parameters as GH and IGF-I levels during treatment of acromegaly. These parameters are definitely linked to

a better outcome and a lower risk of morbidity and mortality (35,76). However, normalized serum GH and IGF-I levels do not necessarily result in complete resolution of signs and symptoms (77,78). Neggers *et al.* reported on the results of a prospective, double blind, placebo controlled crossover study and demonstrated improved quality of life (QoL) in patients using LA-SSA combined with low-dose PEGV (78). QoL was assessed by two acromegaly specific QoL-questionnaires, the AcroQoL and the PASQ. Improved QoL was observed without significant changes in IGF-I levels after addition of PEGV to LA-SSA therapy in patients with normalized IGF-I levels during monotherapy of LA-SSA. An explanation for the observed improvement in QoL by the addition of low-dose PEGV could be a persistent systemic acromegaly disease activity during monotherapy of LA-SSA that has been hypothesized and was called 'extra-hepatic acromegaly' (79). LA-SSA treatment

Table 2. Summary of studies reporting on combination treatment

First author, year, (REF)	Design	Aim of study	N. of patients	Disease control	PEGV dose	Duration study*
Van der Lely 2001 (71)	Case report	IGF-I normalization	1	100	280	18
Neggers 2007 (69)	Retrospective observational study	IGF-I normalization and AEs	32	100	60	35**
Trainer 2009 (66)	Randomized controlled trial	Primary: AEs, secondary: IGF-I normalization	29	73	105	9
Van der Lely 2011 (70)	Prospective observational study	IGF-I normalization and AEs	57	79	60	7
Bianchi 2013 (63)	Retrospective observational study	IGF-I normalization and AEs	27	67	140	30**

Summary of studies reporting on LA-SSA combined with PEGV (mg/week) and the percentage of disease control (%) (normalization of IGF-I levels) and the required PEGV dose in order to control IGF-I levels. The ACROSTUDY™ is not included in this table as it includes patients with monotherapy of PEGV and various other medical combinations with PEGV.

REF: reference, LA-SSA: long-acting somatostatin analogues, PEGV: pegvisomant, IGF-I: insulin-like growth factor I, AEs: adverse events.

* in months

** median

selectively decreases hepatic IGF-I production by approximatly 20% via direct and indirect mechanisms (73,74,80). This might lead to an overestimation of the efficacy of LA-SSAs to normalize IGF-I via a reduction in the pathological GH secretion because of this GH-independent reduction of IGF-I levels. Therefore, GH actions on extra-hepatic tissues can remain elevated despite normalization of serum IGF-I levels. LA-SSA reduces portal insulin levels which only decreases hepatic GHR expression (80). This results in a relatively hyper-GH-sensitive state of the other tissues. Patients report these still excessive GH actions on non-hepatic tissues such as edema, fatigue and headaches comparable to the side-effects of high-dose GH replacement therapy in GH deficient subjects. A clinical example is a Danish study which observed, despite similarly normalized IGF-I levels, that LA-SSA treatment compared with neurosurgery alone was associated with less suppressed GH levels and less symptom relief (81). Blocking non-hepatic GH actions by low-dose PEGV could therefore be useful in treating this 'extra-hepatic acromegaly' (79). Moreover, PEGV has also been shown to improve insulin resistance by several mechanisms (82-87), since these are beneficial in the presence of LA-SSAs, which are known to reduce insulin secretion (88,89).

3.3 Safety aspects

During combination treatment incidence of lipohypertrophy at the injection site occurred in 3% of the patients (63,90). TET >3x the upper limit of normal seems to occur more frequently during the combination of LA-SSA and PEGV, which was observed in 11-15% of the subjects in several studies (63,66,90). To the best of our knowledge, no study has reported a factor that predicts the elevated transaminases during PEGV-treatment, except for one Spanish study that observed that carriers of UGT1A1*28 polymorphism of Gilberts syndrome seem to have a higher risk of developing PEGV-induced TET (91). Another safety aspect is the stability of tumor volume during PEGV treatment. Several studies reported that combination treatment and PEGV monotherapy have similar incidence rates of increased tumor volume cases (63,66,90). In a significant number of patients treated with LA-SSA combined with PEGV, the tumor size even decreased (69,90).

OUTLINES OF THIS THESIS

The combination treatment of LA-SSA and PEGV is in the previous paragraphs described as an attractive treatment modality for severe acromegaly patients, in which transsphenoidal surgery and/or medical treatment were unsuccessful in controlling IGF-I levels. But the combination treatment may also be an option for less severe acromegaly patients regarding QoL-reasons, as stated in paragraph 3.2 of this introduction. The use of PEGV alone or in combination with LA-SSA as a treatment option in acromegaly is increasing, although the drug has only been on the market since 2003. Therefore, long-term data on efficacy rates and safety issues is needed and highly important. This thesis focusses on the efficacy and safety of the combination treatment during the last decade. The Erasmus University Medical Center in Rotterdam currently has the largest single center cohort of acromegaly patients using the combination treatment of LA-SSA and PEGV. These acromegaly patients systematically visit our outpatient clinic, including standard performing of multiple measurements, and therefore is particularly suitable for observational research in a rare disease such as acromegaly. Apart from this rather descriptive clinical approach we focus on the question why some patients needed the addition of PEGV to LA-SSA in order to control their IGF-I levels. Therefore, we observed the SSTR expression on adenomas of patients using the combination treatment. The expression of SSTR2 on the GH-secreting pituitary adenoma cell membrane is significantly and positively correlated with the efficacy of LA-SSAs in suppressing GH and IGF-I levels in vitro, and is also associated with IGF-I normalization in acromegaly patients (92-96). A reduced expression of SSTR2 and/or SSTR5 in untreated GH-secreting pituitary adenomas, might necessitate combination treatment because of (partial) resistance to LA-SSA treatment. Thereafter, we focus on the prediction of PEGV dosing, since PEGV doses vary widely among acromegaly patients, depending on disease activity and individual response to the drug (69,75,97,98). These individual differences might be attributed to a genetic factor, like the common GHR polymorphism lacking exon 3. In this thesis we aim to address the clinically-relevant question: Do clinicians have to take d3-GHR genotyping into account during PEGV dosing? To finish this thesis, a prediction model was created, incorporating several patient-, biochemical- and adenoma characteristics collected during the last years, in order to investigate whether we can individually predict the PEGV dose in addition to LA-SSA needed to control IGF-I levels. Hereafter, the chapters of this thesis are pointed out more in detail.

CHAPTER 2

The combination treatment of LA-SSA and PEGV is used for almost a decade in the Rotterdam cohort, what can we tell about its efficacy parameters and safety profile? Efficacy parameters that are addressed in this chapter are; 1) normalization of IGF-I levels over time; 2) the required PEGV dose in order to normalize IGF-I levels and; 3) control of tumor volume. Safety aspects are focused on the two main side-effects of the combination treatment: lipohypertrophy and elevated transaminases. We also address whether carriers of the UGT1A1*28 polymorphism, causing the Gilbert's syndrome, have a higher risk of developing PEGV-induced elevated transaminases.

CHAPTER 3

The expression of SSTR2 and SSTR5 on GH-secreting pituitary adenomas from acromegaly patients treated with PEGV (together with LA-SSAs) before surgery is currently unknown. In this chapter we address the following hypotheses; 1) various medical pre-treatment modalities can differently affect the SSTR2 and SSTR5 expression on GH-secreting pituitary adenomas and; 2) the SSTR expression could affects postsurgical PEGV dosing in combination with LA-SSA.

CHAPTER 4 AND 5

The differences in individual PEGV dosing might be attributed to the d3-GHR polymorphism, which enhances signal transduction of GH by its receptor. Two main outcome parameters were used to address this hypothesis; 1) the lowest IGF-I level during PEGV-treatment and; 2) the required PEGV-dose to achieve the lowest IGF-I level. This was done in the Rotterdam combination treatment cohort (**chapter 4**), and subsequently pooled in a meta-analysis together with several other European acromegaly cohorts using PEGV (**chapter 5**).

CHAPTER 6

The PEGV dose necessary to achieve disease control differs significantly between individual acromegaly patients. In this chapter, we identify predictors of PEGV dosing during PEGV monotherapy and in combination with LA-SSA. These predictors are used for the development of a multivariate regression model in order to predict the required PEGV dose by patient, biochemical and adenoma characteristics. This study is not designed to predict PEGV-overdosing, but should be considered as a useful clinical tool during PEGV dose titration.

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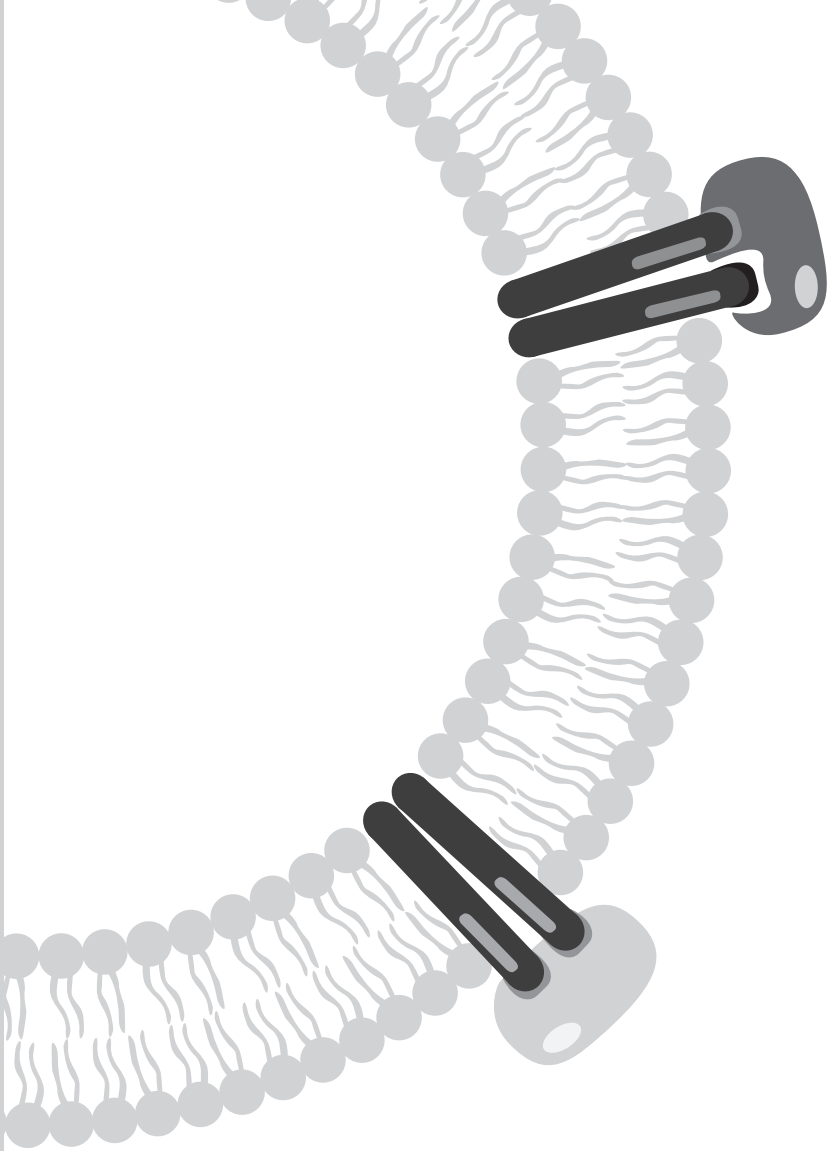

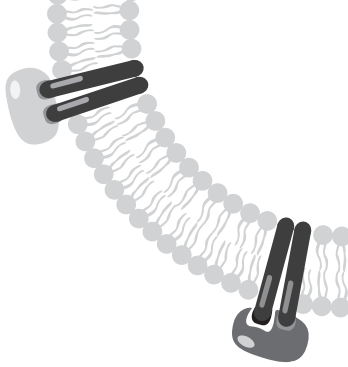

Chapter 2

Long-Term Efficacy and Safety of Pegvisomant in Combination with Long-Acting Somatostatin Analogues in Acromegaly

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ABSTRACT

Background: Treatment for acromegaly patients with long-acting somatostatin analogues (LA-SSA) often does not result in complete normalization of insulin-like growth factor I (IGF-I). Addition of pegvisomant (PEGV), a GH receptor antagonist, could improve this; however, the literature has not described long-term follow-up.

Objective: To assess long-term efficacy and safety of this combined treatment in the largest current single-center cohort of patients, from 2004-2013.

Design: Acromegaly patients were treated for at least 6 months with a high-dose LA-SSA. To patients with persistently elevated IGF-I levels (>1.2 x the upper limit of normal (ULN)) or poor quality of life, PEGV was added as one weekly injection.

Results: The patients (n=141) were treated with PEGV and LA-SSA for a median period of 4.9 years [range: 0.5 – 9.2]. Efficacy, defined as the lowest measured IGF-I level during treatment, was 97.0%. The median PEGV dose to achieve this efficacy was 80 mg/week [interquartile range: 60 – 12]. Combination treatment-related adverse events were recorded in 26 subjects (18.4%). Pituitary tumor size increase was observed in one patient. Injection-site reactions were observed in four subjects. In 19 patients (13.5%), transiently elevated liver transaminases of more than three times the ULN were observed, of which 83% occurred within the first year of combination treatment. Eight patients died, at a mean age of 71 years; none of them were considered treatment-related.

Conclusions: The combination treatment with LA-SSAs and PEGV was effective in 97% of the patients, it appears to be a safe medical treatment and it reduces the required dose of PEGV.

INTRODUCTION

Acromegaly is a rare disease that is almost exclusively caused by a growth hormone (GH)-secreting pituitary tumor that results in signs and symptoms and reduced life expectancy (1). Treatment is focused on improving life expectancy, reducing signs and symptoms, and thereby increasing the patient's quality of life (QoL). These main objectives are considered to be accomplished when serum levels of IGF-I and GH are normalized. This is achieved in less than 60% of patients after surgery (2,3). Normalization after medical treatment occurs in 30% to over 90% of patients (4-7). Current medical treatment modalities focus on the normalization of IGF-I by targeting pituitary GH production or peripheral GH actions. In 1985, studies demonstrated that GH secretion by GH-secreting pituitary tumors could be inhibited by somatostatin analogues (8). Thereafter, many studies on the efficacy of LA-SSA to control pathological GH secretion reported an average normalization of IGF-I and GH (<2.5 µg/l) in 44% of patients treated with LA-SSAs (4).

More recently, PEGV was introduced (9). This drug is a genetically modified analogue of human GH; because it binds to but does not activate the GH receptor, it acts as a competitive GH receptor antagonist (9). The first report on its long-term efficacy and safety was published early this century (10). PEGV was approved in Europe in 2002, followed by the United States in 2003. Although PEGV clinical trials found efficacy rates of 90% (6,9,10), these were not confirmed by observational studies such as the ACROSTUDY™ and the German pegvisomant observational Study (7,11-13). Nevertheless, phase IV non-interventional studies such as the ACROSTUDY™ are designed to gather additional medical information complementary to placebo-controlled, randomized clinical trials (7,14), such as rare adverse events and atypical treatment reactions (15). Therefore, the ACROSTUDY™ is less suitable to assess efficacy. The adverse events reported during PEGV treatment seem relatively mild, the most frequent being transient elevated transaminases (TETs), followed by local lipohypertrophy at the injection site (6,7,16,17).

In 2005, the first study on the combined treatment of acromegaly with weekly PEGV and LA-SSAs reported high efficacy, as well as possible cost reductions due to the lower median required PEGV dose (18). Thereafter, long-term data on efficacy and safety showed an efficacy rate of 90% and the possibility of lowering the necessary dose of PEGV when combined with LA-SSAs (16,17,19). Combined treatment was also able to improve QoL in LA-SSA-controlled patients (20).

Because PEGV competitively blocks the GH receptors in all peripheral tissues, it does not prevent tumor growth of the pituitary adenoma (6). In previous studies with LA-SSAs and PEGV, tumor size increase was not observed, and in a significant number of patients (19%) the tumor size even decreased (16, 17). Although combination treatment and PEGV monotherapy appear to have similar

efficacies (6,7,16), side effects such as TETs seem to occur more frequently during combination therapy (7). No studies could detect any factor that predicts elevations of the liver enzyme alanine aminotransferase (ALT), except one that reported that carriers of UGT1A1*28 polymorphism of Gilbert's syndrome seem to have a higher risk of developing PEGV-induced liver injury (21).

Combination therapy might be an attractive option for treating acromegaly, but the long-term effects outside of the clinical studies such as the ACROSTUDY™ remain uncertain. Here we report on the long-term efficacy and safety of combined treatment with PEGV and high-dose LA-SSA treatment for almost a decade in a single-center tertiary referral hospital.

METHODS

Data was collected from all consecutive patients who were treated with LA-SSA for at least 6 months (n=141) at our Pituitary Center Rotterdam between 2004 and 2013. Permission from the Institutional Review Board of the Erasmus Medical Center Rotterdam was obtained for all the substudies involved, and all patients gave their written informed consent. All patients were initially started on LA-SSA monotherapy in a stable dose, after which PEGV was added by a weekly injection.

Results were derived from two data sets. The first contains data from acromegaly patients (n=112) with elevated IGF-I levels (>1.2 xULN), after at least 6 months of high-dose LA-SSAs (Sandostatin LAR 30 mg or Lanreotide Autogel 120 mg every 28 days). This group is described in this article as the "uncontrolled group." Acromegaly patients in the second group (n=29) were co-treated with PEGV to improve the QoL as add-on-therapy on top of LA-SSAs, which already had normalized their IGF-I levels. They are designated as the "QoL group." In our analyses, this QoL group was only used for the assessment of safety aspects.

In the uncontrolled group, 27 acromegaly patients started with 25 mg PEGV weekly as co-treatment, whereas another 18 started with 40 mg PEGV weekly, and the last 67 patients started with a variable dose of PEGV weekly, guided by their baseline IGF-I. The variable starting dose was based on one of our previous reports (17) (see Figure 2). The formula to calculate the PEGV dose is: 4 + (IGF-I z-score during treatment with highdose LA-SSA*16), which was derived from a method described previously (17). This formula can only be used when IGF-I is elevated after a period of at least 6 months of LA-SSA treatment. Intervals of dose adaptations were 6-8 weeks, until a controlled IGF-I level was achieved on two consecutive occasions. The subjects then visited our outpatient clinic every 16 weeks. For the QoL group, methods were described previously (16). When the once-weekly PEGV dose exceeded 80 mg per injection, patients divided the dosage into two weekly injections. With weekly doses over 200 mg, subjects changed

administration intervals into daily injections or five injections per week. At each visit to our outpatient clinic, efficacy and safety parameters were assessed.

The efficacy parameter IGF-I was assessed using the Immulite 2000 assay (DPC Biermann GmbH/Siemens), a solid-phase, enzyme-labeled, chemiluminescent immunometric assay with an intra-assay variability of 2-5%, and an inter-assay variability of 3-7%. The IGF-I age- and sex-adjusted reference ranges were used from an article by Elmlinger et al (22). PEGV serum levels were assessed in Aarhus, as described in a previous report (23).

Safety assessments included: electrocardiogram, serum concentrations of ALT, aspartate aminotransferase (AST), alkaline phosphatase (Alk phos), γ-glutamyltranspeptidase (γ-GT), total bilirubin (bili), and lactate dehydrogenase. Magnetic resonance imaging (MRI) assessed changes in pituitary tumor volume at least annually. Decrease of tumor size was determined by one radiologist, who was blinded for the outcome. Decrease was defined as more than a 20% reduction of the largest diameter of the tumor during combination treatment compared with the largest diameter of the last MRI before the addition of PEGV.

Genomic DNA was isolated from peripheral blood leukocytes. A 324 11bp fragment of the UGT1A1 gene promoter, which includes the TATA box, was PCR-amplified by using forward (5'-GAGTATGAAATTCCAGCCAG-3') and reverse (5'-GGATCAACAGTATCTTCCC-3') primers and platinum Taq Mix (invitrogen). Cycle conditions were 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and 72°C for 7 minutes. The results were ascertained on the 3500 Genetic analyzer (Applied Biosystems). Gilbert's syndrome was characterized by an additional TA repeat in the TATA sequence of the UGT1A1 promoter region, i.e., A(TA)7 TAA instead of A(TA)6 TAA. Results were first published by Bosma et al (24).

Somatostatin receptor type 2 (SSTR2) mRNA expression was performed in one patient's tumor sample, described in the results. Real-time quantitative PCR was performed as previously published (25). Sequences and concentrations of the SSTR2 primerprobe pairs and of the hypoxanthine phosphoribosyltransferase (HPRT) are described in the same previously published report (25). We used the ABI Prism 7900 Sequence Detection System (Applied Biosystems) to measure the samples and compared it with the housekeeping gene HPRT.

Statistical methods

Data are expressed as median [interquartile range [IQR]] unless otherwise specified. Differences between two or more independent subgroups were analyzed using the Mann-Whitney U test and the one-way ANOVA (multiple comparison), respectively. Paired data were analyzed with the Wilcoxons signed rank test. Nominal variables

were analyzed using the χ^2-test. P-values <0.05 (two-tailed) are considered statistically significant. Statistical analyses were performed with SPSS version 20 and GraphPad Prism version 6 for Windows (IBM SPSS Statistics for Windows, Armonk, NY, USA and GraphPad Software, San Diego, CA, USA).

RESULTS

Efficacy

Patient characteristics are depicted in Table 1. The median duration of PEGV treatment was 4.9 years [range: 0.5 – 9.2]. Normalization of IGF-I for age and sex, defined as the lowest IGF-I during treatment, was observed in 97.3% of the subjects. The absolute median IGF-I level was 18.0 nmol/l [IQR: 13.4 – 23.6], 0.56 xULN of IGF-I [IQR: 0.43 – 0.74], or expressed as SD score (SDS), -0.16 [IQR: -1.27 – 0.90]. All patients had a lowest IGF-I level below 1.2 xULN. The median weekly PEGV dose to achieve these lowest IGF-I levels was 80 mg [IQR: 60 – 120]. The IGF-I level at

Table 1. Patient characteristics

Sex – male %	58.0
Age – years	48.2 [39.0 – 59.1]
Tumor volume – macro %	81.3
Diabetes mellitus II – %	36.9
Gilbert's polymorphism	
Heterozygous – %	43.1
Homozygous – %	11.5
Previous therapy	
Surgery – %	30.4
Radiotherapy – %	0.9
Surgery and Radiotherapy – %	10.7
IGF-I at start of PEGV – nmol/l	66.5 [46.4 – 87.9]
IGF-I xULN at start of PEGV	1.89 [1.48 – 2.56]
IGF-I expressed as SDS at start of PEGV	8.02 [5.10 – 11.13]

Data are expressed as median [IQR] or percentage.
IGF-I: insulin-like growth factor I, PEGV: pegvisomant, ULN: upper limit of normal, SDS: standard deviation score.

the last visit was 26.0 nmol/l [IQR: 20.0 – 34.4], 0.85 xULN [IQR: 0.67 – 1.09], or expressed as SDS 1.50 [IQR: 0.49 – 2.84]. The median necessary dose of PEGV to achieve this IGF-I level was 80 mg/week [IQR: 60 – 130]. There was no significant difference between the PEGV dose during the lowest IGF-I and the PEGV dose at the last visit (p=0.106).

The weighted median control of IGF-I levels (1.2 xULN) over years 1 to 9 was 89.7%, with a median PEGV dose of 73.8 mg/week. The median control of IGF-I (1.0 xULN) over years 1 to 9 was 78.0%. The annual results from years 1 to 9 of the median IGF-I and PEGV levels are depicted in Figure 1. In Supplemental Figure 1, the normalization rate during the first year and the necessary PEGV dose are depicted. Supplemental materials can be found at the end of this chapter. A clinical tool is depicted in Supplemental Figure 2. This Figure shows the required dose of PEGV that is necessary to normalize the IGF-I level.

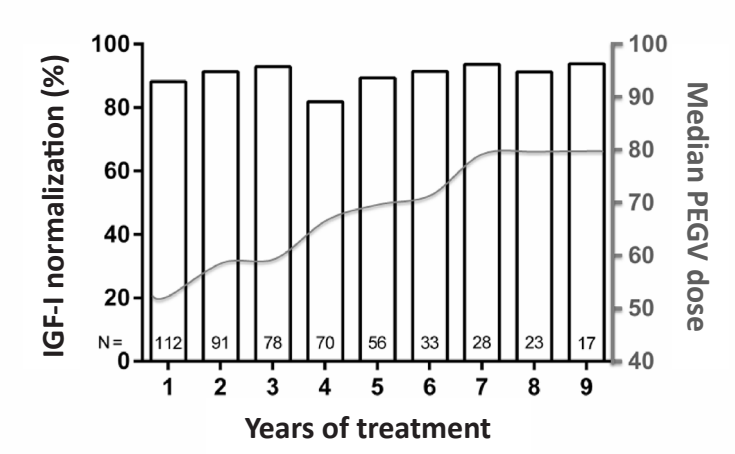


Figure 1. IGF-I serum levels <1.2 xULN years 1-9

Percentage of patients who ar normalized with an IGF-I <1.2 xULN and the median PEGV dose (gray line) are shown in this figure for every individual year during the 9 years of treatment. Cumulative numbers of the included patients at each treatment year are depicted at the bottom of every bar. All patients (n=112) were treated for at least one year, 17 patients were treated for the maximal 9 years of follow up.

IGF-I: insulin-like growth factor I, PEGV: pegvisomant, ULN: upper limit of normal.

Normalization rate, expressed as the lowest IGF-I level, was not significantly different between patients with (95.0%) or without (98.6%) diabetes mellitus type II (DM) (p=0.588). The necessary dose to achieve this was identical for non-DM and DM (p=0.281). Normalization of IGF-I levels was achieved in 95.7% of patients who had undergone prior pituitary surgery, which was comparable to the outcome in patients receiving primary medical treatment (98.5%; p=0.604) and with no difference between the two groups in the PEGV dose (p=0.518). This was also the

case when radiotherapy was excluded from the analysis (p=0.901).

No significant differences in normalization rate and PEGV dose necessary to control IGF-I were observed between sexes (p=0.997, p=0.225, respectively), microadenomas vs macroadenomas (p=0.711, p=0.809, respectively), or carriers vs non-carriers of Gilbert's polymorphism (p=1.000, p=0.789, respectively).

In 11 patients, surgery was performed during combination therapy. In Supplemental Table 1, the reasons for surgery are depicted. The PEGV dose before operation was 120 mg [IQR: 80 – 160]. One patient was cured after transsphenoidal surgery (TSS). Six patients could discontinue PEGV and continued with a high dose of LA-SSAs after significant tumor debulking. The other five patients continued with 80 mg [IQR: 80 – 100] PEGV weekly.

Safety

Liver tests

ALT and AST were normal at baseline in all patients. TETs of more than 3 xULN were observed in 22 patients (15.6%; Table 2). All cases were transient without adaptation of PEGV dose or discontinuation, except for one patient with TET 26.1 xULN, who was previously reported (26) and developed a second period of TET 2 xULN after re-exposure to PEGV monotherapy. The development of TET was not influenced by the PEGV dose (p=0.803). Obstruction of the biliary tract could be an explanation for three of these cases, therefore in 19 patients (13.5%) TET could be linked to PEGV treatment. Re-exposure to PEGV after discontinuation resulted in a second period of TET >3 xULN in two patients during combination therapy. TET >3 xULN occurred after a median period of 5.2 months [IQR: 3.2 – 13.3] (see Supplemental Figure 3A). In a median period of 5.5 months [IQR: 3.0 – 14.0], TET normalized again (see Supplemental Figure 3B).

Gilbert's polymorphism

Gilbert's polymorphism (UGT1A1*28) was assessed in 131 (93%) of the 141 patients. No blood could be obtained from 10 patients. UGT1A1*28 was observed in 71 (54.2%) of patients, 11.5% homozygous and 42.7% heterozygous. Of the 22 TET cases, four (18.2%) were homozygous and eight (36.4%) were heterozygous. No association between UGT1A1*28 and TET was found in patients with heterozygous (p=1.000) or homozygous polymorphism (p=0.827). The same lack of association applied to heterozygous UGT1A1*28 compared to homozygous (p=0.752). Neither sex (p=0.393) nor DM (p=0.956) was associated with TET.

Table 2. TET patients during pegvisomant treatment of acromegaly

			Peak TET (xULN)							
Patient number	**Sex**	**PEGV dose during TET (mg/week)**	**Bili**	**Alk. phos.**	**ɣ-GT**	**AST**	**ALT**	**DM**	**Gilbert's Polymorphism**	**Clinical features**
1	M	30	0.7	0.6	2	4.0	4.7	-	Normal	
2	M	300	-	1.0	1.8	5.0	5.5	-	Normal	
3	M	40	0.4	1.2	0.9	4.3	7.0	-	Normal	
4	M	160	0.4	1.2	1.8	4.6	6.5	+	Normal	
5	M	80	1.1	0.5	2	3.5	3.9	-	Heterozygous	
6	M	60	2.3	2.9	16.7	16.7	25.8	+	Homozygous	MRCP: Cholecystolithiasis
7	M	60	0.6	0.8	1.8	2.4	3.6	-	Heterozygous	
8	M	40	5.3	0.9	5.1	4.9	8.0	-	Homozygous	Ultra-sound: Cholecystolithiasis
9	M	80	-	0.9	0.8	3.1	1.2	+	Heterozygous	
10	M	40	1.6	1.1	4.6	3.1	4.3	-	ND	
11	M	40	2.0	1.4	8.8	9.5	8.3	-	Homozygous	
12	M	60	0.9	1.2	4.8	6.3	13.2	-	Normal	
13	M	80	1.3	2.6	11.8	5.6	3.7	-	Heterozygous	Ultra-sound: Cholecystolithiasis
14	M	60	1.3	1.3	6.9	13.7	26.1	+	Heterozygous	
15	F	80	0.8	0.8	1.5	2.9	4.5	+	Normal	
16	F	20	1.0	0.6	0.9	7.5	10.0	-	Heterozygous	
17	F	60	0.7	0.7	2.7	2.8	4.0	-	Heterozygous	
18	F	60	-	-	3.9	-	4.6	-	Homozygous	
19	F	160	-	1.1	4.1	2.4	3.7	-	Normal	
20	F	20	-	0.9	2.3	2.8	3.7	+	Normal	
21	F	40	1.3	0.7	1.3	8.4	11.7	-	Normal	
22	F	60	-	1.0	1.5	4.0	4.8	-	Heterozygous	
Second period of TET										
16	F	40	-	0.5	0.5	6.1	7.1	-	Heterozygous	
20	F	60	-	2.0	9.9	2.4	4.0	+	Normal	

TET: transient elevated transaminases, PEGV: pegvisomant, xULN: times upper limit of normal, Bili: total bilirubin, Alk. Phos: alkaline phosphatase, ɣ-GT: ɣ-glutamyltranspeptidase, AST: aspartate aminotransaminase, ALT: alanine aminotransferase, DM: diabetes mellitus type II, M: male, F: female, ND: not determined, MRCP: magnetic resonance cholangiopancreatography.

Pituitary tumor size

Decrease in tumor size, defined as a decrease of more than 20% during combination treatment, was observed in 13 patients (16.9%), whereas size could not be determined in patients with the presence of an empty sella (n=8) or in whom radiotherapy was performed (n=13). During combined treatment, pituitary apoplexy occurred in two patients without a necessity for surgical intervention. In one patient, surgery was needed due to true tumor size increase. This case is described at the end of the *Results, Two exceptional patients (tumor growth).*

Injection-site reactions

Injection-site reactions were observed in four subjects (2.8%). In three patients, lipohypertrophy appeared to be reversible by a more frequent rotation of the injection site. Nevertheless, one of these patients decided for TSS (see Supplemental Table 1) in order to be able to stop PEGV treatment. After TSS, the patient was able to stop the PEGV and to lower the LA-SSA dose. One patient underwent cosmetic surgery due to lipohypertrophy.

Mortality

During the 9 years of follow-up, eight patients died (5.6%). All deaths were considered to be unrelated to the treatment. In Supplemental Table 2, the causes of death are listed. The average age of the patients who died was 71 years [range: 51 – 86]. All patients had significant comorbidities such as cardiovascular, cerebrovascular, malignant, or pulmonary disease.

Two exceptional patients

Tumor growth

The first patient, a 25-year-old female, showed significant tumor growth during the combination therapy. In 2011, the patient presented with bitemporal hemianopia; therefore, TSS was performed. Retrospectively, her symptoms were presented 2 years before the diagnosis. Before surgery, the IGF-I level was 127 nmol/l (2.9 xULN). After surgery, the bitemporal hemianopia resolved, and other pituitary axes remained unaffected. Octreotide LAR 20 mg monthly was started, and the IGF-I level dropped to 67 nmol/l (1.6 xULN) 5 months after surgery. Octreotide LAR was increased to 30 mg every 3 weeks because IGF-I was still elevated. Despite the change in dose, the IGF-I level rose to 100 nmol/l (2.3 xULN). Pituitary MRIs (Figure 2, A-C) performed 3 and 8 months after surgery showed a large tumor remnant. After 11 months, the LA-SSA IGF-I level was 87 nmol/l (2.0 xULN), and GH was 105 g/l; at this stage, the patient was transferred to our hospital. IGF-I normalized

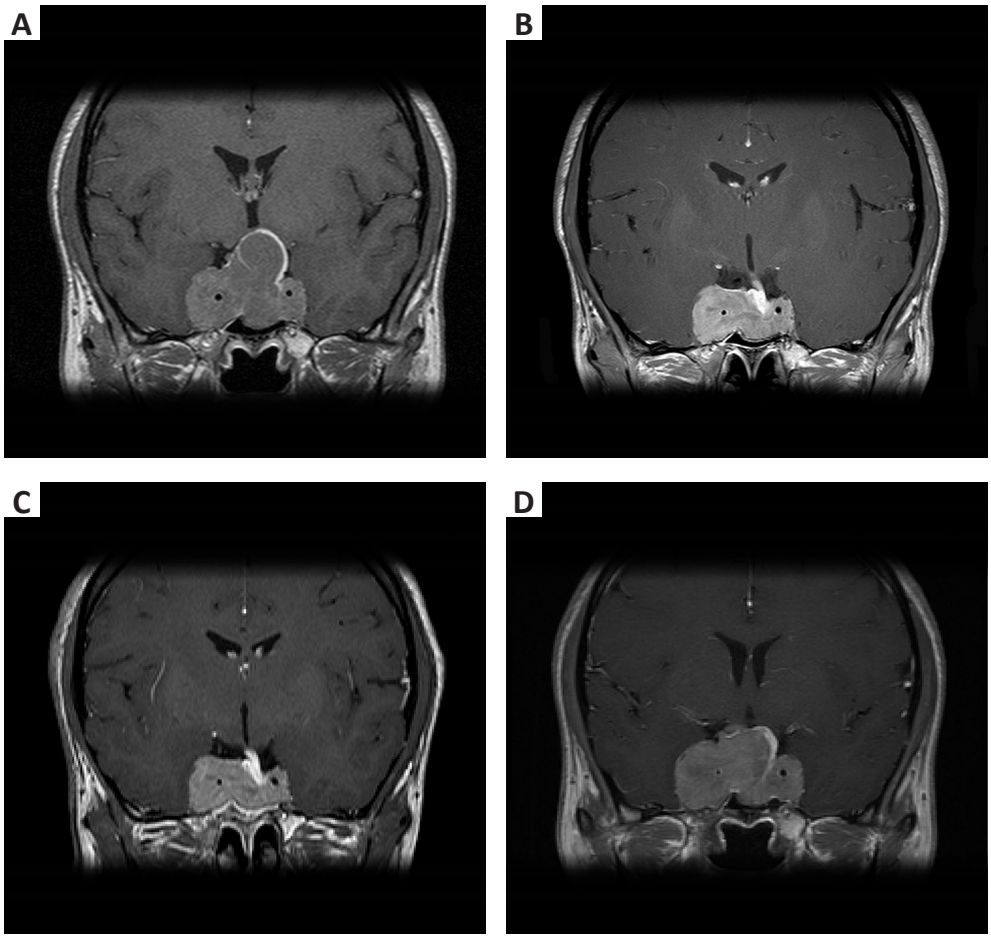


Figure 2. Magnetic resonance images of first patient (tumor growth)
A) At diagnosis.
B) Three months after the 1st surgery, no medical treatment.
C) Eight months after the 1st surgery, Sandosatin Lar 20 mg every 4 weeks.
D) Seven months after the addition of PEGV, Sandostatin Lar 30 mg every 4 weeks + PEGV 160 mg/week.

with Sandostatin LAR 30 mg and PEGV 80 mg twice weekly. Seven months after the addition of PEGV, an increase in tumor size was observed (Figure 2D) and a second TSS was needed, which was followed by radiotherapy. PEGV was restarted after surgery in combination with Sandostatin LAR 30 mg. A weekly dose of 100 mg of PEGV normalized the IGF-I level. To date, IGF-I remains normal, and the tumor size is stable. The pathology report after the first surgery revealed a GH-secreting adenoma with a Ki-67 index of 1%. After the second surgery, the pathology reported

sparsely granulated GH-secreting adenoma with a Ki-67 index of 1-2%; additionally, receptor expression was determined on the tumor specimen (Figure 3A). SSTR2 mRNA expression could be demonstrated (0.3; expressed as relative expression of HPRT), as well as SSTR1 and SSTR5 mRNA expression. Incubation of primary cultures of dispersed somatotroph tumor cells of this patient with octreotide resulted in significant reduction of GH secretion (15.5%; p<0.01; Figure 3B).

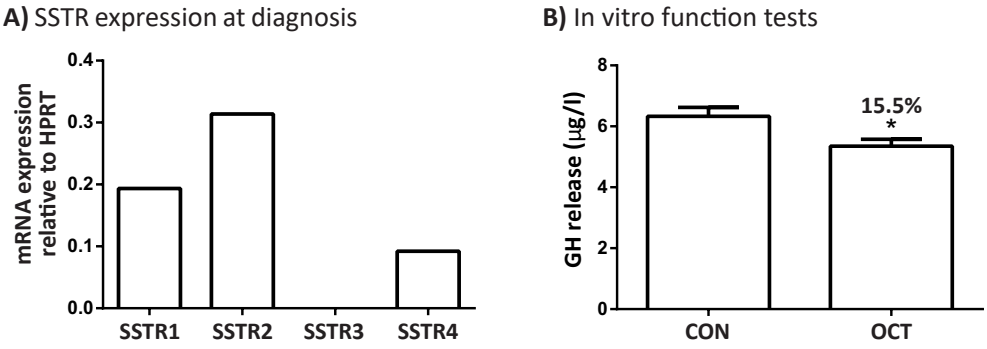


Figure 3. Tumoral mRNA receptor expression
A) Tumoral SSTR2 mRNA expression is 0.3, expressed as relative to the expression of HPRT.
B) Incubated primary cultures of dispersed somatrotroph tumor cells with octreotide. Tumor cells were available after transspenoidal surgery, because of tumor growth in a patient during combination therapy. Shown here a significant decrease of GH secretion by 15.5%.
SSTR: somatostatin receptor, CON: control, OCT: octreotide (10 nM), * p<0.001.

Increasing demand for pegvisomant

The second patient, a 29-year-old male, started combination therapy in 2007 after TSS and transfrontal surgery. Normalization of IGF-I was achieved after 9 months with a PEGV dose of 210 mg/week (Figure 4). Eight months after normalization, the IGF-I increased again above the ULN. In the subsequent 3 years (2008-2010), the IGF-I level continued to increase despite a stepwise doubling of the PEGV dose to 420 mg/week. PEGV was still given in combination with a high-dose LA-SSA (Lanreotide Autogel, 120 mg every 3 weeks). At the end of 2010, the combination therapy was sufficient to decrease the IGF-I below the ULN for 2 years, but the IGF-I level rose again in 2012. During these periods of IGF-I increase, additional MRIs were assessed but did not show any tumor size increase. PEGV injections were supervised at the hospital on several occasions in order to check the administration procedures and compliance. Retrospectively, PEGV levels were measured (Figure 4). GH levels did not increase significantly, and no PEGV antibodies were detected. Serum PEGV level in relation to injected doses and IGF-I levels are depicted in Figure 4.

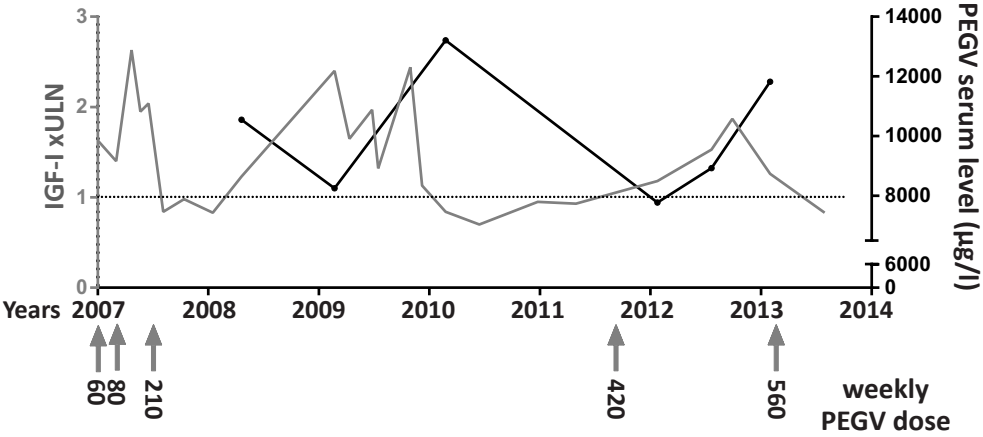


Figure 4. IGF-I serum level and PEGV serum level during time
IGF-I serum levels expressed as upper limit of normal and PEGV serum levels (µg/l) of one patient during years of combination treatment. The arrows show the moment of PEGV dose increase (mg/week).
IGF-I: insulin-like growth factor I, PEGV: pegvisomant, ULN: upper limit of normal.

DISCUSSION

Combined treatment has a high efficacy, similar to the clinical registration trials, and seems to be safe. Almost all of the adverse events occurred within the first year and were transient.

The efficacy and safety of the combination therapy that we report here are based on data that we obtained from a single tertiary referral center. All patients attended the outpatient clinic on a regular basis, under the supervision of experienced pituitary endocrinologists. This approach could explain the superior outcome as compared to the results reported in the ACROSTUDY™, which contains patients treated at centers with less experience. Recent reports suggest that the efficacy of PEGV treatment (7,13) is not as high as reported by the clinical trials using mono-therapy of PEGV (9,10). Our data, however, show that PEGV in combination with LA-SSA is as effective as in clinical trials, provided that optimal dosing is applied.

In our opinion, rare diseases should be treated in dedicated expert centers only. This has been clearly shown for neurosurgery in acromegaly (27). Therefore, it would make sense that medication indicated for the treatment of acromegaly is restricted to specialized centers. The large number of PEGV-treated acromegaly patients in our center has brought about more experience and a more structural approach, which might explain in part the higher efficacy that we achieved compared to more recent literature (7). However, dosing strategies could be suboptimal, even in highly experienced centers, due to local legislation or reimbursement issues.

Studies in the field have used different criteria for the assessment of IGF-I levels. Some use the lowest IGF-I and end-of-study IGF-I, and others the lowest annual IGF-I. The current data underline the fact that efficacy numbers are a matter of definition. The cotreatment study of PEGV with Lanreotide Autogel used end-of-study and lowest IGF-I, and efficacy was 58 and 79%, respectively (28). The initial registration studies (10) and our combined treatment study (6) used the lowest IGF-I, and the efficacy rates were over 90%. With the current data set, a similar efficacy of 97.3% is observed, assessing lowest IGF-I recorded during treatment in a specialized center with standardized methods and not from a clinical trial with irregular follow-up.

Surgery before combined treatment did not significantly reduce the required dose of PEGV to normalize IGF-I. This is in line with our previous observations (6,16,17). In the current study, 11 patients underwent surgery during combined treatment. In five patients, the pre- and post-surgery PEGV doses were more or less similar. The decision to perform surgery in such cases therefore remains difficult.

The incidence of TETs and local effects of PEGV at the injection site have been previously reported by several groups (7,10,11,13,17,29,30). The incidence of TET during combination therapy seems to be higher compared to PEGV monotherapy. Reported incidences during combination therapy range from 11-15% when an ALT cutoff of 2 xULN or 3 xULN is used (16,28). During PEGV monotherapy, an incidence of elevated transaminases was reported to be 5.2% (13). In the ACROSTUDY™, only 30 patients (2.5%) had an AST or ALT above 3 xULN (7). Most of the patients with TET were on combination treatment (7). However, the real frequency of PEGV-related TET during monotherapy in this cohort might be underestimated. Elevated transaminases are usually transient, patients are usually not seen in a systematic, repetitive way, and therefore TET will go unnoticed. This can explain part of the difference in TET between our 15% and the 2.5% in the ACROSTUDY™. Our data support the notion that TET seems to occur more frequently during combined treatment. We recommend careful monitoring of patients with TET 3 xULN. Cholelithiasis must be excluded by an ultrasound of the liver. In patients with TET 10 xULN, we also recommend doing a liver biopsy and discontinuing PEGV in case of drug-induced hepatitis.

We found an association between DM and TET in 2007 (17), but it was not found in a more recent study (16) or in the current evaluation of the long-term treatment data. A few years later, a Spanish group observed an association between a common polymorphism (UGTA1A*28) of Gilbert's syndrome and male sex and TET (21). In our large single-center cohort, we detected this polymorphism in 54.2% of our patients. However, we could not find a dose effect between wild-type and homo- or heterozygous carriers of this polymorphism of Gilbert's syndrome. Moreover, we could not confirm any association with TET and the polymorphism.

No other association for TET could be observed in this database. Therefore, there is still no explanation for TET during PEGV treatment, except for the hypothesis that combined LA-SSA and PEGV increases the intrahepatic lipid content assessed by MRI that could lead to TET (31).

From the current data, it is clear that combined treatment usually stabilizes or decreases pituitary tumor size, which is in line with our previous observations (16). In some patients, tumor size decrease was observed; however, in one patient tumor size was increased. This was an exceptional patient with a short duration of symptoms before diagnosis and a significant SSTR2 expression, but only an octreotide-mediated GH decrease in vitro of 15.5%. In most GH-adenomacultures with similar SSTR2 expression, GH secretion decreases 50% or more after treatment with a similar dose of octreotide (32). Therefore, it is likely that a postreceptor defect was present in this tumor, explaining the lack of biochemical control during LA-SSA alone and tumor size control during combined treatment.

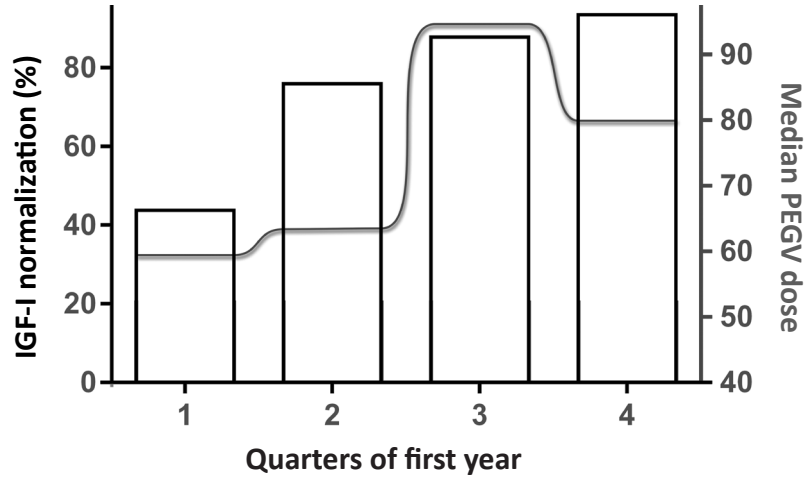
During long-term control of IGF-I with combination therapy, PEGV dose does not increase. In one exceptional case, dose adaptation was needed to treat the escape in IGF-I levels over time, without any change in tumor volume. PEGV injection instruction was repeated several times, as was supervised injections. We could not observe any aberrant injection pattern at any of these supervised moments. The single Dutch pharmacist who provides PEGV throughout The Netherlands was asked to review the amount of delivered PEGV. The pharmacist could not find any disparity between the amount of PEGV delivered to the patient and the amount prescribed. Certain studies report large interindividual differences in PEGV serum levels with similar PEGV dose administrations (23,33). However, there is only limited data on serum PEGV levels during long-term use. The patient from our series who needed a very high PEGV dose to obtain disease control also exhibited very high serum PEGV levels, suggesting adequate compliance and no obvious evidence of increased clearance of the drug.

CONCLUSION

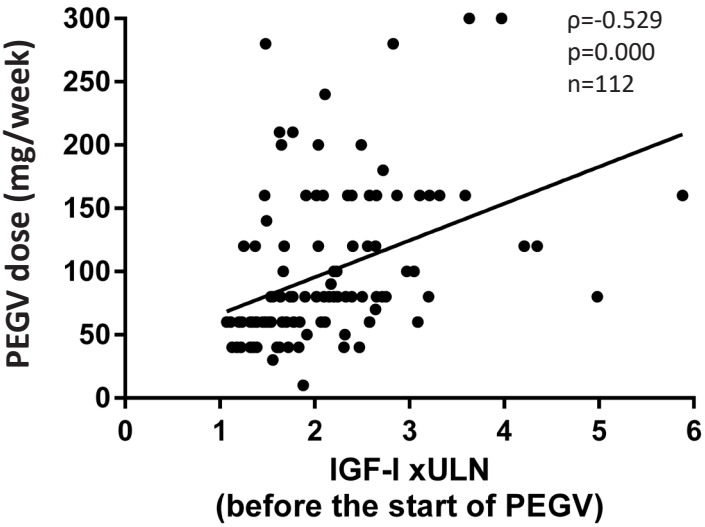
Combined treatment for acromegaly for almost a decade appears highly effective and comparable to the original registration trials, providing that the proper PEGV dose is used. Side effects were mild and transient, and we could not confirm Gilbert's polymorphism as a cause of the observed transient elevated liver enzymes. Tumor size decrease was observed in 16.9% of the patients. However, one patient had continued growth of the pituitary adenoma despite normalization of IGF-I. The only way to identify these patients is to continue periodic monitoring by radiological imaging

We found that in combination with high-dose LA-SSAs, the median dose of PEGV necessary to normalize IGF-I levels (80 mg/week) was considerably less than the dose of PEGV in those patients in the ACROSTUDY™ who did not normalize their IGF-I during long-term treatment with PEGV only (140 mg/week). This suggests that, for a significant number of patients, the combination of PEGV and LA-SSAs could be considerably less expensive. However, in one patient, dose increments of PEGV were required over time.

SUPPLEMENTAL MATERIALS



Supplemental Figure 1. IGF-I serum levels <1.2 ULN during the first year
Percentages of patients with IGF-I normalization (<1.2 xULN) an median PEGV doses in mg/week (gray line) are shown for the first year of treatment.
IGF-I: insulin-like growth factor I, PEGV: pegvisomant.

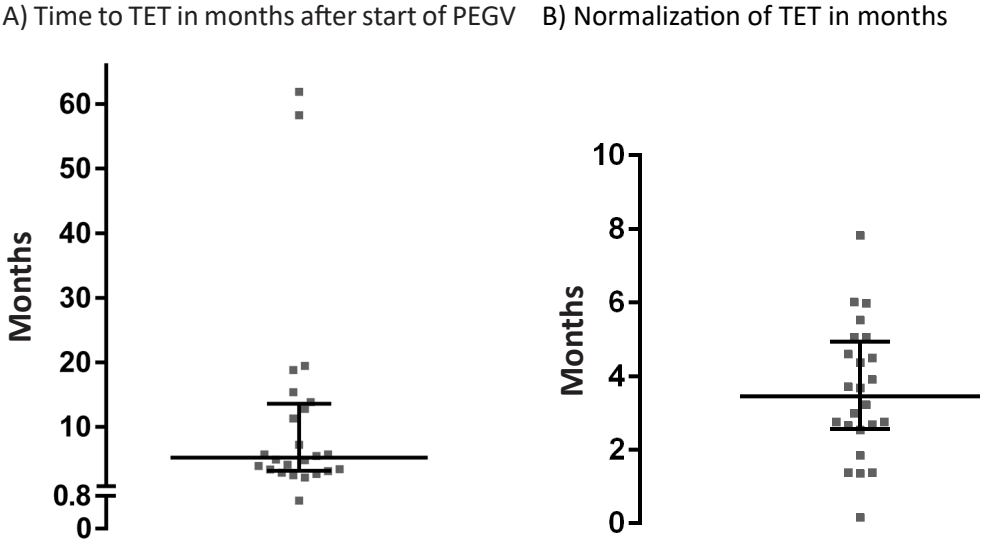


Supplemental Figure 2. A clinical tool for PEGV dosing
This is a clinical tool to calculate the required dose of PEGV to normalize the IGF-I level based on the IGF-I level before the start of PEGV. Formula of the required PEGV dose = 37.28 + (29.11* (IGF-I expressed in ULN)), (p<0.001). This formula can only be used when IGF-I is elevated after a period of at least 6 months of LA-SSA treatment. This Figure can only be used if the IGF-I level is measured by the Immulite 2000 assay, as was done in this article (DPC Biermann GmbH/Siemens, Fernwald, Germany).
IGF-I: insulin-like growth factor I, PEGV: pegvisomant, xULN: x the upper limit of normal.

Supplemental Table 1. Surgical patients during combination therapy

	Indication for transsphenoidal surgery	Curation
1	Request patient for debulking	-
2	Tumor volume reduction due to LA-SSA, candidate for curation/ debulking	-
3	Request patient for debulking	-
4	Request patient for debulking	-
5	Request patient for debulking	-
6	Request patient for debulking	-
7	Request patient for debulking	-
8	Candidate for curation	+
9	Side effect of PEGV (lipohypertrophy)	-
10	Tumor growth and visual field loss	-
11	Request patient for debulking	-

PEGV: pegvisomant.



Supplemental Figure 3.

PEGV: pegvisomant, TET: transient elevated transaminases.

Supplemental Table 2. Cause of death during combination treatment

Cause of death	Number of patients	Percentage
Cardiovascular failure	3	37.5
Sepsis	2	25.0
Malignancy	2	25.0
Pulmonary failure	1	12.5
Total	8	100.0

In the total cohort 5.6% of the subjects deceased.

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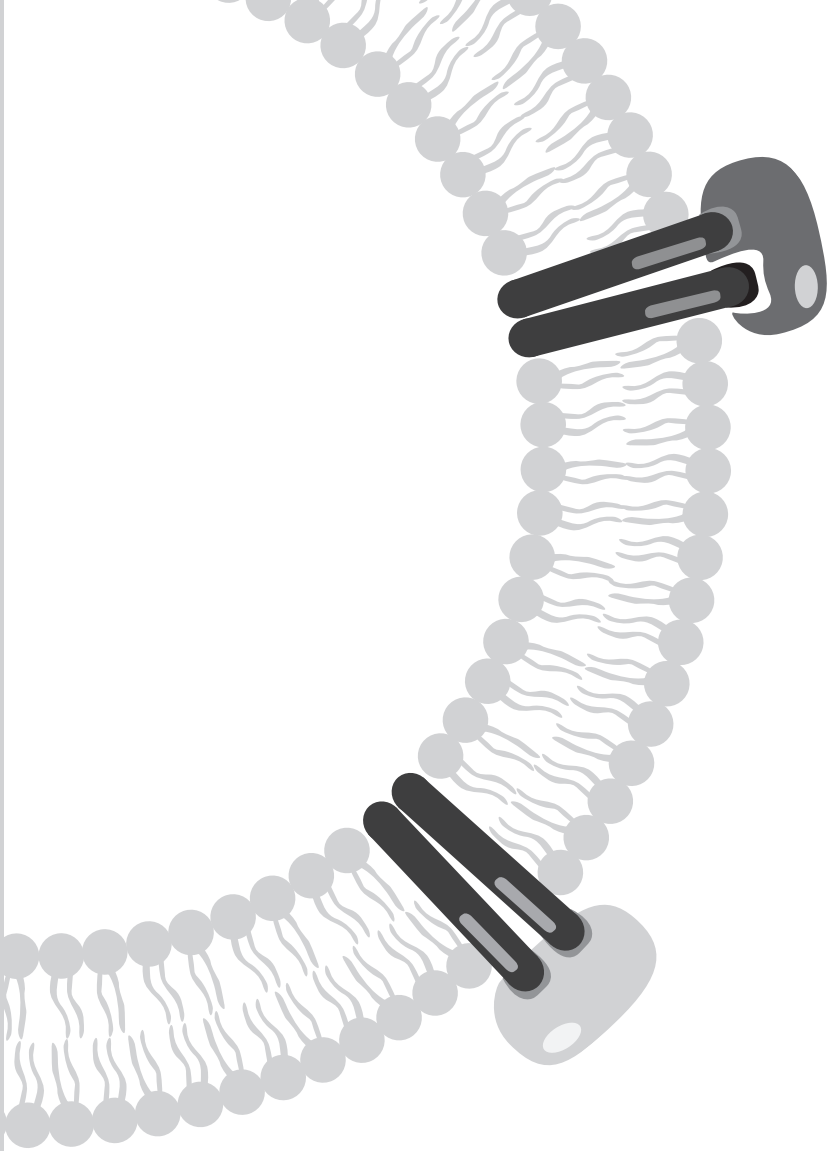

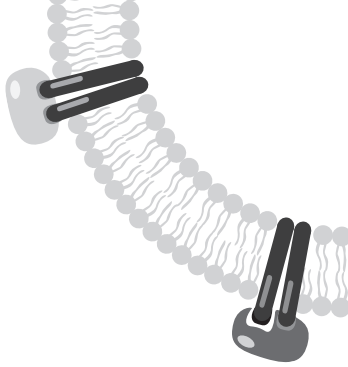

Chapter 3

Somatostatin Receptor Expression in Growth Hormone-Secreting Pituitary Adenomas Treated with Long-Acting Somatostatin Analogues in Combination with Pegvisomant

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ABSTRACT

Background: Growth hormone secreting pituitary adenomas (somatotroph adenoma) predominantly express somatostatin receptors (SSTRs) subtypes 2 and 5. Higher SSTR2 expression on somatotroph adenomas results in a better response to somatostatin analogues (SSAs), which preferentially bind, but also downregulate, SSTR2. The effect of the combined treatment with SSAs and the growth hormone (GH) receptor antagonist pegvisomant (PEGV) on SSTR expression in somatotroph adenomas is currently unknown.

Objective: To assess SSTR2 and SSTR5 expression in three groups of somatotroph adenomas: drug-naive, treated with long-acting (LA) SSA monotherapy, or LA-SSA and PEGV combination therapy before surgery. Additionally, we evaluated the required PEGV dose to achieve insulin-like growth factor I (IGF-I) normalization in relation to the SSTR expression.

Design: At our Pituitary Center Rotterdam, we selected acromegaly patients who underwent transsphenoidal neurosurgery. All patients were eventually treated with LA-SSA and PEGV combination therapy during their medical history. SSTR2 and SSTR5 expression in somatotroph adenomas tissues was determined using immunohistochemistry.

Results: Out of 39 somatotroph adenoma tissue samples, 23 were drug-naive, 9 received pre-treatment with LA-SSA and 7 LA-SSA and PEGV combined treatment. SSTR2 expression was significantly higher in treatment-naive compared to combined treatment somatotroph adenomas ($p=0.048$), while SSTR5 expression did not differ. Noteworthy, SSTR2 expression in naive somatotroph adenoma tissues was inversely correlated to the required PEGV dose to achieve insulin-like growth factor I (IGF-I) normalization during post-surgical medical treatment ($\rho=-0.538$, $p=0.024$).

Conclusions: In our specific cohort, the SSTR2 expression is lower in patients pre-treated with LA-SSA and PEGV compared to the drug-naive acromegaly patients. Additionally, the SSTR2 expression in treatment naive somatotroph adenoma tissues was inversely correlated with the required PEGV dose to achieve IGF-I normalization.

INTRODUCTION

Somatotroph adenomas express different SSTR subtypes, predominantly SSTR subtypes 2 and 5 (1), which play a major role in reducing GH secretion and thereby IGF-I levels. LA-SSAs have high-binding affinity for SSTR2 and a moderate affinity for SSTR5. According to current guidelines, LA-SSAs are considered to be the first line medical treatment modality after unsuccessful surgery as well as primary treatment option in selected cases (2,3). However, a number of studies published on the efficacy of LA-SSAs in acromegaly, show that LA-SSA treatment alone fails to reach complete normalization of IGF-I levels in about 50% of the cases. A recent meta-analysis reported an average control rate of 56 and 55% for GH and IGF-I normalization, respectively, during monotherapy with LA-SSA (4). However, due to selection bias this efficacy rate is probably an overestimation. In unselected treatment-naive patients an LA-SSA efficacy rate of 40% seems to be more common (5,6). Tumor shrinkage with LA-SSA was observed in 63-75% of these primary treated patients (6).

The expression of SSTR2 on somatotroph adenomas cell membrane is significantly and positively correlated with the efficacy of LA-SSAs in suppressing GH and IGF-I levels in vitro, and it is also associated with IGF-I normalization in acromegaly patients (7-11). In this light, a recent study from our group showed that a SSTR2 immunoreactivity score (IRS) of at least 5 had a sensitivity of 86% and a specificity of 91% in predicting IGF-I control during adjuvant LA-SSA treatment (9). Additionally, SSTR2 expression and tumor volume reduction after LA-SSA treatment are positively correlated (11,12). Moreover, partial- and non-responder tumors to monotherapy with LA-SSAs seem to have lower SSTR2 mRNA expression and higher SSTR5 mRNA expression compared to full-responders on LA-SSAs (13). An effective treatment option to normalize IGF-I levels in partial-responder patients is the addition of the GH receptor antagonist PEGV to first-line medical treatment with LA-SSA (14,15). To the best of our knowledge, the expression of SSTR2 and SSTR5 on somatotroph adenomas tissues from acromegaly patients treated with PEGV (together with LA-SSAs) before surgery is currently unknown.

Based on the finding that the response to LA-SSA treatment is mainly driven by the expression of SSTR2, a reduced expression of SSTR2 in untreated somatotroph adenomas at baseline, probably necessitates combined treatment because of (partial) resistance to LA-SSA treatment. However, we cannot exclude feedback mechanisms like the drop in IGF-I levels during medical treatment and an associated increase in hypothalamic growth hormone releasing hormone (GHrH) levels, which theoretically also can influence the expression of SSTR2 and SSTR5 on the somatotroph adenoma cell membrane. To investigate the effects of the addition of PEGV to LA-SSAs on SSTR expression, we assessed the expression of

SSTR subtypes 2 and 5 in three groups of acromegaly patients: drug-naive (naive group), treated with LA-SSA monotherapy (mono LA-SSA group) before surgery, and treated with LA-SSA and PEGV (combined group) before surgery. Since all patients included in the present study received LA-SSA and PEGV combined treatment during their clinical history, we were able to assess the required PEGV dose (added to LA-SSAs) needed after surgery to achieve the normalization of IGF-I levels. Therefore, we additionally evaluated the required PEGV dose in relation to the expression of SSTR2 and SSTR5, in order to observe indirectly the partial-resistance to LA-SSA. The underlying hypotheses are; 1) various medical pre-treatment modalities can differently affect the SSTR2 and SSTR5 expression on somatotroph adenomas and; 2) the SSTR expression can affect post-surgical PEGV dosing in combination with LA-SSA.

METHODS

Patient and somatotroph adenoma tissue selection

Data collection of acromegaly patients was performed at our Rotterdam Pituitary Center and a retrospective evaluation was carried out. We used a database including acromegaly patients which were all eventually treated with PEGV in combination with LA-SSA (n=141), and we selected those patients who underwent transsphenoidal neurosurgery (n=66), representing a total of 71 transsphenoidal surgeries. Five patients underwent a second surgery, of these patients we only included the tissue samples of the first surgery. Besides medical history data, a somatotroph adenoma paraffin-embedded tissue sample in order to perform immunohistochemistry, had to be available. We selected 39 somatotroph adenoma tissues obtained from 39 patients. A flowchart representing the selection procedure of the somatotroph adenoma tissues for our study is depicted in Figure 1. No patient underwent radiotherapy before surgery. Three groups of different medical pre-treatment modalities before surgery were identified: drug-naive patients, patients on monotherapy with LA-SSAs and patients treated with LA-SSAs in combination with PEGV. Table 1 shows patient characteristics of the three pre-treatment groups before surgery. The mono LA-SSA group was treated with LA-SSAs for a median of 6 months, the combined group was treated for a median of 20 months with LA-SSAs and for a median of 13 months with PEGV before surgery. PEGV treatment was added to the highest dose of LA-SSA by weekly injections. For starting doses of PEGV and the protocol of PEGV dose titration to achieve normal IGF-I levels see Franck & Neggers *et al.* (14). Magnetic resonance imaging was used to assess tumor volume as macro vs. micro adenomas at time of diagnosis.

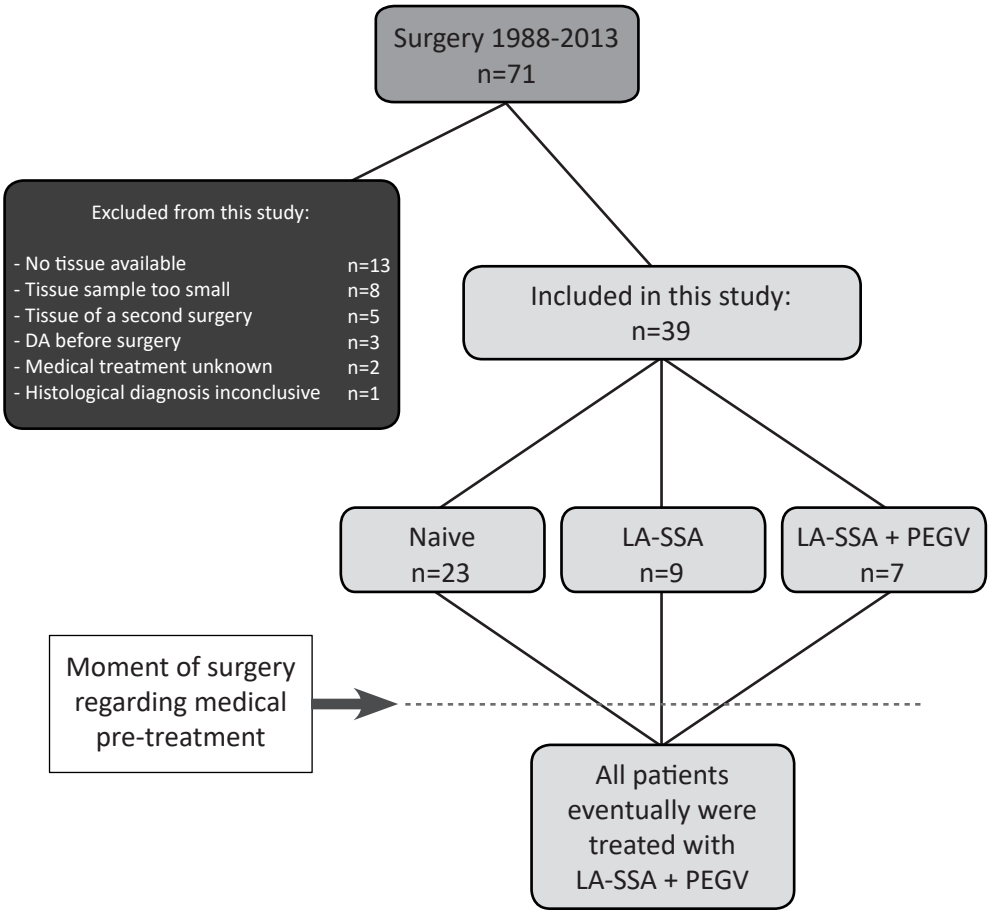


Figure 1. Flowchart depicting tissue selection

Flowchart of this study selection procedure. Patients who underwent transsphenoidal surgery and eventually were treated with LA-SSA in combination with PEGV, were selected and tissues were collected. N is the number of tissues, which represents the number of patients, as we excluded tissues from a second surgery.

DA: dopamine agonist, Naive: no previous medical treatment, LA-SSA: long-acting somatostatin analogues, PEGV: pegvisomant.

All patients selected for this study were eventually treated with LA-SSA in combination with PEGV after surgery, and were subdivided in partial- and full-responder to monotherapy with LA-SSAs. Most of the patients included in this study were partial-responders to LA-SSAs (n=31) and were considered to have a minimal decrease of 15% in their GH and IGF-I levels and still have elevated IGF-I serum levels (>1.2x upper limit of normal (ULN)) after at least six months on the highest dose of LA-SSAs (Sandostatin LAR 30 mg or Lanreotide Autogel 120 mg every 28 days). These patients required PEGV in combination with LA-SSA in order to achieve normalized IGF-I levels. Partial-responders had a median IGF-I decrease of -28.4% [IQR: -21.2 – -43.4] after LA-SSA monotherapy. Full-responders to LA-SSAs (n=8) achieved normal IGF-I levels during monotherapy with LA-SSAs and received PEGV during a clinical trial aimed to investigate aspects of quality of life (16). Full-responders had a median IGF-I decrease of -61.9% [IQR: -53.5 – -67.3] after LA-SSA monotherapy. The response to monotherapy with LA-SSA in order to subdivide the total cohort in partial- and full-responder patients, could have been evaluated before or after surgery. Table 2 provides general, biochemical and somatotroph adenoma characteristics of partial- and full-responder patients to monotherapy of LA-SSAs. When investigating the required PEGV dose to achieve normalization of IGF-I in relation with the SSTR2 expression, we included only the partial-responder patients, not pre-treated before surgery, in order to achieve a more clear indication from a homogeneous cohort. Permission from the Institutional Review Board of the Erasmus Medical Center Rotterdam was obtained and all patients gave their written informed consent.

Hormone assays

Serum IGF-I levels were measured with a Radioimmunoassay (Medgenix Diagnostics, Fleurus, Belgium, intra-assay coefficient of variation (CV) 6.1%, inter-assay CV 9.9%), an Immunoradiometric assay (Diagnostic Systems Laboratories, Webster, Texas, USA, intra-assay CV 3.9%, inter-assay CV 4.2%), and the Immulite 2000 assay, a solid-phase, enzyme-labelled chemiluminescent immunometric assay (DPC Biermann GmbH/Siemens, Fernwald, Germany, intra-assay variability of 2-5%, inter-assay variability of 3-7%). IGF-I age-adjusted reference ranges were used in accordance with an earlier report (17). IGF-I levels were evaluated just before surgery, during monotherapy of LA-SSA and after surgery when every patient eventually was treated with LA-SSA in combination with PEGV. Over time, IGF-I assays were replaced by one another. We therefore chose to express IGF-I levels only as ULN and not by the absolute values.

Immunohistochemistry

Thirty-nine somatotroph adenoma tissues were available for haematoxylin staining and immunostaining of SSTR2 and SSTR5. Formalin-fixed paraffin-embedded tumor samples were cut into sequential 4 µm thick sections, deparaffinized and stained using a fully automated Ventana BenchMark ULTRA Stainer (Ventana, Tucson Arizona, USA) according to manufacturers' instructions at the pathology department. Binding of peroxidase-coupled antibodies was detected using 3,39-diaminobenzidine as a substrate and the sections were counterstained with haematoxylin. The rabbit monoclonal anti-SSTR2 antibody (BioTrend, Köln, Germany) was used at a dilution of 1:25, whereas the rabbit monoclonal anti-SSTR5 (Abcam, Cambridge, UK) antibody at a dilution of 1:50.

Normal pancreatic tissue served as a positive control for both SSTR2 and SSTR5 staining. For negative controls, the primary antibody was omitted. Immunostaining

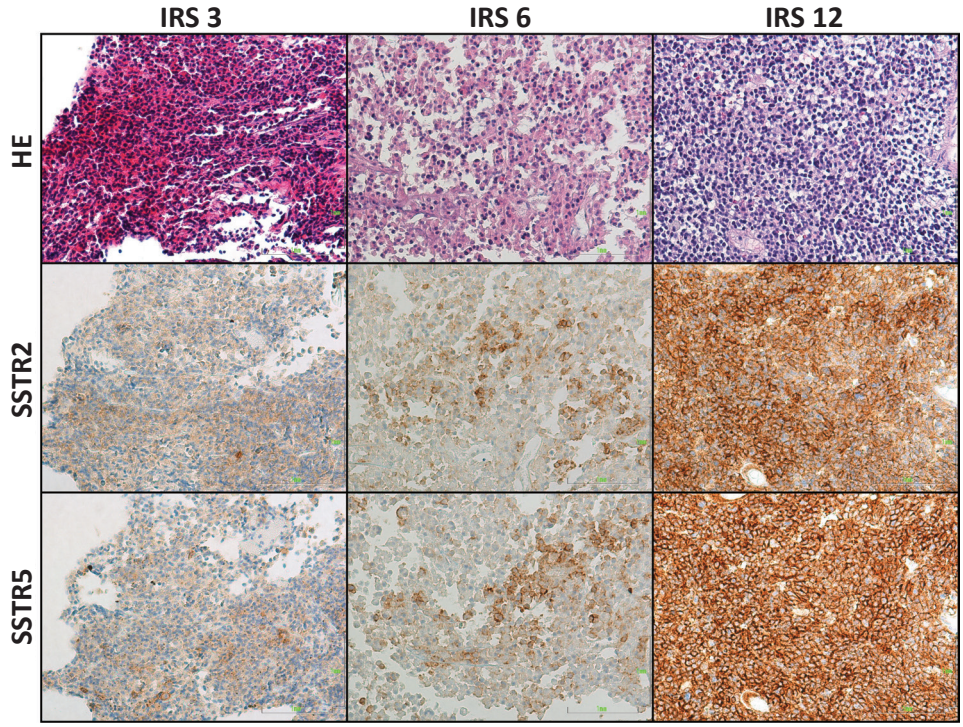


Figure 2. Immunohistochemical expression patterns of SSTR2 and SSTR5

Heterogeneous expression patterns of SSTR2 and SSTR5 on somatotroph adenomas, scored by the immunoreactivity score (IRS). IRS 3 represents a low, IRS 6 represents an intermediate and IRS 12 represents an high SSTR2 and SSTR5 expression pattern. Photography was performed during magnification of 200x.

HE: haematoxylin, SSTR2: somatostatin receptor subtype 2, SSTR5: somatostatin receptor subtype 5.

of the somatotroph adenoma tissues was scored by a semi-quantitative IRS (18), and is the product of the percentage of positive stained cells (0: no positive cells; 1: <10%; 2: 10-50%; 3: 51-80%; 4: 80%) and the staining intensity (0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining), shown in Figure 2. The IRS ranges between 0 and 12. The somatotroph adenoma tissue scoring was performed by two independent investigators (S.E.F and F.G.), who were blinded for each other's findings, for patient characteristics and their treatment regimes.

Statistical methods

Data are expressed as median [interquartile range [IQR]]. Differences between two subgroups were analysed using an unpaired t-test or the Mann-Whitney U test (in case of non-parametric data). Differences between three independent subgroups were analysed using one-way ANOVA or Kruskal-Wallis test (in case of non-parametric data). Nominal variables were analysed using Fisher's exact test. Results of correlation analyses are expressed as Spearman's rank correlation coefficients (rho). P-values <0.05 (two-tailed) are considered statistically significant. Statistical analyses were performed with SPSS version 20 and GraphPad Prism version 6 for Windows (IBM SPSS Statistics for Windows, Armonk, NY, USA and GraphPad Software, San Diego, CA, USA).

RESULTS

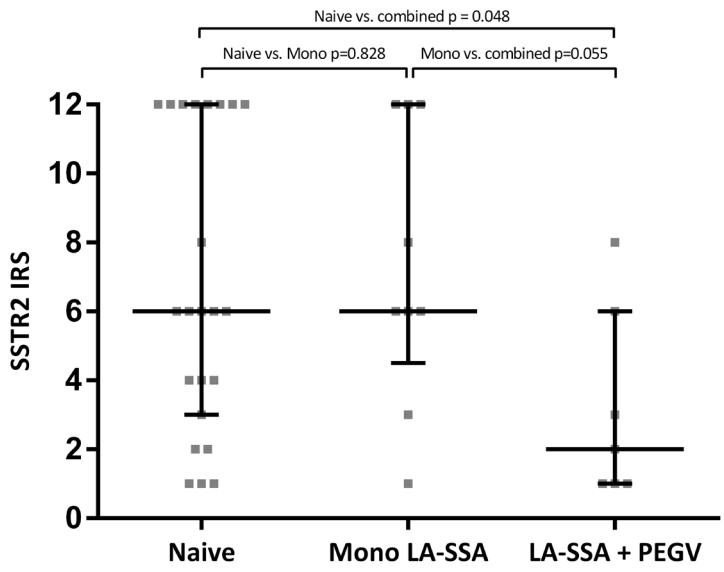
Patient characteristics before surgery

Patient characteristics before surgery are presented in Table 1. Out of the 39 evaluated somatotroph adenoma tissue samples, 23 were collected during a drug-naive state, 9 during mono LA-SSA treatment and 7 during LA-SSAs combined with PEGV therapy. No significant differences were present between the three medical pre-treatment groups before surgery when considering: sex, tumor volume assessed as macro vs. micro adenomas, IGF-I levels at diagnosis, age at time of surgery, IGF-I levels before surgery and the duration of LA-SSA treatment before surgery. IGF-I levels are expressed as xULN.

Expression of SSTR2 and SSTR5 after different medical pre-treatment options

The SSTR2 IRS in the naive group has a median of 6.0 [IQR: 2.0 – 12.0], the mono LA-SSA group has a median of 6.0 [IQR: 6.0 – 12.0], and the group pre-treated with LA-SSA and PEGV has a median of 2.0 [IQR: 1.0 – 4.5], depicted in Figure 3A. A pairwise comparison showed that the median SSTR2 IRS on somatotroph adenomas was statistically significant higher in the treatment naive group compared to the

A) SSTR2



B) SSTR5

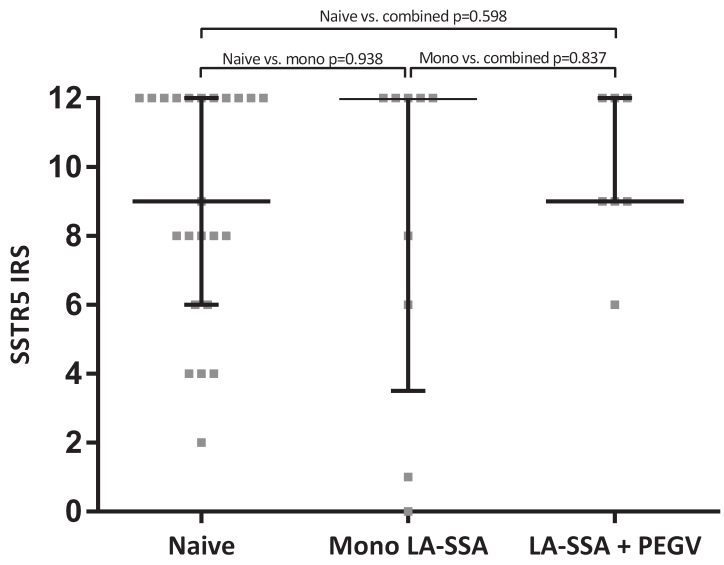


Figure 3. SSTR2 and SSTR5 expression per medical pre-treatment group

A) The SSTR2 IRS is significantly lower in the combination group compared to the naive group.

B) The SSTR5 IRS is not significantly different between the different medical pre-treatment groups. SSTR IRS is expressed as median [interquartile range]. Pre-treatment groups before surgery were tested reciprocally with the Mann-Whitney U test (Naive: n=23; Mono LA-SSA: n=9; LA-SSA + PEGV: n=7).

Naive: no previous medical treatment, LA-SSA: long-acting somatostatin analogues, PEGV: pegvisomant, IGF-I: insulin-like growth factor I, ULN: upper limit of normal, SSTR2: somatostatin receptor subtype 2, SSTR5: somatostatin receptor subtype 5, IRS: immunoreactivity score.

Table 1. Patient characteristics; medical pre-treatment at time of surgery

	Naive	Mono LA-SSA	LA-SSA + PEGV	p-value
No. of tissues – n (%)	23 (59.0)	9 (23.1)	7 (17.9)	
Baseline characteristics				
Males – n (%)	13 (56.5)	7 (77.8)	4 (57.1)	0.652
Tumor volume – macro-n (%)	22 (95.7)	9 (100)	6 (85.7)	0.379
Tumor volume-largest diameter[a] – mm	27.5 [17.0 – 40.0]	26.5 [25.0 – 27.0]	14.5 [13.0 – 16.0]	0.061
IGF-I xULN at diagnosis[b]	2.8 [2.7 – 3.1]	3.8 [2.7 – 5.5]	3.1 [2.8 – 4.5]	0.247
Time of surgery				
Age at time of surgery – years	43.2 [29.9 – 49.8]	43.8 [36.2 – 46.3]	43.2 [36.7 – 47.1]	0.773
IGF-I xULN level before surgery	2.7 [2.5 – 3.2]	2.6 [1.7 – 3.8]	1.9 [1.3 – 2.4]	0.098
Duration of LA-SSA before surgery[c] – months	N/A	6.0 [4.0 – 9.0]	20.0 [18.5 – 24.0]	0.094
Immunohistochemistry				
SSTR2 IRS[d]	6.0 [2.0 – 12.0]	6.0 [6.0 – 12.0]	2.0 [1.0 – 4.5]	
SSTR5 IRS	12.0 [7.0 – 12.0]	12.0 [6.0 – 12.0]	9.0 [9.0 – 12.0]	

Numerical data are expressed as median [interquartile range].
Naive: no previous medical treatment, LA-SSA: long-acting somatostatin analogues, PEGV: pegvisomant, IGF-I: insulin-like growth factor I, ULN: upper limit of normal, IRS: immunoreactivity score, SSTR2: somatostatin receptor subtype 2, SSTR5: somatostatin receptor subtype 5.
a. The largest diameter was missing in 56.5% of the patients in the naive group, 33.0% in the mono LA-SSA group and in 14. 3% in the LA-SSA + PEGV group.
b. The IGF-I level expressed as ULN at baseline was missing in 21.7% of the patients in the naive group. In the other two groups all data were available.
c. The mono LA-SSA group includes 7 patients that used the maximum dose of LA-SSA and 2 patients which used Sandostatin LAR 20 mg monthly for 5 and 6 months. All patients in the combined treatment group used the maximum dose of LA-SSA.
d. Scoring system which takes into account both the percentage of positive cells and the intensity of the staining.

combined group (p=0.048). We did not find any statistically significant difference for the SSTR2 IRS between treatment naive and the mono LA-SSA group. However, a trend for a lower median SSTR2 IRS was observed in the combined group when compared to the mono LA-SSA group (p=0.055).

The SSTR5 IRS in the naive group has a median of 12.0 [IQR: 7.0 – 12.0], the mono LA-SSA group has a median of 12.0 [IQR: 6.0 – 12.0], and the group pre-treated with LA-SSA and PEGV has a median of 9.0 [IQR: 9.0 – 12.0]. The SSTR5 IRS did not significantly differ between LA-SSA and PEGV treatment and the other two groups, depicted in Figure 3B.

Partial- and full-responder patients to monotherapy with LA-SSAs

All patients selected for this study were eventually treated with LA-SSAs combined with PEGV after surgery, and were subdivided in partial- and full-responder to monotherapy with LA-SSAs. Partial-responder patients have elevated serum IGF-I levels after at least six months on LA-SSAs, and needed PEGV in combination with LA-SSAs in order to achieve normalized IGF-I levels. Full-responders to LA-SSA achieved normal IGF-I levels during monotherapy with LA-SSA. Patient characteristics of partial- (n=31) and full-responders (n=8) to monotherapy with LA-SSA are presented in Table 2. No major differences were observed between partial- and the full-responders to monotherapy with LA-SSAs, regarding sex, age at time of surgery, tumor volume assessed as macro vs. micro adenomas, medical pre-treatment before surgery and IGF-I xULN at time of surgery. IGF-I xULN during monotherapy with LA-SSA was significantly different, as we selected for this variable. As for the SSTR expression at time of surgery, when only partial-responder patients were included, we observed that the SSTR2 and SSTR5 IRS followed a similar distribution between the medical pre-treatment groups as the one showed in the previous paragraph. However, possibly due to the loss of statistical power (eight patients less were included in the analysis), the difference of the SSTR2 IRS between the naive and the combined group was not significant anymore (p=0.135), depicted in Supplemental Figure 1A and B. Supplemental materials can be found at the end of this chapter.

Table 2. Patient characteristics; partial- and full-responders during monotherapy with LA-SSA

	Partial-LA-SSA-responders	Full-LA-SSA-responders	p-value
No. of tissues – n (%)	31 (79.5)	8 (20.5)	
Males – n (%)	19 (61.3)	5 (62.5)	0.640
Age at time of surgery – years	43.2 [30.7 – 49.9]	43.5 [42.1 – 49.7]	0.306
Tumor volume – macro-n (%)	30 (96.8)	8 (87.5)	0.372
Medical pre-treatment before surgery:			
Naive – n (%)	16 (51.6)	7 (87.5)	
Mono LA-SSA – n (%)	8 (25.8)	1 (12.5)	0.225
LA-SSA + PEGV – n (%)	7 (22.8)	0	
IGF-I xULN before surgery	2.3 [1.8 – 3.2]	2.8 [2.4 – 3.2]	0.853
IGF-I xULN during mono LA-SSA[a]	2.0 [1.5 – 2.3]	0.7 [0.5 – 0.8]	≤0.001

Partial-responders to LA-SSA were considered to have elevated serum IGF-I levels after at least six months on the highest dose of LA-SSAs and needed PEGV for disease control. Full-responders to LA-SSA achieve normal IGF-I levels during monotherapy of LA-SSA and PEGV was added because of quality of life reasons during a clinical trial. Monotherapy of LA-SSA could have been administered to the patient before or after surgery. Numerical data are expressed as median [interquartile range].
Naive: no previous medical treatment, LA-SSA: long-acting somatostatin analogues, PEGV: pegvisomant, IGF-I: insulin-like growth factor I, ULN: upper limit of normal.
a. The response of IGF-I levels to monotherapy with LA-SSA in order to subdivide the total cohort in partial- and full-responder patients, could have been evaluated before or after surgery.

IGF-I levels and PEGV dosing in relation to the SSTR2 and SSTR5 expression

IGF-I levels (xULN) during monotherapy with the highest approved dose of LA-SSAs were inversely correlated with the SSTR2 expression (ρ=-0.495, p=0.002, n=39, Figure 4). The SSTR5 expression was not correlated with the IGF-I (xULN) during monotherapy with LA-SSA (ρ=0.145, p=0.405, n=39). As mentioned in the methods section, when observing the required PEGV dose to achieve normalization of IGF-I in relation with the SSTR2 and SSTR5 expression, we included only the partial-responder patients, not pre-treated before surgery (drug-naive status). In this context, we observed that the required PEGV dose was inversely correlated to the SSTR2 expression (ρ=-0.538, p=0.024, n=16, Figure 5A), while it did not correlate to the SSTR5 expression (ρ=-0.071, p=0.792, n=16, Figure 5B). Correlation analyses tested in the medical pre-treatment groups before surgery; monotherapy with LA-SSA (n=9) and LA-SSA in combination with PEGV (n=7), as described in the first part of this results section, were not statistically significant between the required PEGV dose and the SSTR2 and SSTR5 expression.

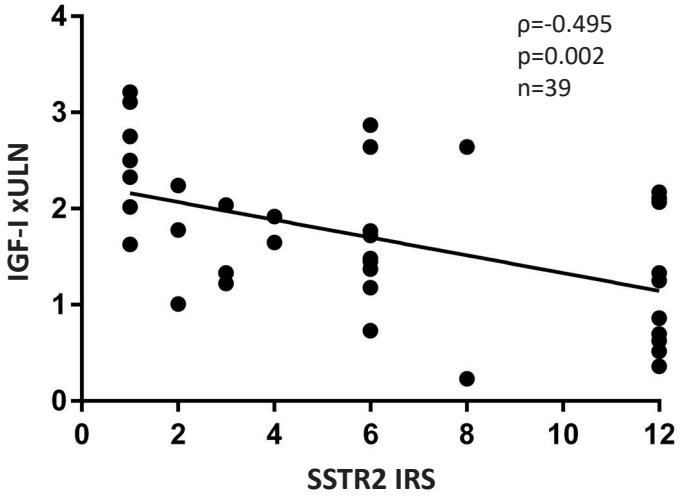


Figure 4. IGF-I during monotherapy with LA-SSA is inversely correlated with the SSTR2 expression

IGF-I xULN during the highest dose of monotherapy with LA-SSA is inversely correlated with the SSTR2 expression (Spearman correlation analyses).

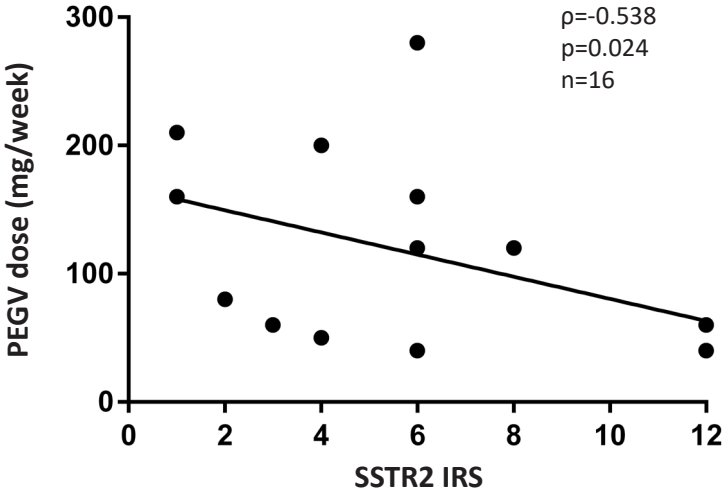
LA-SSA: long-acting somatostatin analogues, IGF-I: insulin-like growth factor I, ULN: upper limit of normal, SSTR2: somatostatin receptor subtype 2, IRS: immunoreactivity score.

DISCUSSION

This is the first study aimed to investigate the expression of SSTR2 and SSTR5 in somatotroph adenoma tissues of acromegaly patients treated with LA-SSAs in combination with PEGV. In our specific cohort, the combined group (LA-SSA and PEGV) at time of surgery showed a lower median SSTR2 IRS compared to the drug-naive group, while the IRS of the SSTR5 was not different between the pre-treated groups. Additionally, we observed that the required PEGV dose to achieve normalization of IGF-I levels was inversely correlated with the SSTR2 expression, but not with the IRS of SSTR5 in drug-naive acromegaly patients. In the following discussion, we focus on all the possible explanations for the finding that in our cohort the SSTR2 expression is lower in the combined group compared with the naive group. The most likely explanation for this finding is that somatotroph adenomas (partial) resistant to LA-SSA, that need combined treatment, have lower SSTR2 expression at baseline. However, we cannot exclude that other factors, such as feedback mechanisms of the GH-IGF-I axis and the down-regulation of SSTR2 due to prior LA-SSA treatment could affect the SSTR expression as well.

As far as the GH-IGF-I axis feedback mechanisms play a possible role in the

A) SSTR2



B) SSTR5

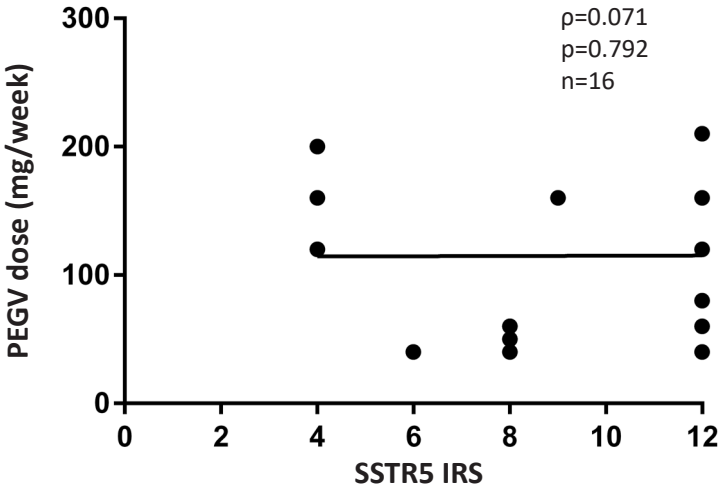


Figure 5. The PEGV dose in combination with LA-SSA is inversely correlated with the SSTR2 expression

The required PEGV dose (mg/week) in combination with the highest dose of LA-SSA needed to control IGF-I levels is inversely correlated with the expression of SSTR2 (Spearman correlation analyses) and was not correlated with the SSTR5 expression. This Figure only contains partial-responders to monotherapy with LA-SSA and the SSTR expression was estimated during a drug-naive state (n=16).

PEGV: pegvisomant, LA-SSA: long-acting somatostatin analogues, SSTR2: somatostatin receptor subtype 2, SSTR5: somatostatin receptor subtype 5, IRS: immunoreactivity score.

modulation of SSTR expression, we assume that GH itself does not have a direct effect on the modulation of SSTRs at the pituitary level during combination treatment, since PEGV blocks GH receptors (GHRs) also at the level of the pituitary (19). More complex is the prediction of growth hormone releasing hormone (GHrH) levels via the hypothalamus in presence of PEGV-treatment. PEGV generally causes a further elevation of serum GH levels (20), which could result in a decrease of GHrH via some areas in the hypothalamus and might consequently result in down-regulation of SSTR2. On the other hand, the drop of IGF-I and the associated increase in hypothalamic GHrH levels might have an influence on the expression of SSTR2 and SSTR5 at the level of the somatotroph adenoma as well. In our study IGF-I levels of patients treated with LA-SSA in combination with PEGV were relatively lower compared to the naive group. However, Park *et al.* reported that in spontaneous dwarf rats the expression of all SSTR subtypes was not directly influenced by exogenous IGF-I treatment (21). Moreover, in the same study the authors observed that GHrH has a direct stimulatory effect on SSTR2 expression, both in vivo and in vitro. If these observations can be extrapolated to humans, we could speculate that the decrease of IGF-I levels probably does not play a major role in the explanation of the observed decrease of SSTR2 expression during the combination treatment, while the modulation of GHrH levels seems to be more involved. However, based on the current knowledge, our results demonstrating a lower SSTR2 expression in the combined group compared to the naive group, cannot clearly be explained by the effect of PEGV on the modulation of the GH-IGF-I-pituitary-hypothalamus axis. Finally, to the best of our knowledge, a direct effect of PEGV on SSTR expression has not been reported, although it cannot be excluded a priori.

The down-regulation of cell surface SSTR2 by endogenous somatostatin and LA-SSA treatment has been reported in several studies (7,22,23), possibly through ligand-induced receptor internalization. Casar-Borota & Heck *et al.* demonstrated this down-regulation of SSTR2 expression by LA-SSA therapy also in a randomized subset of acromegaly patients (n=13 mono LA-SSA, n=13 direct surgery), to exclude a possible clinical selection bias (7). However, we did not observe a statistically significant difference in our acromegaly cohort between the naive and mono LA-SSA pre-treatment group regarding SSTR2 expression (p=0.828). A possible explanation for this finding may reside in the selection of our patient group. Indeed, our cohort is most likely represented by acromegaly patients with more disease activity, since they were all referred to us as a tertiary referral hospital and, most importantly, the majority of these patients needed PEGV in addition to LA-SSAs to normalize IGF-I levels (n=31). In this light, as shown in the result section, even the drug-naive group showed a relatively low SSTR2 IRS (median IRS 6, which means about 50% moderately stained cells) compared to staining observed in a previous study using comparable techniques and scoring system (median IRS 9) (7). This finding may

result in a lower down-regulation of SSTR2 after LA-SSA treatment alone, and/or contribute to “mask” the down-regulation of a receptor pattern already relatively low at baseline. However, considering all these limitations we observed that the combined treatment group in our study had a lower SSTR2 IRS compared to the naive group. Noteworthy, the SSTR2 IRS between the mono LA-SSA and combined group was almost significantly different (p=0.055). Furthermore, the duration of LA-SSA treatment was remarkably shorter in the mono LA-SSA group compared to the combined treatment group by a median difference of 14 months, however this difference did not reach statistical significance. This difference could be a possible explanation (besides the concomitant treatment with PEGV) for the observed lower expression of SSTR2 in the combined treatment group compared to the mono LA-SSA and naive group. In conclusion, the lower expression of SSTR2 in somatotroph adenomas in the combined treatment group could simply be related to the fact that these patients necessitate combined treatment due to their (partial) resistance to LA-SSA treatment. Therefore, we can hypothesize that these partial responders to monotherapy with LA-SSA have already lower SSTR2 expression at baseline, which could be the reason these patients need PEGV in addition to LA-SSA for disease control. Previously, it was already reported that full-responders to LA-SSA showed significant higher SSTR2 expression compared to the partial LA-SSA responders (9, 12). Furthermore, for the first time, we observed that in drug-naive somatotroph adenomas a lower SSTR2 expression correlates with a higher required PEGV dose in order to achieve normalized IGF-I levels, which also reflects more severe disease activity. This observation may have clinical implications for the postoperative treatment in acromegaly patients and, therefore, represents an important finding of our study.

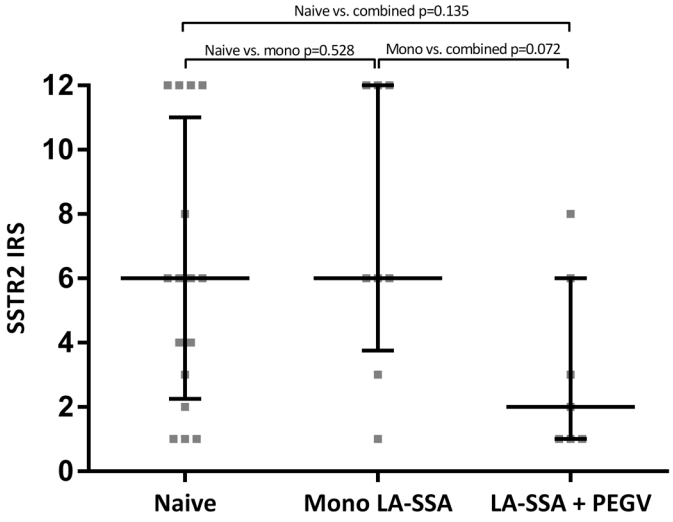
The main limitations of this study are; 1) the retrospective design; 2) the relative small sample size and; 3) the peculiar patient group in which the study has been conducted (all treated with combination medical therapy during their clinical history). However, this can be expected by the rarity of acromegaly as well as the fact that only a subset of the patients is treated with LA-SSA in combination with PEGV. These limitations of the present study could be overcome in the future by the design of a large prospective randomized study aimed to evaluate the impact of different treatment modalities on SSTR expression. Furthermore, assessment of IGF-I and GH levels can also be improved, and, in particular, GH measurement should be assessed by a non-commercial assay in order to distinguish between endogenous GH and PEGV, which is recently introduced in our clinical practice.

CONCLUSION

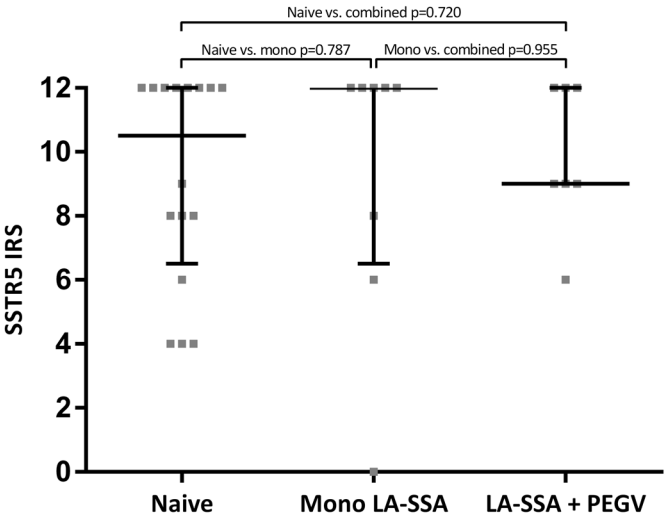
This is the first study that assessed the effect of the combined medical treatment with LA-SSAs and PEGV on SSTR expression in somatotroph adenoma tissue of acromegaly patients. In our specific cohort, SSTR2 expression is lower in patients pre-treated with LA-SSA and PEGV compared to the drug-naive acromegaly patients. This finding is in line with the evidence that LA-SSA and PEGV-treated patients are usually (partial) resistant to LA-SSA treatment alone (dependent on SSTR2 expression). Moreover, we observed that patients with a lower SSTR2 expression need a higher required PEGV dose in combination with LA-SSA to achieve normalized IGF-I levels after surgery, when assessed in drug-naive somatotroph adenoma tissues.

SUPPLEMENTAL MATERIALS

A) SSTR2



B) SSTR5



Sublemental Figure 1A and 1B. SSTR2 and SSTR5 expression per medical pre-treatment group in partial-responders to LA-SSA

SSTR2 IRS (A) and SSTR5 IRS (B) per medical pre-treatment group in only partial-responders to LA-SSA. SSTR2 IRS and SSTR5 IRS were not statistically significant different when medical pre-treatment groups before surgery were tested reciprocally with the Mann-Whitney U test (Naive: n=16; Mono LA-SSA: n=8; LA-SSA + PEGV: n=7). SSTR IRS is expressed as median[interquartile range].

Naive: no previous medical treatment, LA-SSA: long-acting somatostatin analogues, PEGV: pegvisomant, IGF-I: insulin-like growth factor I, ULN: upper limit of normal, SSTR2: somatostatin receptor subtype 2, SSTR5: somatostatin receptor subtype 5, IRS: immunoreactivity score.

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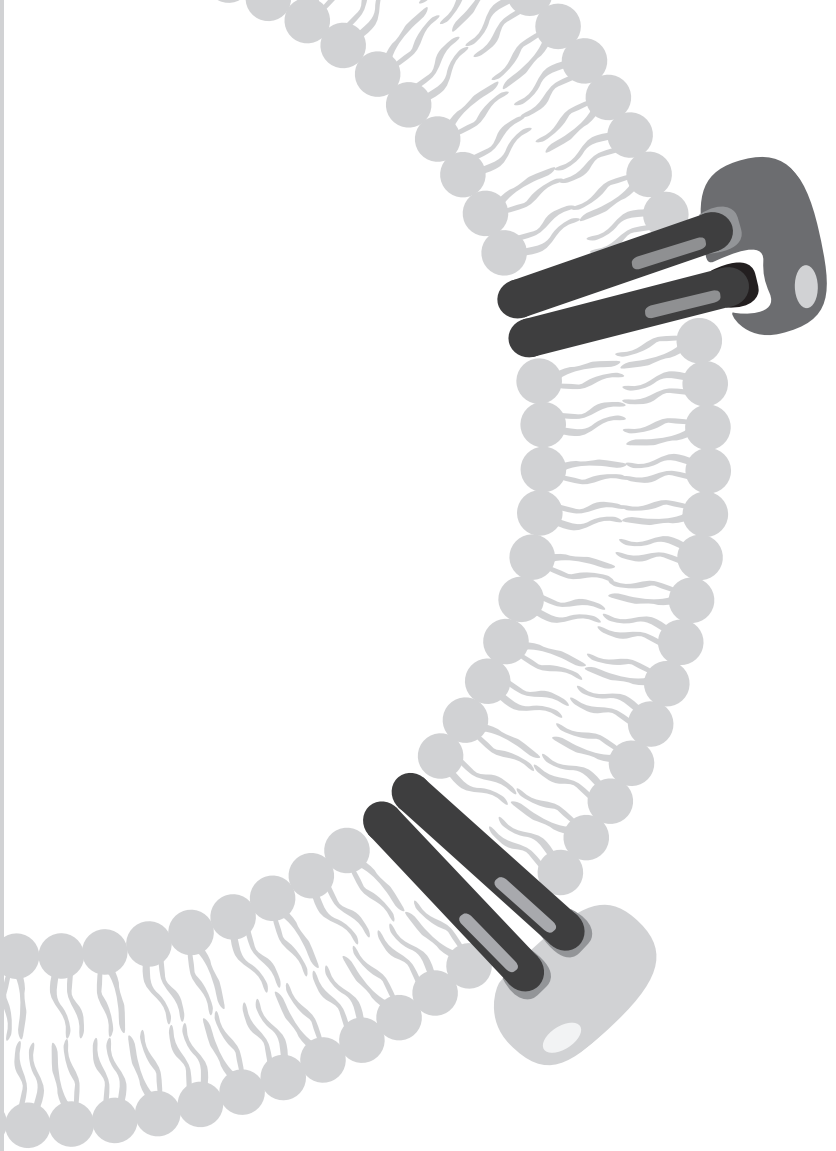

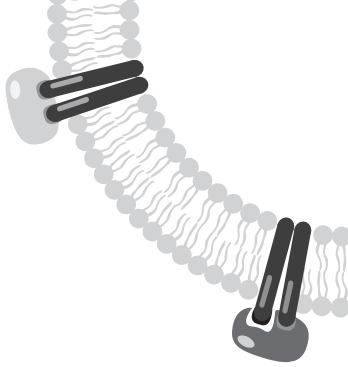

Chapter 4

Pegvisomant in Combination with Long-Acting Somatostatin Analogues in Acromegaly: the Role of the Growth Hormone Receptor Deletion of Exon 3

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ABSTRACT

Background: Doses of the growth hormone receptor (GHR) antagonist pegvisomant (PEGV) that normalize insulin like growth factor I (IGF-I) levels vary widely among acromegaly patients. Predictors for PEGV response are baseline IGF-I levels, sex, body weight, and previous radiotherapy. A GHR polymorphism lacking exon 3 (d3-GHR) is frequent in the general population. The influence of d3-GHR on PEGV responsiveness in acromegaly is unclear.

Objective: To assess the influence of d3-GHR on IGF-I levels and PEGV responsiveness in acromegaly patients using combined PEGV and long-acting somatostatin analogues (LA-SSA) treatment.

Design: Data was collected at the Rotterdam Pituitary Centre between 2004 and 2013. Patients with elevated IGF-I levels (>1.2 upper limit of normal (ULN); n=112) and over 6 months of high-dose LA-SSA treatment were co-treated with PEGV. GHR genotype was assessed using genomic DNA in 104 patients.

Results: D3-GHR was observed in 51 (49.0%) of the patients (7.7% homozygous, 41.3% heterozygous) and was in Hardy-Weinberg equilibrium (p=0.859). Baseline characteristics were similar in d3-GHR and full-length (fl)-GHR genotypes. During PEGV and LA-SSA treatment IGF-I levels were not different between d3-carriers and non-carriers. Similarly, no difference in PEGV dose required to normalize IGF-I (p=0.337) or PEGV serum levels (p=0.433) was observed between the two groups. However, adenoma size decreased significantly (>20% of largest diameter) in 25.6% of the fl-GHR genotype but only in 7.5% of d3-carriers (p=0.034, OR: 4.6 [CI: 1.1 – 18.9]).

Conclusions: GHR genotype does not predict the IGF-I normalizing dose of PEGV in acromegaly patients using PEGV in combination with LA-SSA. However, fewer d3-carriers showed significant reductions in adenoma size.

INTRODUCTION

Disease activity and phenotype is diverse among patients with acromegaly. Comorbidities such as hypertension, cardiomyopathy, diabetes mellitus type II, sleep apnea and osteoarthritis are influenced by the severity and the duration of GH hypersecretion (1). PEGV is a competitive GHR antagonist that is used in the treatment of acromegaly (2). The required dose of PEGV to achieve disease control as assessed by the normalization of IGF-I levels differs significantly between individual patients (2). Baseline IGF-I appears to be a predictor for the required dose (3). Other factors known to influence the required dose are sex, body weight and previous radiotherapy (4). However, GHR polymorphisms seem to have an influence as well (5,6).

A polymorphism of the GHR that lacks exon 3 during splicing is common in the general population. About half of the population is homozygous for the fl-GHR, 30-40% is heterozygous for d3-GHR and 10-20% is homozygous for this deletion (7,8,9). It has been reported that the d3-GHR polymorphism shows a comparable distribution between different cohorts of acromegaly patients (6,10,11,12). Multiple studies show that the fl-GHR and d3-GHR have comparable binding properties, and that internalization of fl-GHR is as effective as d3-GHR (13,14,15). However, Dos Santos *et al.* (8) showed in transfection experiments that the lack of exon 3 results in an enhanced signal transduction by the STAT-5-dependent pathway, which increases the expression of IGF-I and other GH-dependent genes. Scientific attention on d3-GHR was in the beginning focused on the outcome of recombinant GH replacement therapy in GH-deficient children and later in adults, in which the studies often show different conclusions (16,17,18). Thereafter research was focused on the severity of acromegaly regarding the GHR-genotype.

Previous research showed that d3-GHR carriers with acromegaly have a more severe clinical and biochemical phenotype, however, inconsistent results have also been reported. For example, Wassenaar *et al.* (19) reported an increased prevalence of osteoarthritis, dolichocolon and adenomatous colonic polyps in d3-GHR carriers with acromegaly, but no difference in cardiovascular risk and bone mineral density. Mercado *et al.* (11) observed that diabetes mellitus type II was more prevalent in patients with the d3-GHR genotype apart, whereas several other phenotypical features were independent of GHR genotype. The authors also observed a significantly higher serum IGF-I concentration after treatment (surgery, radiotherapy and/or pharmacological therapy) in d3-GHR carriers (11). Cinar *et al.* (20) reported that d3-GHR genotype did not have an effect on clinical features nor on comorbidities in acromegaly patients.

A meta-analysis on GH-deficiency concluded that the presence of d3-GHR increases the response to recombinant GH treatment in GH-deficient children (21).

This pharmacogenetic phenomenon could be important for PEGV treatment in acromegaly. In theory, carriers of d3-GHR might need less PEGV than non-carriers to reach a comparable decrease in IGF-I levels. Indeed, two studies reported that the required PEGV dose for normalization of IGF-I levels was significantly lower in acromegaly patients with a d3-GHR genotype (5,6). A later study, however, did not observe a better response of d3-GHR carriers during monotherapy PEGV nor during combination treatment with PEGV and somatostatin analogues (10). These inconsistent findings indicate that larger cohorts of acromegaly patients are needed to investigate whether the response to PEGV differs between d3-GHR and fl-GHR genotypes. We, therefore, examined whether there were differences in the clinical and biochemical responses during PEGV treatment between both genotypes in our cohort of 104 patients using somatostatin analogues combined with PEGV.

METHODS

Patients

Data of acromegaly patients was collected at our Rotterdam Pituitary Centre between 2004 and 2013. Inclusion criteria were; 1) elevated serum IGF-I levels (>1.2 xULN) after at least 6 months on the highest dose of LA-SSAs (Sandostatin LAR 30 mg or Lanreotide Autogel 120 mg every 28 days) and; 2) genomic DNA could be obtained (n=104). After the initial start with monotherapy of LA-SSA, co-treatment with PEGV was added by weekly injections. For starting doses of PEGV and the protocol of PEGV dose titration to achieve normal IGF-I levels, see Franck & Neggers *et al.* (22). All patients gave their written informed consent. The study was approved by the local Institutional Review Board.

Hormone assays

Serum levels of IGF-I and GH were measured with the Immulite 2000 assay (DPC Biermann GmbH/Siemens, Fernwald, Germany), a solid-phase, enzyme-labeled chemiluminescent immunometric assay, with an intra-assay variability of 2-5%, and an inter-assay variability of 3-7%. The IGF-I age-adjusted reference ranges were used in accordance with earlier reports (23). PEGV serum levels were assessed in Aarhus, as described previously (24).

Assessment of side effects included serum concentrations of alanine aminotransferase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase, γ-glutamyltranspeptidase and total bilirubin. Magnetic resonance imaging (MRI) was used to assess changes in pituitary tumor volume at least every 2 years. Changes in tumor size were assessed by a single radiologist who was blinded for patient characteristics and treatment regimens. A ‘significant decrease’ was defined

as a reduction of more than 20% of the largest diameter of the tumor during combination treatment compared with the largest diameter of the last MRI before the addition of PEGV.

Q-PCR of GHR deletion of exon 3

The exon 3-deleted GHR polymorphism could be assessed in 104 patients. Genomic DNA was extracted from peripheral blood leukocytes by standard procedures. Analysis of the d3-GHR polymorphism was carried out using quantitative PCR (Q-PCR) as previously described (5). Briefly, primer/probe sets binding to exons 3 and 10 of the human GHR gene were added to the DNA samples. GHR exon 10 served as an internal positive control. Q-PCR was performed in triplicate 384-wells plates with 20 ng genomic DNA in a volume of 5 µl using 1x Taqman Universal PCR Master Mix (Life Technologies), 3 µM GHR exon 3 primers/probe and 9 µM GHR exon 10 primers/probe. Amplification was performed using a real-time Taqman 7900 HT instrument (Applied Biosystems) with the following cycle conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Differences in cycle threshold (ΔCt) between exon 3 and exon 10 amplicons were used to determine the exon 3 copy number for each sample. A ΔCt value of 1 indicates two exon 3 copies (genotype fl/fl), a ΔCt value of 2 indicates one exon 3 copy (genotype fl/d3) and no signal for exon 3 in the presence of a normal exon 10 signal indicates an absent exon 3 (genotype d3/d3). To validate the genotyping accuracy, 25 samples were randomly selected to determine the GHR exon 3 polymorphism for a second time using a multiplex PCR assay (7). No discrepancies were found between the genotypes obtained by either method.

Statistical analysis

Data are expressed as medians [interquartile range [IQR]] unless otherwise specified. Nominal variables were analyzed using the χ^2-test. Differences between two or more independent subgroups were analyzed using the Mann-Whitney *U* test or the Kruskal-Wallis ANOVA respectively. Hardy-Weinberg equilibrium was analyzed with the χ^2-test via the observed and expected genotype frequencies. Results of correlation analyses are expressed as Spearman's rank correlation coefficient. P-values <0.05 (two-tailed) are considered statistically significant. Statistical analyses were performed with SPSS version 20 and GraphPad Prism version 6 for Windows (SPSS software, Chicago, IL, USA and GraphPad Software, San Diego, CA, USA).

RESULTS

Patient characteristics

Characteristics of the 104 patients are presented in Table 1. The median age of all patients at time of diagnosis was 45.4 years and the majority of the patients were male. The median time between the diagnosis and the start of PEGV was 1.4 years. The median duration of PEGV treatment was 5 years. The majority of the patients had a macroadenoma. Diabetes mellitus type II was present in one-third of subjects. Previous therapies were surgery (42.3%) and radiotherapy (12.5%). Before the start of PEGV in combination with LA-SSAs, the median absolute IGF-I level was 63.8 nmol/l or 1.81 expressed as times the ULN of IGF-I. The median GH level at the start of PEGV was 4.0 µg/l. For 18 patients, GH levels were missing at the start of PEGV.

Table 1. Patient characteristics of fl-GHR and d3-GHR genotypes

		GHR genotype		
	All patients	**fl-GHR**	**All d3-carriers**	**p-value[a]**
n – (%)	104	53 (51.0)	51 (49.0)	
Males – %	58.7	54.7	62.7	0.432
Age at diagnosis – years	45.4 [35.4 – 56.0]	43.0 [35.4 – 52.7]	46.8 [36.8 – 56.2]	0.400
Time between diagnosis and start of PEGV – years	1.4 [0.9 – 3.5]	1.4 [0.9 – 4.1]	1.6 [0.9 – 3.5]	0.995
Age at start PEGV – years	47.8 [37.8 – 59.2]	46.8 [37.2 – 59.0]	48.9 [40.1 – 59.2]	0.309
Years of PEGV treatment	5.0 [2.4 – 6.3]	4.2 [2.0 – 7.4]	5.2 [3.2 – 7.2]	0.181
Tumor volume – macro %	81.7	79.2	84.3	0.614
Diabetes mellitus type II – %	36.5	30.2	43.1	0.222
Previous therapy:				
Surgery – %	42.3	43.4	41.2	0.845
Radiotherapy – %	12.5	7.5	17.6	0.146
GH at start of PEGV – µg/l	4.0 [2.3 – 10.3]	4.5 [2.5 – 10.2]	3.5 [2.2 – 7.3]	0.206
IGF-I at start of PEGV – nmol/l	63.8 [46.5 – 86.6]	68.8 [46.1 – 94.0]	62.6 [48.9 – 78.3]	0.558
IGF-I xULN at start of PEGV	1.81 [1.48 – 2.50]	2.03 [1.47 – 2.72]	1.77 [1.52 – 2.31]	0.521

Expressed as median [interquartile range].
ULN: upper limit of normal, PEGV: pegvisomant, IGF-I: insulin-like growth factor I, GHR: growth hormone receptor, d3-GHR: growth hormone receptor polymorphism lacking exon 3, fl-GHR: full-length growth hormone receptor.
a. fl/fl genotype vs d3 genotype (fl/d3 and d3/d3).

Patient characteristics and GHR genotype

The d3-GHR polymorphism assessed in 104 (92.9%) of the 112 patients. The 8 patients, from whom genomic DNA was not retrieved, did not exhibit phenotypical features different from the genotyped patients (data not shown). D3-GHR was observed in 51 (49.0%) of the patients, of which 7.7% were homozygous and 41.3% were heterozygous. This distribution of the d3-GHR genotype followed the Hardy-Weinberg equilibrium (p=0.859). Patient characteristics for both groups are depicted in Table 1. No statistically significant differences were present at baseline between the GHR genotypes, regarding age at diagnosis and at start of PEGV, years of treatment, sex, tumor volume assessed as macro- vs. microadenomas, presence of diabetes mellitus type II, kind of previous therapy, and GH and IGF-I levels before start of PEGV.

Clinical and biochemical response during treatment

IGF-I levels

Normalization of IGF-I corrected for age, defined as a dichotomous variable based on the lowest IGF-I during treatment, was observed in almost all fl-GHR patients and d3-GHR carriers, and was not significantly different between the groups (p=0.587, Table 2). The lowest median absolute IGF-I level during treatment (18.0 nmol/l) in the fl-GHR genotype was not significantly different from that in the d3-GHR genotype (18.4 nmol/l, p=0.592). Furthermore, IGF-I xULN and decrease of IGF-I during treatment was not different between d3-carriers and non-carriers (p=0.780 and 0.728, respectively, Figure 1).

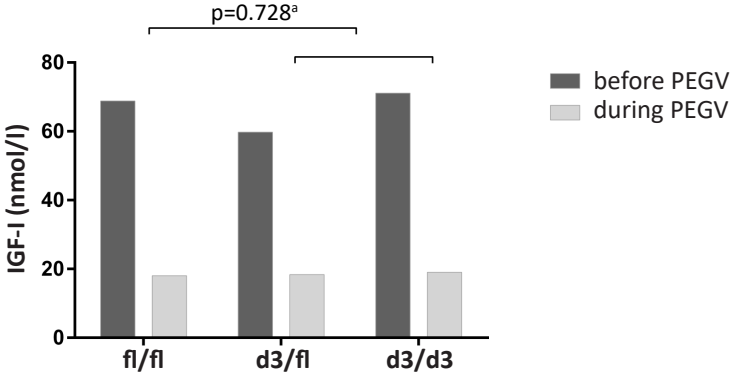

Figure 1. Decrease of IGF-I during PEGV treatment

Data is expressed as median. Decrease of IGF-I is based on the IGF-I leven before the start of PEGV and the lowest IGF-I during PEGV treatment.

PEGV: pegvisomant, IGF-I: insulin-like growth factor I, GHR: growth hormone receptor, d3-GHR: growth hormone receptor polymorphism lacking exon 3, fl-GHR: full-length growth hormone receptor.

a. fl/fl genotype vs d3 genotype (fl/d3 and d3/d3).

Table 2. Clinical and biochemical response during treatment of fl-GHR and d3-GHR genotypes

		GHR genotype		
	All patients	**fl-GHR**	**All d3-carriers**	**p-value[a]**
n – (%)	104	53 (51.0)	51 (49.0)	
Normalization of IGF-I <1.0 xULN[b] – %	97.1	96.2	98.0	0.587
Lowest IGF-I during treatment – nmol/l	18.5 [14.5 – 23.5]	18.0 [14.7 – 24.3]	18.4 [12.1 – 23.1]	0.592
Lowest IGF-I xULN during treatment	0.57 [0.43 – 0.75]	0.56 [0.43 – 0.76]	0.56 [0.40 – 0.71]	0.780
Decrease IGF-I[c] – %	69.5 [59.6 – 78.3]	75.4 [59.6 – 79.7]	72.0 [63.5 – 78.1]	0.728
PEGV dose[d] – mg/week	80.0 [60.0 – 120.0]	80.0 [60.0 – 110.0]	80.0 [60.0 – 140.0]	0.337
PEGV dose – mg/kg/week	0.90 [0.66 – 1.28]	0.82 [0.59 – 1.24]	0.91 [0.66 – 1.40]	0.655
PEGV serum level – µg/l	4625 [2975 – 11962]	4913 [3025 – 12500]	4625 [2925 – 8025]	0.433
Ratio PEGV serum/dose	63.0 [38.5 – 100.0]	68.0 [39.0 – 99.0]	56.0 [36.0 – 93.0]	0.293
Transient elevated transaminases – %	16.3	15.1	17.6	0.725
Decrease of tumor size[e,f] during treatment – %	16.5	25.6	7.5	0.034[g] OR: 4.6 [CI: 1.1 – 18.9]
Change in tumor size during treatment[f]				
Decrease of tumor size[e] – n (%)	13 (12.5)	10 (18.9)	3 (5.9)	
No change of tumor size – n (%)	66 (63.5)	29 (54.7)	37 (72.5)	0.036[h]
Increase of tumor size – n (%)	1 (1.0)	1 (1.9)	0	

Expressed as median [interquartile range].
ULN: upper limit of normal, PEGV: pegvisomant, IGF-I: insulin-like growth factor I, GHR: growth hormone receptor, d3-GHR: growth hormone receptor polymorphism lacking exon 3, fl-GHR: full-length growth hormone receptor.
a. fl/fl genotype vs d3 genotype (fl/d3 and d3/d3).
b. Dichotomous variable based on the lowest IGF-I during treatment period.
c. Difference between IGF-I before the start of PEGV and Lowest IGF-I during combination treatment.
d. Required PEGV dose to achieve normalization of the IGF-I level.
e. More than 20% volume reduction.
f. Patients with an empty sella were not included in the analyses, as decrease and increase are not applicable.
g. Tested by logistic regression, correction for radiotherapy did not influence the significance.
h. Tested by Fisher's exact test.

PEGV dose and PEGV serum level

The median required PEGV dose to achieve the lowest IGF-I level was 80.0 mg/week [60.0 – 110.0] for non-carriers and 80.0 mg/week [60.0 – 140.0] for the d3-GHR genotype. No significant difference between the two groups was found for the PEGV dose (p=0.337), as presented in Figure 2. Correlation analysis showed a positive correlation between the decrease in IGF-I level and the PEGV dose (ρ=0.211, p=0.030), depicted in Figure 3. The median required PEGV dose between men and women was not significantly different (p=0.650). Weight correlated positively with the PEGV dose (ρ=0.265, p=0.007). The PEGV dose in mg/kg/week was not

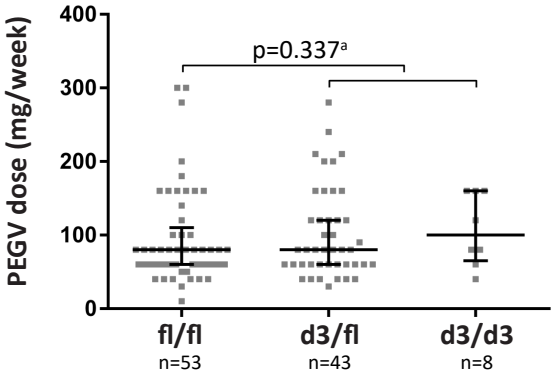

Figure 2. Required PEGV dose for normalization of IGF-I

Expressed as median [interquartile range].

a. fl/fl genotype vs d3 genotype (fl/d3 and d3/d3).

PEGV: pegvisomant, IGF-I: insulin-like growth factor I, GHR: growth hormone receptor, d3-GHR: growth hormone receptor polymorphism lacking exon 3, fl-GHR: full-length growth hormone receptor.

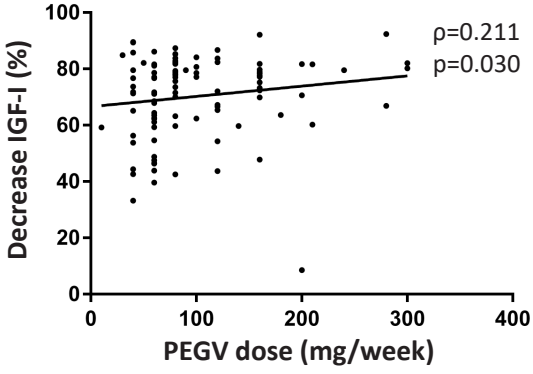

Figure 3. Correlation between IGF-I and PEGV dose

The decrease of IGF-I between the start of PEGV and the lowest IGF-I level during treatment of the total acromegaly cohort during combination treatment. PEGV dose is the required dose to achieve this lowest IGF-I level during treatment. Adjustment for GHR variant, age and sex did not influence correlation analysis.

PEGV: pegvisomant, IGF-I: insulin-like growth factor I.

significantly different between fl-GHR and d3-GHR (p=0.655).

The PEGV serum levels (n=83, genotyped cohort), measured during the end of the inclusion period of this study, were also not significantly different between the two genotypes (p=0.433), as shown in Figure 4. PEGV serum levels were 4913 µg/l [IQR: 3025 – 12500] for non-carriers and 4625 µg/l [IQR: 2925 – 8025] for

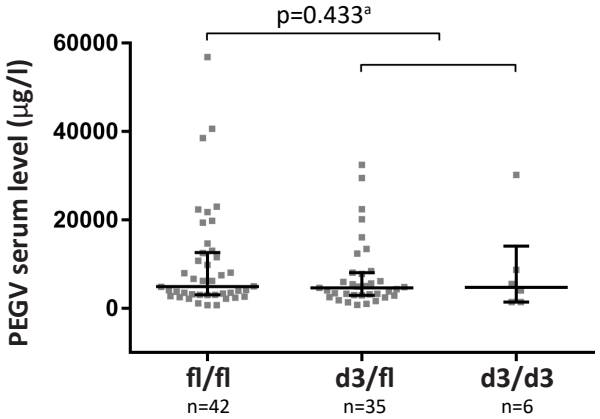

Figure 4. PEGV serum levels

PEGV serum levels were measured during the end of the inclusion period of this study. Expressed as median [interquartile range].

PEGV: pegvisomant, IGF-I: insulin-like growth factor I, GHR: growth hormone receptor, d3-GHR: growth hormone receptor polymorphism lacking exon 3, fl-GHR: full-length growth hormone receptor.

a. fl/fl genotype vs d3 genotype (fl/d3 and d3/d3).

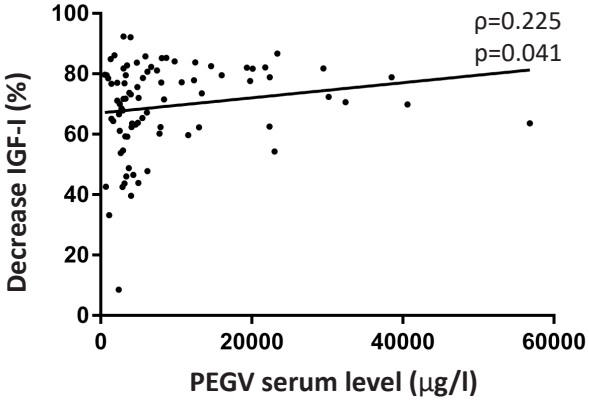

Figure 5. Correlation between IGF-I and PEGV serum level

The decrease of IGF-I between the start of PEGV and the lowest IGF-I level during combination treatment. PEGV serum levels were measured during the end of the inclusion period of this study. Adjustment for GHR variant, age and sex did not influence correlation analysis.

PEGV: pegvisomant, IGF-I: insulin-like growth factor I.

the d3-GHR carriers. Correlation analysis showed a positive correlation between the decrease of IGF-I and the PEGV serum level (n=86 (total cohort), ρ=0.225, p=0.041, Figure 5). The median PEGV serum levels were not different between men and women (n=86, p=0.870). Weight was not correlated to the PEGV serum level (n=79, ρ=-0.027, p=0.814). The ratio of PEGV serum levels over the PEGV dose was not significantly different between the d3-GHR carriers and non-carriers (n=83, p=0.293). Samples of 29 patients (25.9%) were not available for the assessment of PEGV concentrations. In 7.8% of the samples, a PEGV serum level of 0 mg/l was measured during PEGV treatment and was left out of the analysis.

Tumor shrinkage and liver enzyme tests

A significant reduction of the adenoma size was observed in 25.6% of the fl-GHR genotype and in 7.5% of the d3-carriers, which is a significant difference between the groups (p=0.034, OR: 4.6 [CI: 1.1 – 18.9] tested by logistic regression). When tested by Fisher's exact test, p-value was 0.036. Of the d3-carriers, 17.6% had transiently elevated transaminases (TET), the most common side effect of the combination treatment with PEGV and LA-SSA, compared to 15.1% of patients with the fl-GHR genotype (p=0.725).

DISCUSSION

The results of our study did not show a significant difference in patient characteristics or treatment response to PEGV between fl-GHR and d3-GHR genotypes in patients with acromegaly. Carrying the d3-GHR polymorphism did not affect the PEGV dose nor the PEGV serum levels during treatment. However, a significantly larger reduction in tumor volume during treatment was observed in patients with the fl-GHR genotype compared to d3-carriers (p=0.034).

Transfection studies have shown that the lack of exon 3 in the GHR enhances GH signal transduction (8) and there are clinical data to suggest that this polymorphism confers a better response to GH replacement therapy and also impacts on patients with acromegaly (5,6,25,26). The group of acromegaly patients in our study is suitable to further analyze the clinical relevance of the d3-GHR genotype for two reasons. First, acromegaly patients using LA-SSAs and PEGV have a more severe disease activity, as LA-SSA monotherapy was not effective enough to normalize IGF-I. Secondly, d3-GHR carriers, having a higher GHR signal transduction, are considered to respond better to PEGV compared to patients with the fl-GHR. Therefore, the d3-GHR carriers can be hypothesized to need a lower dose of PEGV to achieve disease control. Indeed, studies during PEGV monotherapy by Bianchi *et al.* (6) (n=19) and Bernabeu *et al.* (5) (n=44) revealed that the required PEGV dose to normalize IGF-I levels was significantly lower in acromegaly patients with d3-GHR

genotype. However, Filopanti *et al.* (10) studied two groups of acromegaly patients using monotherapy PEGV (n=64) and LA-SSAs in combination with PEGV (n=63) and could not confirm the superior treatment response of d3-GHR carriers in either group. It could be argued that the sample sizes of these two cohorts in the last negative study were too small to observe an effect. However, in our current study, with a reasonable sample size, an effect of the d3-GHR was also not observed.

In our cohort of acromegaly patients, the d3-GHR polymorphism was observed in 49% of the patients and followed the Hardy-Weinberg equilibrium. This demonstrates the absence of a selection benefit in our acromegaly cohort using combination therapy. Acromegaly cohorts in the studies of Bernabeu *et al.* and Filopanti *et al.* did not follow the Hardy-Weinberg equilibrium. The latter study suggested an association between d3-GHR genotype and a more severe phenotype of acromegaly patients.

In our study, we measured PEGV serum levels to investigate whether a discrepancy might exist between the PEGV dose and the serum levels of PEGV between the genotypes. For both PEGV serum levels and PEGV doses, we could not detect a significant difference between the two genotypes. PEGV serum levels of 0 µg/l (n=5) measured during PEGV treatment were left out of the analysis. These undetectable PEGV serum levels could be explained by a false negative error of the assay, noncompliance of the patient in taking the drug or the absence of PEGV due to the half-life of the drug ($T_{1/2el}$=74-172 h (27)). Although the half-life of the drug is probably increased during combination treatment, since PEGV serum levels increase by 20% (24,28), in our opinion it is the loss of PEGV from the circulation that is the most likely explanation for the undetectable PEGV serum levels. The majority of patients with undetectable PEGV serum levels were using a low PEGV dose, between 10 and 60 mg once weekly.

Apart from differences in sample sizes, the discrepancies between our data and those previously published could be due to an IGF-I-independent pathway. Binder *et al.* (25,26) published two studies on the use of recombinant human GH (rhGH) in children short for gestational age with remarkable outcomes. They observed a higher growth velocity in d3-GHR carriers during rhGH therapy, although this was not reflected by increases in IGF-I levels, and may allow cautious speculation about an IGF-I-independent pathway. If this perspective held true, our study and previous research may not have been suitable to assess the influence of d3-GHR genotype on IGF-I levels during PEGV treatment in acromegaly patients.

We could not find a significant difference in baseline characteristics, clinical or biochemical response between fl-GHR and d3-GHR carriers. However, we did observe a difference between the decrease in adenoma volume during treatment between patients with fl-GHR genotype and the d3-carriers. An explanation for the higher decrease in the fl-GHR group is not readily available as PEGV dose and PEGV

serum levels were not significantly different between carriers and non-carriers. Moreover, Veldhuis *et al.* (29) observed that PEGV does not cross the human blood-brain barrier. However, many areas of the hypothalamus and the pituitary lack a blood-brain barrier (30,31). A direct effect of PEGV on the difference between fl-GHR and d3-GHR genotype in tumor shrinkage is thus difficult to estimate. LA-SSAs are more likely to have influence on the different rates of tumor shrinkage between the two genotypes. A meta-analysis by Giustina *et al.* (32) reported that LA-SSAs induced relevant tumor shrinkage in more than half of the acromegaly patients studied. Predictors of tumor shrinkage by LA-SSAs are age, decrease of GH and IGF-I levels, treatment-naive patients and duration of LA-SSA-treatment (32,33,34). Age at diagnosis, age at start of PEGV, decrease of IGF-I levels during treatment and previous therapy (such as neurosurgery and radiotherapy) were not significantly different in our study between fl-GHR and d3-GHR genotype. The other studies about d3-GHR and PEGV treatment (Bianchi, Bernabeu and Filopanti) did not describe tumor shrinkage (5,6,10). Bernabeu *et al.* did report two cases of tumor increase during PEGV monotherapy, but genotypes were not specified. Filopanti *et al.* reported four cases of tumor increase in the PEGV monotherapy group, of which one patient had the fl-GHR genotype and three patients were carriers of d3-GHR. In a single-center study in Mexico City (11), the authors observed more severe disease activity with lower efficacy of treatment (radiotherapy, surgery and/or pharmacological therapy) in acromegaly subjects with the d3-GHR genotype. Therefore, it might be expected that carriers of the d3-GHR have less tumor regression, since biochemical response and tumor regression seem to be linked.

CONCLUSION

The clinical data in our study do not support a role for GHR genotype in the treatment response to PEGV combined with LA-SSA in patients with acromegaly. Our observation that the reduction in pituitary tumor volume during combination therapy was smaller in d3-carriers was unexpected and merits further attention.

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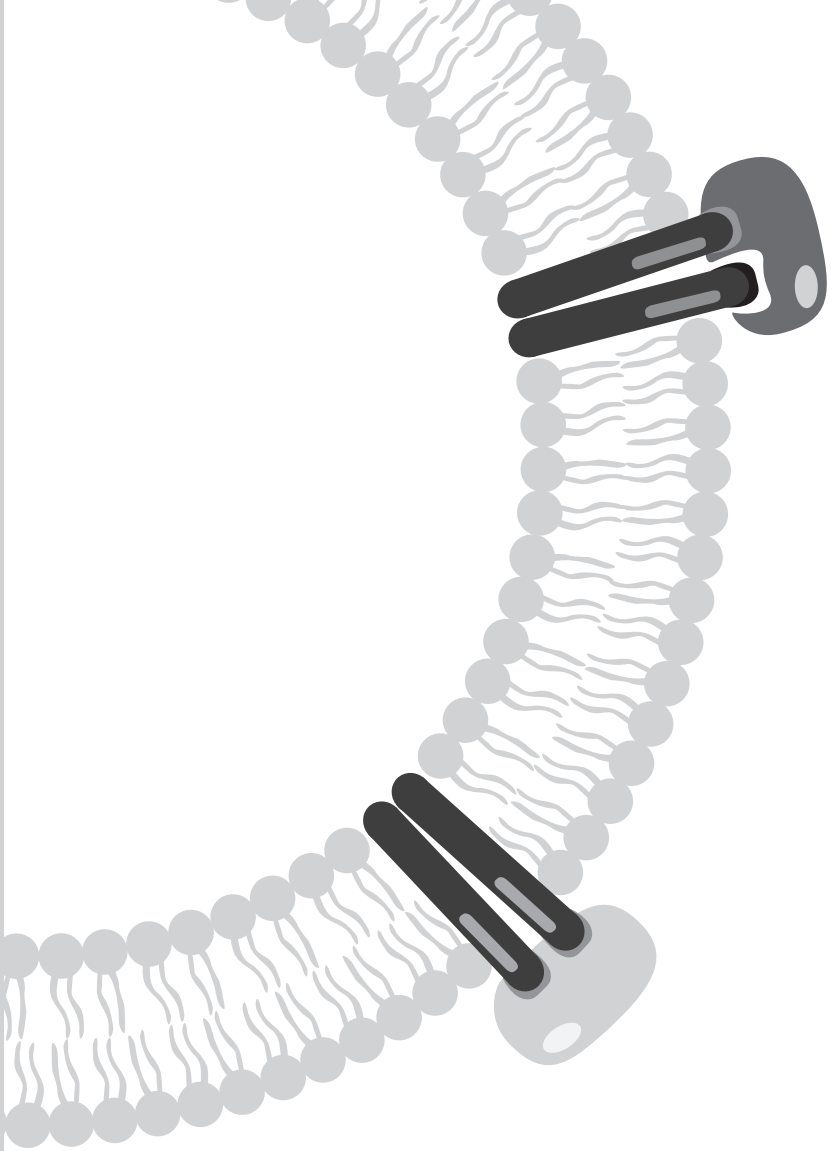

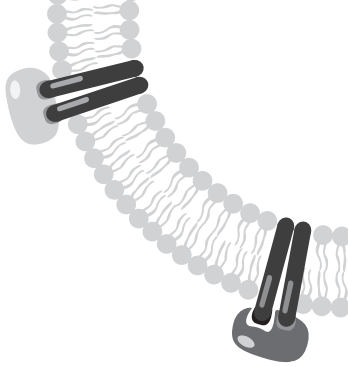

Chapter 5

The effect of the Exon 3-Deleted Growth Hormone Receptor in Pegvisomant-Treated Acromegaly: a Systematic Review and Meta-Analysis

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ABSTRACT

Background: The common exon 3 deletion polymorphism of the growth hormone receptor (d3-GHR) is associated with disease severity in acromegaly patients. The GHR antagonist pegvisomant (PEGV) is highly effective in treating severe acromegaly. Response to PEGV treatment seems to be influenced by d3-GHR and appears to be more responsive to PEGV, although available results remain conflicting.

Objective: To assess the influence of d3-GHR on the responsiveness of acromegaly patients to PEGV by compiling the evidence derived from the largest available studies.

Design: A systematic review of the literature identified three published studies and one conference abstract. Acromegaly patients (n=324, 49.7% d3-GHR carriers) were treated with either PEGV monotherapy or PEGV combined with long-acting somatostatin analogues (LA-SSA) and/or cabergoline. A meta-analysis of raw data from these studies was performed.

Results: No significant effect of the d3-GHR was observed while bringing insulin-like growth factor I (IGF-I) levels below the upper limit of normal with PEGV, which was defined as the lowest IGF-I level during PEGV-treatment (mean difference: -2.3%; 95% CI: -6.5 – 1.8%, p=0.270). The PEGV dose required to achieve the lowest IGF-I levels was also not significantly influenced by individuals carrying d3-GHR (mean difference: 4.1 mg/week; 95% CI: -5.1 – 13.2, p=0.385). For both outcomes, separate analysis of PEGV monotherapy and combination treatment gave similar results.

Conclusions: Our findings suggest that the d3-GHR polymorphism has no effect on biochemical disease control in acromegaly, as it is not of added value for either the prediction of PEGV responsiveness or the determination of the required PEGV dose.

INTRODUCTION

Acromegaly is a rare disease characterized by excessive secretion of growth hormone (GH) resulting in a diversified clinical presentation. The disease is almost exclusively caused by a GH-secreting pituitary adenoma (1). These elevated GH levels subsequently increase IGF-I production, predominantly by the liver, although other tissues also synthesize IGF-I (2). If untreated, the disease is associated with an increase in morbidity and mortality (2). Control of disease activity results in mortality rates similar to the general population (3). Although transsphenoidal surgery remains the first line treatment in most countries (4), it is often unsuccessful for macroadenomas, making additional treatment modalities necessary when GH and IGF-I levels remain elevated. However, primary medical treatment is becoming more and more popular, starting with LA-SSAs with an average efficacy rate in normalizing GH and IGF-I levels in treatment naive patients of 44% (5). A highly effective alternative for patients who are not normalized by LA-SSA monotherapy is the addition of PEGV to LA-SSA, or even PEGV monotherapy, provided that the appropriate PEGV dose is used (6-8).

PEGV is a PEGylated recombinant GH analogue that acts as a competitive GH receptor antagonist in all tissues except the brain, most importantly suppressing GH-dependent production of IGF-I by the liver (9). The PEGV dose required for normalization of IGF-I levels in acromegaly is variable, depending on disease activity and individual response to the drug (6,10). Likewise, a wide inter-individual variation in PEGV serum levels is observed despite identical PEGV dosing (11,12). These differences in individual responses have been partly attributed to a common polymorphism in the GH receptor gene characterized by deletion of exon 3. This in-frame deletion causes loss of 22 amino acids from the extracellular domain. In about half of the general population the polymorphism is homozygous for the full-length GHR (fl/fl-GHR), with the remaining half carrying the d3-GHR polymorphism; 30-40% being heterozygous and 10-20% homozygous for this deletion (13-15). A similar distribution of this GHR variant in cohorts of acromegaly patients has been described in the literature (16-20).

The deletion of exon 3 in GHR is caused by retrovirus-mediated alternative splicing, which results in skipping of coding exons (15). This alternative splicing pattern is human-specific (15). Evolutionary conservation of this GHR variant suggests beneficial effects. Transfections experiments by Dos Santos *et al.* have shown that the lack of exon 3 in the GHR enhances GH signal transduction by approximately 30% (14). More specifically, the deletion of exon 3 leads to greater stimulation of the intracellular JAK-STAT pathway in response to GH, which results in increased transcription of GH-target-genes. Following the report of Dos Santos *et al.*, several studies primarily focused on assessing the role of the d3-GHR polymorphism during

recombinant GH treatment of GH-deficient and non-GH-deficient pre-pubertal children with short stature. Carrying one or more d3-GHR alleles was found to be associated with increased baseline height and growth response to GH, according to a meta-analysis by Wassenaar *et al.* (21). Thereafter, subsequent studies evaluated the influence of d3-GHR on the severity of acromegaly.

Several studies have addressed the influence of the d3-GHR polymorphism on GH and IGF-I levels. The first study included 44 untreated active acromegaly patients, in which a higher baseline GH was observed in d3-GHR carriers whereas IGF-I levels were similar across the three genotypes (22). However, a more recent study in 105 patients with untreated acromegaly could not confirm these findings (18). The impact of the GHR variant on comorbidities was assessed in 86 acromegaly patients during long-term disease control (23). The presence of d3-GHR was associated with an increased prevalence of irreversible long-term complications, such as osteoarthritis, dolichocolon, and adenomatous colonic polyps. However, d3-GHR was not associated with other comorbidities such as metabolic syndrome, diabetes mellitus type II, and vertebral and non-vertebral fractures. A recent Turkish study (n=118) observed no effect of the GHR variant on either clinical features nor comorbidities, but suggested that the polymorphism might play a role in GH/IGF-I level discordance. Posttreatment biochemical characteristics were also assessed by an Italian cohort study, suggesting that more discordant GH/IGF-I levels (high IGF-I and GH ≤2 ng/ml) were observed in d3-GHR carriers, and that this discordance in levels was enhanced after initiation of somatostatin analogue treatment (16).

A previously cited meta-analysis observed an association with increased growth velocity in recombinant human (rh)GH treated GH-deficient children carrying d3-GHR (21). Subsequently, the question emerged whether d3-GHR influences pharmacodynamics of PEGV in acromegaly as carriers of d3-GHR might need less PEGV to normalize IGF-I levels than patients with the fl/fl-GHR genotype in order to normalize IGF-I levels. PEGV directly antagonizes the GHR, and therefore could have a greater impact on exon 3-deleted GHRs. Two studies, indeed, reported a lower required PEGV dose during disease control in acromegaly patients with the d3-GHR genotype (16,24). However, more recent studies in larger acromegaly cohorts could not confirm these findings (25,26). These contradictory reports on the influence of d3-GHR in acromegaly patients regarding PEGV-treatment responses and the PEGV doses required to normalize IGF-I levels, motivated us to conduct a systematic review of the literature to identify studies examining this question and to perform a meta-analysis. The aim of this study is to address the clinically-relevant question: Do clinicians have to take d3-GHR genotyping into account during PEGV dosing?

MATERIALS & METHODS

Inclusion criteria

The two main outcome parameters used by us were; 1) Lowest IGF-I level expressed as upper limit of normal (xULN) during PEGV-treatment and; 2) the required PEGV-dose to achieve the lowest IGF-I level. Studies reporting these main outcomes in acromegaly cohorts concerning the influence of d3-GHR were included. In these studies the exon 3-deleted GHR polymorphism has been reported as fl/fl-GHR, d3/fl-GHR, d3/d3-GHR, and/or d3-GHR, in which d3-GHR could be a combination of the d3/fl-GHR and d3/d3-GHR genotype.

Search strategy

The online literature databases: Embase.com, Medline (OvidSP), Pubmed Publisher, Web of Science, Google Scholar, and the Cochrane Library were used for this systemic search of studies reporting the influence of d3-GHR on the outcome in response to PEGV treatment in acromegaly patients. This was performed under the guidance of a research librarian. The performed search strategy was: (d3GHR OR d3-GHR OR "d3-growth hormone receptor" OR "exon 3" OR d3 OR "exon 3-deleted" OR "exon 3 deletion") AND (GHR OR "GH receptor" OR "growth hormone receptor" OR GHRs OR "GH receptors" OR "growth hormone receptors") AND (Polymorphism OR polymorphisms OR isoform OR isoforms OR genotype OR genotypes OR variant OR variants) AND Acromegaly AND (Pegvisomant OR somavert OR "growth hormone receptor antagonist" OR "GH receptor antagonist" OR "GHR antagonist"). The searches were performed on the 9th of September 2014. Thereafter, the references of relevant articles were revised for additional studies.

Data review and data collection

Data selection was independently assessed by the investigators S.E. Franck and S.J.C.M.M. Neggers. Besides our own cohort, two articles and one conference abstract met our selection criteria. The study of Bianchi *et al.* from 2009 (n=19) was excluded from this meta-analysis as the vast majority of the patients was also included in a larger cohort (n=127) published by the same author-group in 2012 (16, 25). We contacted the principal investigators of these three research groups, in order to collect raw data from these acromegaly cohorts. We were able to obtain from all three selected articles the variables needed to perform the meta-analysis: genotype coded as fl/fl, d3/fl or d3/d3, sex, age at diagnosis, PEGV monotherapy vs. PEGV combined with LA-SSA and/or cabergoline (CAB) (combination treatment), lowest IGF-I levels during PEGV treatment and required PEGV-dose to achieve this lowest IGF-I level. In total 135 patients were treated with PEGV monotherapy and

189 with combination treatment. Medical ethics committees from each hospital approved the protocol, and a written informed consent was obtained from all patients. The paragraph 'Included study characteristics' in the results section describes this approach in more detail.

Statistical analysis

Raw data from each cohort was used to calculate betas (βs) and standard errors (SEs) by linear regression analysis. βs and SEs were calculated per cohort and medication group (PEGV monotherapy, combination treatment and total cohort: monotherapy + combination treatment). The variable lowest IGF-I during PEGV treatment was corrected for sex, age and required PEGV-dose to achieve the lowest IGF-I level. This latter variable itself was corrected for sex and age, when used for meta-analysis individually. Hardy-Weinberg equilibrium was analyzed with the χ^2-test via the observed and expected genotype frequencies.

Statistical analyses were performed with SPSS version 20 (SPSS software, Chicago, IL, USA) and GraphPad Prism version 6 for Windows (GraphPad Software, San Diego, CA, USA). Potential effects of the genotype GHR deletion of exon 3 were calculated by an inverse-variance meta-analyses in R, version 3.2.1 (27). Fixed effects meta-analyses were performed as implemented in the R package 'rmeta' (28). As we test two independent medical treatment variants (PEGV monotherapy and combination treatment) as well as the total cohort, we set the significance threshold of our one-sided p-value's at 0.025 in order to correct for multiple testing (Bonferroni correction).

RESULTS

Literature search

We identified 511 potentially relevant studies by a literature search in Embase, Medline, Pubmed, Web of Science, Google Scholar, and the Cochrane Library, depicted in Figure 1. Of these studies 497 were found not to meet the inclusion criteria on the basis of title and abstract. Fourteen papers were relevant for more detailed examination, of which nine were excluded for different reasons: four papers presented a description/citation of an included study in our meta-analysis in a book, three papers presented an abstract of an included study in our meta-analysis, and two studies did not report original data. One original study (16) was excluded as the majority of the patients were included in a larger more recent study, which was already included in this meta-analysis (25). Finally, we included three published original studies and one conference abstract describing one relevant acromegaly cohort (24-26,29). All four selected papers included

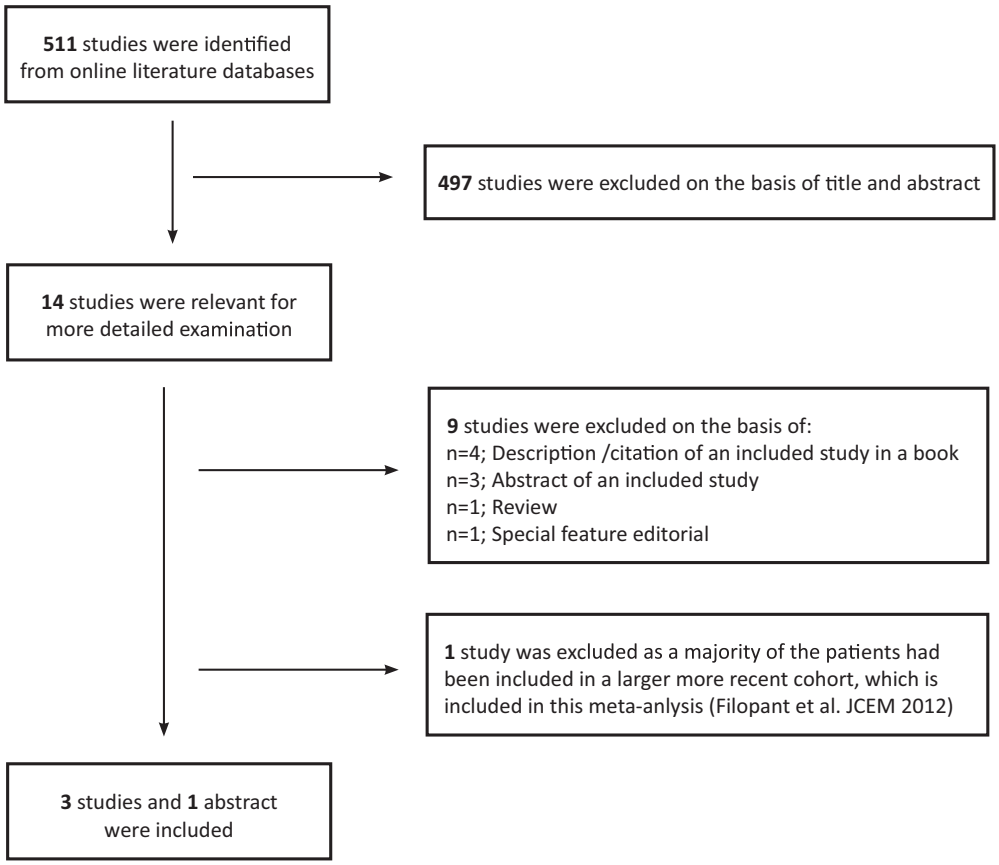

Figure 1. Flow diagram of study selection and exclusion stages

data of both requested outcomes; 1) Lowest IGF-I level expressed as ULN during PEGV-treatment and; 2) the required PEGV-dose to achieve the lowest IGF-I level.

Included study characteristics

Characteristics of the four included studies are summarized in Table 1. The reports were published between 2010 and 2015, and the range of the number of included patients varied between 44 and 127. All reports included only acromegaly patients with elevated IGF-I levels after at least 6 months of the maximum tolerated dose of LA-SSA. Kamenický *et al.* included 53 patients, of which one patient was excluded because LA-SSA pre-treatment was not administered, and GHR genotype was not available in three patients, resulting in 49 acromegaly patients relevant for analysis (29).

Table 1. Characteristics of included cohorts

First author, year [REF]	n	males – %	age – years	GHR genotype (%) fl/fl	d3/fl	d3/d3		Effect of d3-GHR
Bernabeu 2010 [24]	44	40.9	46 [36 – 56]	40.9	56.8	2.3	Quantitative PCR	
Normalization of IGF-I during PEGV								No effect
Months to normalization of IGF-I								Effect: ↓ months
Required PEGV dose								Effect: ↓ PEGV dose
Kamenický 2011 [29]	49	57.1	36 [26 – 48]	49.0	26.5	24.5	Multiplex PCR	
Required PEGV dose								No effect
Filopanti 2012 [25]	127	57.5	42 [32 – 50]	53.5	32.3	14.2	Multiplex PCR	
IGF-I SDS during PEGV								No effect
Required PEGV dose								No effect
Franck 2015 [26]	104	58.7	45 [36 – 56]	51.0	41.3	7.7	Quantitative PCR	
IGF-I xULN during PEGV								No effect
Required PEGV dose								No effect

Description of patient characteristics of the included studies and the effect of d3-GHR on IGF-I during PEGV treatment and required PEGV dose. Effect indicates p=≤0.05; no effect indicates p=>0.05; The required PEGV dose is provided to normalize IGF-I levels. Age was noted at diagnosis and expressed as median [interquartile range].

REF: reference, GHR: growth hormone receptor, fl: full-length, d3: deletion of exon 3, n: number of patients, IGF-I: insulin-like growth factor I, SDS: standard deviation score, ULN: upper limit of normal, PEGV: pegvisomant, PCR: polymerase chain reaction.

In total 135 patients were treated with PEGV monotherapy and 189 with combination treatment (177 PEGV + LA-SSA, 8 PEGV + CAB, 4 PEGV + LA-SSA + CAB). Treatment modalities were different per study. Bernabeu *et al.* (n=44) and Kamenický *et al.* (n=49) predominantly included patients treated with PEGV monotherapy (Bernabeu: 29 PEGV alone; 7 PEGV + LA-SSA; 7 PEGV + CAB; 1 PEGV + LA-SSA + CAB, Kamenický: 42 PEGV alone; 3 PEGV + LA-SSA; 1 PEGV + CAB; 3 PEGV + LA-SSA + CAB). Filopanti *et al.* (n=127) included an almost equal number of patients treated with PEGV monotherapy and combination treatment (64 PEGV alone, 63 PEGV + LA-SSA). Franck *et al.* (n=104) included only patients treated with LA-SSA in combination with PEGV.

The frequency distribution of the d3-GHR genotype showed some variation between studies and ranged between 46.5% in the cohort of Filopanti *et al.* to 59.1% in the cohort of Bernabeu et al, shown in Table 1. The total sample of this meta-analysis included the following genotypes: fl/fl: 163 (50.3%); d3/fl: 122 (37.7%); d3/d3: 39 (12.0%). With the exception of Franck *et al.* (p=0.859), none of the included cohorts had genotype frequencies following Hardy-Weinberg equilibrium (HWE) proportions. When all cohorts (n=324) were pooled together the genotype distribution was not in accordance with HWE (p=0.034).

In one of the four studies, an effect of d3-GHR was observed on the duration of successful PEGV treatment, as carriers required less time to reach IGF-I normalization (24). However, no difference was observed in genotype distribution between normalized and non-normalized patients regarding IGF-I levels during PEGV treatment. Meanwhile, the required PEGV dose per kg bodyweight was approximately 20% lower in d3-GHR carriers (24). The other three studies did not report an influence of the genotype on PEGV-treated acromegaly patients regarding either IGF-I levels or the required PEGV dose.

META-ANALYSIS

Lowest IGF-I during PEGV treatment

The effects of d3-GHR on the lowest IGF-I during PEGV treatment could be assessed in all four cohorts using the raw data sets obtained from the principal investigators and the individual effects of the studies are summarized in Table 2. The mean difference in lowest IGF-I (xULN) between d3-GHR carriers and fl/fl-GHR in the total cohort was -2.3% [95% CI: -6.5 – 1.8%], which reflects a small negative effect in d3-GHR carriers when compared with fl/fl-GHR in combined data from the four studies. However, this effect was not significant (p=0.270, heterogeneity p=0.535, Figure 2). Similar results were observed when the total cohort was subdivided into patients using either PEGV monotherapy or combination treatment and analyses were performed separately (PEGV monotherapy; mean difference: -0.2%, 95% CI: -0.07 – 0.07, p=0.961 and combination treatment; mean difference: -2.2%, 95% CI: -0.07 – 0.03, p=0.417; heterogeneity both not significant).

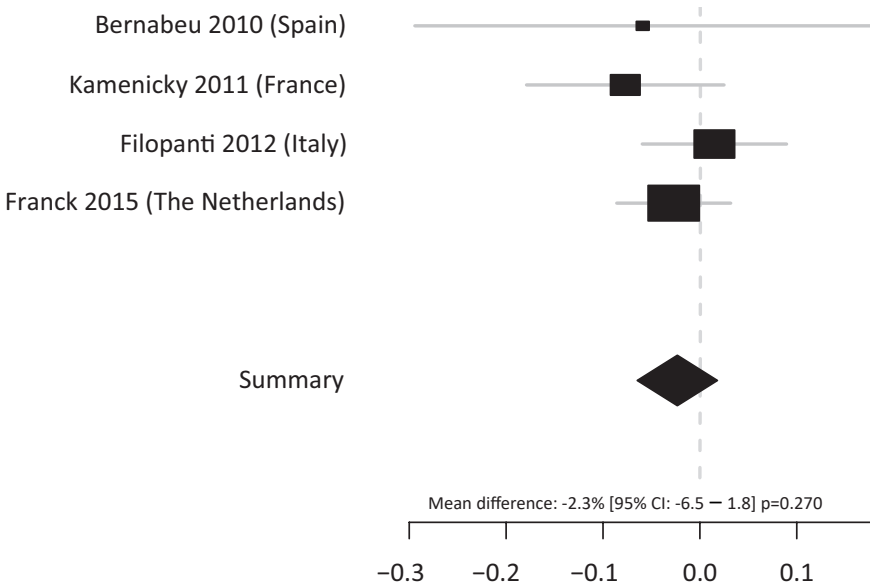

Figure 2. Forest plot of meta-analysis: effect of d3-GHR on IGF-I during PEGV

The summary effect of d3-GHR genotype with respect to fl/fl-GHR on lowest IGF-I xULN during PEGV treatment in patients treated with PEGV alone or in combination with LA-SSA and/or CAB (n=43) has a mean difference of -2.3% relative to the ULN of IGF-I [95% CI: -6.5 – 1.8%], p=0.270.

GHR: growth hormone receptor, fl: full-length, d3: deletion of exon 3, IGF-I: insulin-like growth factor I, ULN: upper limit of normal, PEGV: pegvisomant, LA-SSA: long-acting somatostatin analogues, CAB: cabergoline, CI: confidence interval.

Table 2. Effect of d3-GHR on the lowest IGF-I xULN during PEGV treatment

First author, year	β of ULN	SE of β	p-value	Heterogeneity p-value
PEGV monotherapy				
Bernabeu 2010	0.017	0.147	0.907	
Kamenický 2011	-0.052	0.060	0.393	
Filopanti 2012	0.026	0.046	0.576	
Meta-analysis PEGV monotherapy	-0.002	0.035	0.961	0.582
Combination treatment				
Bernabeu 2010	-0.193	0.309	0.547	
Kamenický 2011	-0.185	0.193	0.439	
Filopanti 2012	0.025	0.062	0.684	
Franck 2015	-0.027	0.030	0.371	
Meta-analysis combination treatment	-0.022	0.027	0.417	0.654
Total cohort				
Bernabeu 2010	-0.059	0.120	0.626	
Kamenický 2011	-0.077	0.052	0.145	
Filopanti 2012	0.015	0.038	0.702	
Franck 2015	-0.027	0.030	0.371	
Meta-analysis total cohort	-0.023	0.021	0.270	0.535

Meta-analyses of the effect of d3-GHR vs. fl-GHR on lowest IGF-I xULN during PEGV treatment in patients treated with PEGV alone, in combination with LA-SSA and/or CAB and in the total cohort (PEGV monotherapy + combination treatment). βs and SEs are corrected for sex, age at diagnosis and required PEGV dose. Effect indicates p≤0.025; no effect indicates p≥0.025;
GHR: growth hormone receptor, fl: full-length, d3: deletion of exon 3, IGF-I: insulin-like growth factor I, SE: standard error, ULN: upper limit of normal, PEGV: pegvisomant, β: beta, SE: standard error.

Required PEGV dose to achieve the lowest IGF-I levels

The effect of d3-GHR on the required PEGV dose needed for the lowest IGF-I level during treatment and the individual effects of the studies are summarized in Table 3. The mean difference in required PEGV dose was 4.1 mg/week [95% CI: -5.1 – 13.2 mg/week], which suggests a small positive effect in d3-GHR carriers when compared with fl/fl-GHR in the total cohort from the four studies, however this effect was not significant (p=0.385, heterogeneity p=0.535, Figure 3). Similar results were observed when the total cohort was subdivided in patients using PEGV monotherapy or in combination with LA-SSA and analyses were perform separately

Table 3. Effect of d3-GHR on the required PEGV dose

First author, year	β of ULN	SE of β	p-value	Heterogeneity p-value
PEGV monotherapy				
Bernabeu 2010	21.880	15.523	0.171	
Kamenický 2011	-22.323	19.305	0.255	
Filopanti 2012	9.213	7.424	0.219	
Meta-analysis PEGV monotherapy	7.930	6.327	0.210	0.193
Combination treatment				
Bernabeu 2010	-73.166	26.187	0.017	
Kamenický 2011	-39.367	106.970	0.737	
Filopanti 2012	3.061	10.123	0.763	
Franck 2015	9.440	9.200	0.312	
Meta-analysis combination treatment	1.350	6.577	0.837	0.029
Total cohort				
Bernabeu 2010	-10.624	15.288	0.419	
Kamenický 2011	-19.448	18.215	0.291	
Filopanti 2012	6.696	6.127	0.277	
Franck 2015	9.440	9.200	0.312	
Meta-analysis total cohort	4.060	4.675	0.385	0.374

Meta-analyses of the effect of d3-GHR vs. fl-GHR on the required PEGV dose needed to achieve normalization of IGF-I levels in patients treated with PEGV alone, in combination with LA-SSA and/or CAB and in the total cohort (PEGV monotherapy + combination treatment). βs and SEs are corrected for sex and age. Effect indicates p≤0.025; no effect indicates p≥0.025.
GHR: growth hormone receptor, fl: full-length, d3: deletion of exon 3, IGF-I: insulin-like growth factor I, SE: standard error, ULN: upper limit of normal, PEGV: pegvisomant, β: beta, SE: standard error.

(PEGV monotherapy; mean difference: 7.9 mg/week, 95% CI: -4.5 – 20.3, p=0.210 and combination treatment; mean difference: 1.4 mg/week, 95% CI: -11.5 – 14.2, p=0.837; heterogeneity both not significant). In a multivariate linear regression model, the required PEGV dose was not different between the three genotypes with adjustment for sex, age and the different cohorts included in this meta-analysis (β=-0.5, SE=5.2, p=0.923). When the total cohort was subdivided in monotherapy PEGV or combination treatment with LA-SSA and separately analyzed for the required PEGV dose, the linear multivariate regression model showed similar results.

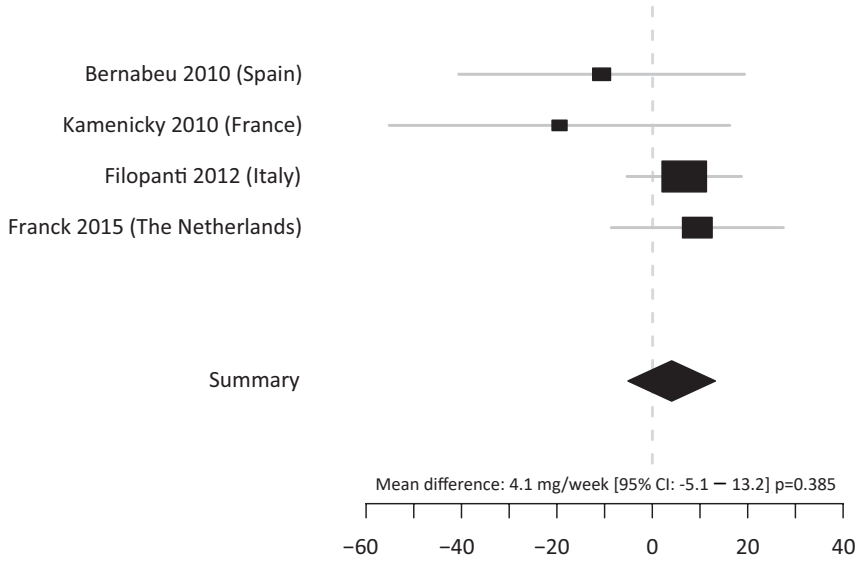

Figure 3. Forest plot of meta-analysis: effect of d3-GHR on the required PEGV dose
The summary effect of d3-GHR genotype with respect to fl/fl-GHR on the required PEGV dose needed to achieve normalization of IGF-I levels in patients treated with PEGV alone or in combination with LA-SSA and/or CAB (n=43) has a mean difference of 4.1 mg/week [95% CI: -5.1 – 13.2], p=0.385].
GHR: growth hormone receptor, fl: full-length, d3: deletion of exon 3, IGF-I: insulin-like growth factor I, ULN: upper limit of normal, PEGV: pegvisomant, LA-SSA: long-acting somatostatin analogues, CAB: cabergoline, CI: confidence interval.

DISCUSSION

The aim of this meta-analysis was to answer the question whether clinicians should take into account d3-GHR genotyping during PEGV prescription, as previous studies of its function are contradictory. Our results indicate that d3-GHR does not influence the pharmacodynamics of PEGV in acromegaly, at least not clinically-relevant. No effect was observed concerning the response of PEGV treatment as the mean change in IGF-I levels was not significantly different between the GHR genotypes. Furthermore, the required PEGV dose to achieve normalization of IGF-I levels did not differ between carriers of the d3-GHR and the fl/fl-GHR genotypes.

The first two published studies reporting the effect of d3-GHR genotype on PEGV-pharmacodynamics in acromegaly observed beneficial effects regarding d3-GHR carriers. An Italian study (n=19) observed lower IGF-I levels in d3-GHR carriers compared to those with the fl/fl-GHR genotype after 3 and 6 months of PEGV (16). However, after 12 months of PEGV treatment this difference was lost, although the final PEGV dose was significantly lower in d3-GHR carriers compared to the fl/fl-GHR genotype. Similar results were observed in a Spanish cohort (n=44); no difference was observed in genotype distribution between IGF-I controlled and non-controlled patients, but the required PEGV dose per kilogram of weight to normalize IGF-I levels was 20% lower (p=0.033) in patients with the d3-GHR polymorphism compared with the fl/fl-GHR genotype (24). The previous 19 Italian patients were later included in a larger cohort (n=127) in which the same research group reported that they could not confirm their previous results (25). Similarly, we did not observe a difference between the two genotypes regarding IGF-I levels during PEGV treatment. Concerning PEGV dosing, we only observed a significant difference in a subset of the patients undergoing combination treatment (n=15, Table 3). This effect was not observed in patients receiving PEGV monotherapy (n=29) or in the total cohort (monotherapy and combination treatment tested together). More recent studies, reporting larger cohorts (n=49, n=127, n=104), were also unable to confirm the beneficial effects on PEGV-pharmacodynamics in acromegaly patients carrying the d3-GHR polymorphism (25, 26, 29). This phenomenon could be due to the presence of a publication bias in the beginning of this publication series about the effect of d3-GHR in acromegaly, the so-called ‘winner’s curse’.

This meta-analysis included 324 acromegaly patients, the d3-GHR polymorphism was observed in 161 (49.7%) of the patients, of which 122 (37.7%) were heterozygous and 39 (12.0%) were homozygous. As previously mentioned, half of the general population is homozygous for the fl/fl-GHR; 30-40% is heterozygous for d3-GHR and 10-20% is homozygous for this deletion (13-15). A similar distribution of the d3-GHR polymorphism is reported in several acromegaly cohorts (16-20), including the total cohort in this meta-analysis, although the distribution was not

in accordance with HWE. When HWE was tested in the individual cohorts, only the Dutch cohort was in HWE. An explanation for this deviation from HWE in the smaller cohorts may indicate a possible random genetic drift related to the small sample size. The smaller French cohort (18) (n=49) has an increased prevalence of the d3/d3 genotype and a lower prevalence of the d3/fl genotype. When three d3/3 patients are shifted to the d3/fl genotype the distribution is in HWE. Interestingly, when only these three French patients are shifted, the total cohort of 324 patients is also in accordance with the HWE. These three patients from a smaller cohort, that could explain the disbalance in the HWE distribution, are not expected to affect the results of this meta-analysis. In the larger cohort of Filopanti *et al.* the deviation from HWE could be explained by a genotyping error, however this seems to be unlikely as these data were obtained following the same methods and by the same laboratory analysts that found consistent distributions of the d3-GHR genotype following HWE in healthy individuals and several series of acromegaly patients (20), which was also additionally monitored and confirmed by an independent staff.

Since acromegaly is a rare disease and patients treated with PEGV are limited, we could only include a relatively small number of large studies in this meta-analysis. To date however, this is the largest dataset available for this specific group of acromegaly patients. Furthermore, GHR genotyping was done locally in several laboratories using multiplex and quantitative PCR techniques. Similarly, different assays were used to measure IGF-I levels, and therefore it was chosen to express the IGF-I level as the upper limit of normal. In addition, a comparison between fl/fl-GHR and d3-GHR regarding baseline GH and IGF-I levels in untreated acromegaly patients is missing, which would have given a more complete overview. Especially, as there are data reporting an association between d3-GHR and discordant GH and IGF-I levels (high IGF-I vs. normal GH) (22,30), however conflicting data regarding this topic are also reported (31). Despite these limitations, we were able to obtain all the available raw data from the studies derived from our systematic literature search on d3-GHR and PEGV treatment. Moreover, data were all collected using similar definitions, as response to PEGV treatment was objectified by the lowest IGF-I level during the PEGV treatment period, as was the associated required PEGV dose linked to these IGF-I levels. Furthermore, PEGV monotherapy and combination therapies showed similar results for both the response to PEGV treatment and the required PEGV dose. If d3-GHR carriers were to exhibit greater biological activity of GH, a dose effect for PEGV per haplotype is also to be expected. In this meta-analysis, we aimed to evaluate a dose effect regarding the required PEGV dose in a pairwise comparison between the three genotypes. No difference in PEGV dose was observed between the three genotypes either in the PEGV monotherapy or in the combination treatment group, making it highly unlikely that there is a clinically-relevant effect of d3-GHR on the pharmacodynamics of PEGV in acromegaly. This

outcome leaves room for discussion about the effect of the d3-GHR polymorphism in healthy individuals. D3-GHR carriers present in the general population, should either maintain normal GH activity despite less circulating GH, and therefore have similar GH end-targets such as metabolic state, body composition and final height or exhibit an increase in these GH end-targets. In this respect, it is interesting to note that genome-wide association studies on final height (32,33) and metabolic state (34) did not report any association with the GHR locus in healthy individuals, corroborating our findings. In summary, we believe that our study demonstrates that the presence of the d3-GHR genotype in acromegaly patients has no impact on clinical practice. Moreover, we are convinced that this meta-analysis provides us a final conclusion regarding the d3-GHR polymorphism and its lack of effect on PEGV response and dosing in acromegaly.

CONCLUSION

In our meta-analysis of a combined group of 324 acromegaly patients obtained from four separate study cohorts, the presence of one or two copies of the d3-GHR polymorphism had no significant effect on the lowest IGF-I levels during PEGV treatment nor on the required PEGV dose to achieve these levels. Similarly, no difference between subgroups of subjects that used PEGV either as monotherapy or in combination with LA-SSAs were observed. Our results indicate that there is no evidence supporting a role for the d3-GHR polymorphism in either predicting responses to PEGV therapy or determining PEGV dosing during treatment of acromegaly.

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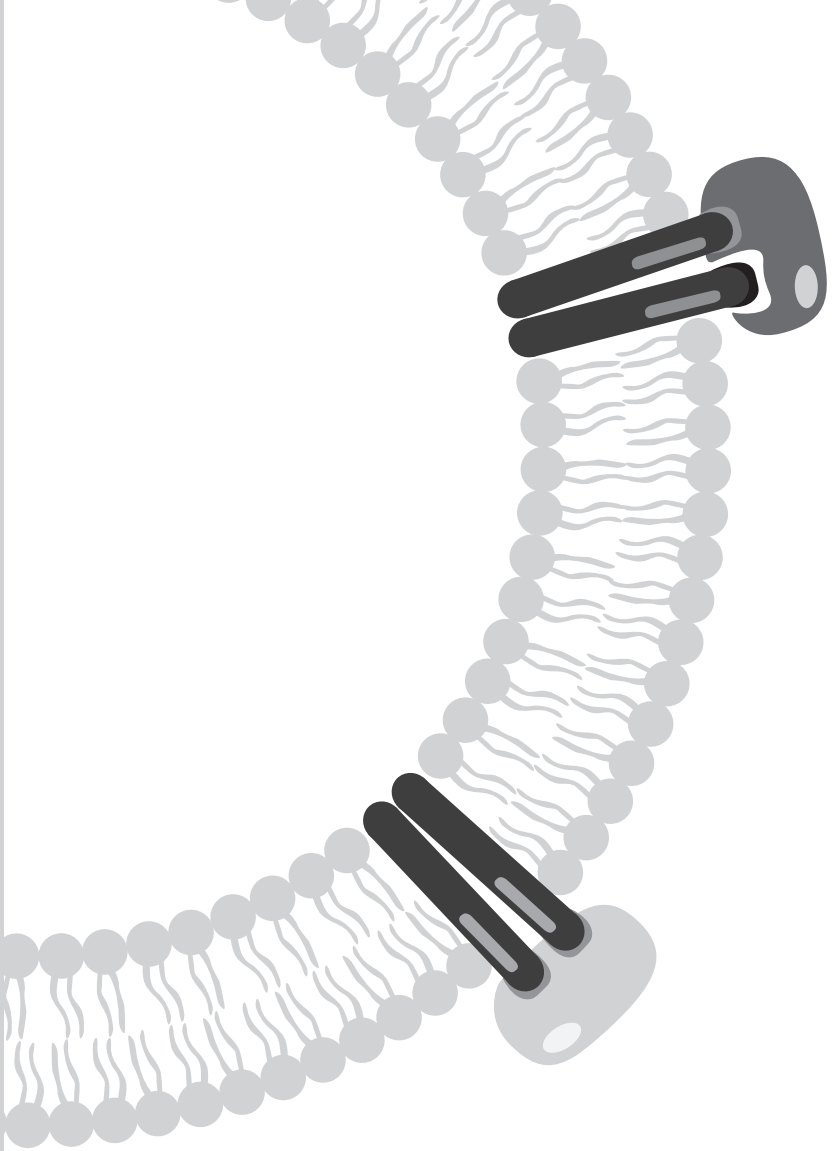

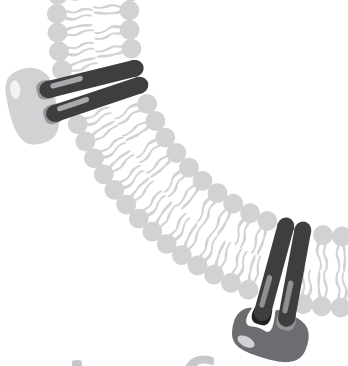

Chapter 6

A Multivariable Prediction Model for Pegvisomant Dosing: Monotherapy and in Combination with Long-Acting Somatostatin Analogues

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ABSTRACT

Background: Effective treatment of acromegaly with pegvisomant (PEGV), a growth hormone receptor antagonist, requires an appropriate dose titration. PEGV doses vary widely among individual patients, and various covariates may affect its dosing and pharmacokinetics.

Objective: To identify predictors of the PEGV dose required to normalize insulin-like growth factor I (IGF-I) levels during PEGV monotherapy and in combination with long-acting somatostatin analogues (LA-SSAs).

Design: Two retrospective cohorts (Rotterdam + Liège acromegaly survey (LAS), total n=188) were meta-analysed as a form of external replication to study the predictors of PEGV dosing in addition to LA-SSA, the LAS (n=83) was used to study the predictors of PEGV monotherapy dosing. Multivariable regression models were used to identify predictors of the PEGV dose required to normalize IGF-I levels.

Results: For PEGV dosing in combination with LA-SSA, IGF-I levels, weight, height and age, were associated with the PEGV normalization dosage ($p \leq 0.001$, $p \leq 0.001$, $p=0.028$ and $p=0.047$, respectively). Taken together, these characteristics predicted the PEGV normalization dose correctly in 63.3% of all patients within a range of $^{+}/_{-}$ 60 mg/week (21.3% within a range of $^{+}/_{-}$ 20 mg/week). For monotherapy, only weight was associated with the PEGV normalization dose ($p \leq 0.001$) and predicted this dosage correctly in 77.1% of all patients within a range of $^{+}/_{-}$ 60 mg/week (31.3% within a range of $^{+}/_{-}$ 20 mg/week).

Conclusions: In this study, we show that IGF-I levels, weight, height and age can contribute to define the optimal PEGV dose in order to normalize IGF-I levels in addition to LA-SSA. For PEGV monotherapy, only the patient's weight was associated with the IGF-I normalization PEGV dosage.

INTRODUCTION

Acromegaly is a rare disease caused by excessive secretion of growth hormone (GH), and a subsequent increase in IGF-I production (1). The disease is almost exclusively caused by a GH-secreting pituitary adenoma (2). Severity and phenotype of the disease varies among acromegaly patients. Uncontrolled acromegaly is associated with an increase in morbidity and mortality (1). The control of IGF-I levels results in mortality rates similar to the general population (3). Although often unsuccessful in macroadenomas, transsphenoidal surgery generally is considered as the first treatment modality (4,5). Additional treatment after surgery is necessary when GH and IGF-I levels remain uncontrolled. LA-SSAs, as adjuvant medical treatment or as primary medical treatment, are regularly prescribed. Several studies addressed the response of LA-SSA, and show that LA-SSA treatment alone reaches control of the disease in about 40% of the patients (6,7). A highly effective alternative for patients who are not normalized by LA-SSA monotherapy is the addition of PEGV to LA-SSA, or PEGV monotherapy, provided that the appropriate PEGV dose is given (8-12). PEGV is a PEGylated recombinant analogue of GH which competitively blocks the GH receptor, and thereby reduces the excessive GH actions in the liver and peripheral tissues (13,14). PEGV is slowly absorbed from the subcutaneous depot (T_{max} of 33-77 hours, $T_{1/2\ el}$ 74-172 hours) (15). The mode of PEGV-clearance is still not understood. We do not know whether the kidneys and/or the liver metabolizes the drug.

The dose of PEGV required to achieve disease control, defined as normalization of IGF-I levels, differs between individual acromegaly patients, both during PEGV monotherapy and in combination with LA-SSA (8,12). PEGV doses range widely between 20-200 mg/week during combination treatment with LA-SSA (16). A study by Freda *et al.* observed that patients using PEGV monotherapy in the ACROSTUDY™ with persistently elevated IGF-I levels needed a higher mean PEGV dosage (17). Defining the optimal starting dose for PEGV is difficult as the pharmacokinetics remain to be elucidated and data on pre-treatment determinants of the PEGV dosage required for biochemical disease control is sparse. Currently, IGF-I levels are most commonly used during PEGV titration, which is in line with a previous study from our group reporting a positive correlation between baseline IGF-I levels and the PEGV dose required for normalization of IGF-I during combination treatment of LA-SSA and PEGV (8,18). Other predictors that have been reported are GH levels, sex, body weight and previous radiotherapy (19,20). Two studies previously reported about a GH receptor polymorphism lacking exon 3, which seemed to have an influence as well during PEGV dosing (21,22). However more recent studies in larger acromegaly cohorts clearly state that this polymorphism has no clinical effect on the PEGV response nor the determination of the required PEGV dose (23-25).

Given the importance of swift biochemical control in acromegaly but the lack of studies investigating pre-treatment predictors we aimed to develop a multivariate regression model for predicting the required PEGV dose to achieve normalization of IGF-I levels in acromegaly patients.

MATERIALS & METHODS

Cohorts description

Patients (n=271) were included from two retrospective cohorts; 1) the Rotterdam cohort and; 2) the Liège acromegaly survey (LAS) cohort (26). The Rotterdam cohort contains data from acromegaly patients using LA-SSA in combination with PEGV (n=112) collected in the Pituitary Center Rotterdam between 2004 and 2013, previously published in 2014 (8). The LAS cohort (n=3194 from 14 centers), was created using a software tool which enables hospitals throughout Europe to include acromegaly patients and report patient, biochemical and adenoma characteristics (26). For this study, only patients using PEGV monotherapy (n=83) or PEGV in combination with LA-SSA (n=76) were enrolled from 10 different centers. The inclusion period was between 2010 and 2015.

Rotterdam cohort

Clinical and biochemical data were collected from acromegaly patients with elevated IGF-I levels (>1.2x upper limit of normal (ULN)), after at least 6 months of the highest dose of LA-SSAs (octreotide LAR 30 mg or lanreotide Autogel 120 mg every 28 days). In this group, 27 acromegaly patients started with 25 mg PEGV weekly as co-treatment, while another 18 started with 40 mg PEGV weekly, and the last 67 patients started with a variable PEGV dose, guided by their baseline IGF-I levels. This variable PEGV starting dose was based on one of our previous reports (Figure 2, (18)). The formula to calculate the PEGV dose is 4 + (IGF-I z-score during treatment with high dose LA-SSA*16) and was deducted from a method described previously (18). This formula can only be used when IGF-I levels are elevated after a period of at least 6 months of LA-SSA treatment. Intervals of dose adaptations were 6-8 weeks until a controlled IGF-I level was achieved on two consecutive occasions. The subjects then visited our outpatient clinic every 16 weeks. When the once weekly PEGV dose exceeded 80 mg per injection, patients divided the dosage to two weekly injections. With weekly doses over 200 mg, subjects changed administration intervals into daily injections or 5 injections per week. At each visit to our outpatient clinic, standard measurements were performed including assessments of IGF-I levels. Permission from the Institutional Review Board of the Erasmus Medical Center Rotterdam was obtained and all patients gave their written informed consent.

LAS cohort

Acromegaly patients from the LAS database treated with PEGV were selected and divided in two groups; PEGV in combination with LA-SSA and PEGV monotherapy. From the LAS-database, we were able to select 141 potential patients using the combination treatment. We excluded 65 patients, because of two reasons; 1) no IGF-I normalization during LA-SSA + PEGV treatment was achieved (n=16) and; 2) follow-up data during LA-SSA and PEGV-treatment were missing (n=49). The remaining patients (n=76) were selected for this study. The same exclusion criteria applied for the PEGV monotherapy patients. We were able to select 122 potential patients using PEGV monotherapy. We excluded 39 patients (no IGF-I normalization during PEGV monotherapy was achieved (n=6) and follow-up data during PEGV-treatment were missing (n=33)). The remaining patients (n=83) were selected for this study. The medical ethics committee from the Liège University hospital approved the protocol, and was covering the other European centers.

Hormone assays

In the Rotterdam cohort, the GH and IGF-I level measurements were assessed with the Immulite 2000 assay (DPC Biermann GmbH/Siemens, Fernwald, Germany), a solid-phase, enzyme-labeled chemiluminescent immunometric assay, with an intra-assay variability of 6%, and an inter-assay variability of 5-6% for GH and with an intra-assay variability of 2-5%, and an inter-assay variability of 3-7% for IGF-I. The IGF-I age and sex-adjusted reference ranges were used from an article by Elmlinger *et al.* (27). In the LAS cohort, containing acromegaly patients from several European hospitals, the GH and IGF-I level measurements were assessed locally, and consequently performed with different assays. Therefore, the IGF-I levels were chosen to be expressed as the ULN of the reference ranges used in the local hospitals. In this study, GH levels were measured as a single random sample and expressed as absolute values.

Candidate predictors

Variables that were considered as possible predictors for PEGV normalization dosage were selected based on the literature (8,18-20), biological plausibility, and availability of robust data ascertainment in both cohorts and included: age at diagnosis, sex, weight, height, tumor size (micro vs. macroadenoma at diagnosis), presence of diabetes mellitus type II, IGF-I levels (expressed as ULN), random GH levels and previous treatment modalities (transsphenoidal surgery, radiotherapy and the duration of LA-SSA monotherapy before the addition of PEGV). Weight, IGF-I levels (expressed as ULN) and random GH levels were collected between

6 months before and at the time of PEGV-addition. Other data was collected at baseline (as indicated), was fixed data in the patient's record, or was established during disease process.

Outcome

The outcome used in this study was the PEGV dose (mg/week) needed for the normalization of IGF-I levels either during the addition to LA-SSA (highest tolerable dose) or as PEGV monotherapy.

Statistical analysis

Data are expressed as median [interquartile range]. Differences between two subgroups were analysed using an unpaired t-test or the Mann-Whitney U test (in case of non-parametric data). Nominal variables were analysed using Fisher's exact test. For subjects in which PEGV was added to LA-SSA therapy, the distribution of the PEGV dose required for normalization of IGF-I levels was not comparable between the two cohorts, therefore we meta-analyzed the data as a form of external replication. For all regression models, log-transformation of the outcome variable (required PEGV dose) was performed to normalize residuals and non-linearity was assessed utilizing restricted cubic splines with 3-4 knots. We used univariable linear regression models to assess the association between each candidate predictor and the required PEGV dose. The decision for linear regression models instead of multiple models for the identification of predictors was based on Akaike information criterions and log-likelihood tests comparing multilevel models with random intercepts and/or slope per cohort versus standard linear regression correcting for cohort. To allow for optimal generalizability of effect estimates that predict the required PEGV dose, we performed multivariable multilevel modelling with a random intercept per cohort for the final model. We selected useful predictors using backward selection based on the change in regression coefficients and residual explained variability of the model, with a p-value <0.20 as to keep predictors liberally in the model. Other p-values are considered statistically significant when lower than 0.05 (two-tailed). For subjects switching from LA-SSA to PEGV monotherapy, we used univariable linear regression models to assess the association between each potential predictor and the required PEGV dose. We subsequently calculated the predicted normalization dosage for each subject using the outcomes of the final (multivariable) regression models. In addition, we also calculated more conservative and more progressive models to cope with potential under or overtreatment by adding or subtracting the equivalent of 40 mg/week from the outcome of the regression formula. To cope with (differentially) missing values of the candidate predictors, missing data on candidate predictors were multiple imputed (five times). The imputation model included all

candidate predictor variables, the outcome variable and several relevant variables descriptive for the study subjects. There was no difference between the original or any of the imputed datasets. All analyses were performed in each of the completed datasets and final results were pooled. All statistical analyses were performed using Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc. Chicago, IL, USA) or using R statistical software version 3.2.43 (packages rms, MASS and lm4).

RESULTS

Cohort characteristics

Patient characteristics and previous treatment modalities of the two combination treatment cohorts and the PEGV monotherapy cohort are depicted in Table 1. Acromegaly patients treated with the combination treatment included in the LAS-database are younger (39.0 vs. 45.5 years), more likely to be diagnosed with a macroadenoma (90.8 vs. 81.3%) and suffered from diabetes mellitus type II more frequently (43.4 vs. 36.6%). Patients from the Rotterdam cohort are taller (178 vs. 170 cm). Patients who were included in the LAS-database needed higher PEGV doses in order to achieve normalized IGF-I levels both during combination treatment with LA-SSA and during PEGV monotherapy and had a higher IGF-I level (xULN) before the addition of PEGV. Other descriptive data and measurements such as weight, height, and biochemical data are depicted in Table 1, as well as comparisons between the combination treatment group and the PEGV monotherapy group. No significant differences were observed in the combination treatment cohort between excluded (all originated from the LAS database) and included patients, except for the percentage of performed surgeries, radiotherapy and height, the excluded patients were smaller in stature. No significant differences were observed in the PEGV monotherapy cohort between excluded and included patients.

Predictors of PEGV dosing required for disease control in combination treatment with LA-SSA

All univariate analyses of the candidate predictors are depicted in Figure 1. A positive linear association was observed between IGF-I (xULN) and the PEGV dosage required for disease control. There was a positive non-linear association of weight with PEGV normalization dosage, suggesting an effect threshold from approximately 100 kg (Figure 1), results were similar after adjustment for age and height (data not shown). There was a negative linear association of age with PEGV normalization dosage and a positive linear association of height with PEGV normalization dosage. In multivariable analyses, the association of age and height were no longer statistically significant after adjustment for weight, yet age did meet the pre-specified criteria of

Table 1. Descriptive characteristics of the combination treatment and PEGV monotherapy cohorts

	Combination treatment LA-SSA + PEGV			p-value[a]	PEGV monotherapy	p-value[b]
	Total cohort	Rotterdam	LAS		LAS	
No. of patients	188	112	76		83	
Patient characteristics:						
Age at diagnosis – yrs	42.0 [33.0 – 53.0]	45.5 [36.0 – 56.0]	39.0 [29.5 – 47.0]	0.000	41.0 [29.0 – 51.0]	0.001
Sex – male %	58.0	58.0	57.9	1.000	53.0	0.000
Weight before addition of PEGV – kg	90.0 [77.0 – 104.0]	91.5 [79.0 – 104.0]	89.0 [74.5 – 105.0]	0.107	83.0 [71.0 – 93.0]	0.000
Height before addition of PEGV – cm	175.0 [168.0 – 182.0]	178.0 [170.0 – 184.0]	170.0 [166.0 – 180.0]	0.000	170.0 [163.0 – 180.0]	0.000
Tumor size – macro %	85.1	81.3	90.8	0.000	83.9	0.276
Diabetes mellitus type II – %	39.4	36.6	43.4	0.025	34.9	0.050
IGF-I xULN before addition of PEGV	2.0 [1.5 – 2.7]	1.9 [1.5 – 2.6]	2.1 [1.6 – 2.8]	0.000	2.1 [1.5 – 3.2]	0.001
GH before addition of PEGV – µg/l	7.9 [3.1 – 17.8]	8.4 [3.2 – 17.5]	7.5 [2.2 – 18.6]	0.617	5.9 [2.0 – 11.0]	0.000
Previous treatment:						
Surgery – total %	51.0	28.6	84.2	0.000	81.9	0.000
Once debulked – %	48.6	28.6	78.1		71.1	
Twice debulked – %	2.4	N/A	6.1		8.4	
>Twice debulked – %	N/A	N/A	N/A		2.4	
RTx – %	16.0	10.7	23.7	0.000	40.2	0.000
Duration of LA-SSA before addition of PEGV – months	16.0 [8.3 – 39.0]	12.0 [7.2 – 26.8]	25.0 [11.5 – 62.0]	0.000	34.4 [13.4 – 86.4]	0.000
Outcome:						
Required PEGV dose – mg/week	105.0 [65.0-200]	80.0 [60.0-120.0]	210.0 [105.0-280.0]	0.000	105.0 [105-140]	0.000

Descriptive characteristics of the three cohorts: Rotterdam cohort using LA-SSA + PEGV, LAS cohort using LA-SSA + PEGV and the LAS cohort using PEGV monotherapy. Missing data were imputed in the original datasets by multiple imputation. Continues variables are expressed in median [interquartile range] and categorical variables in percentages. LA-SSA: long-acting somatostatin analogues, PEGV: pegvisomant, LAS: Liège acromegaly survey, kg: kilogram, cm: centimeter, Macro: macroadenoma, IGF-I: insulin-like growth factor I, GH: growth hormone, RTx: radiotherapy,mg: milligram, N/A: not applicable.

a. Combination treatment (Rotterdam) vs. combination treatment (LAS)

b. Combination treatment (Rotterdam and LAS) vs. PEGV monotherapy (LAS)

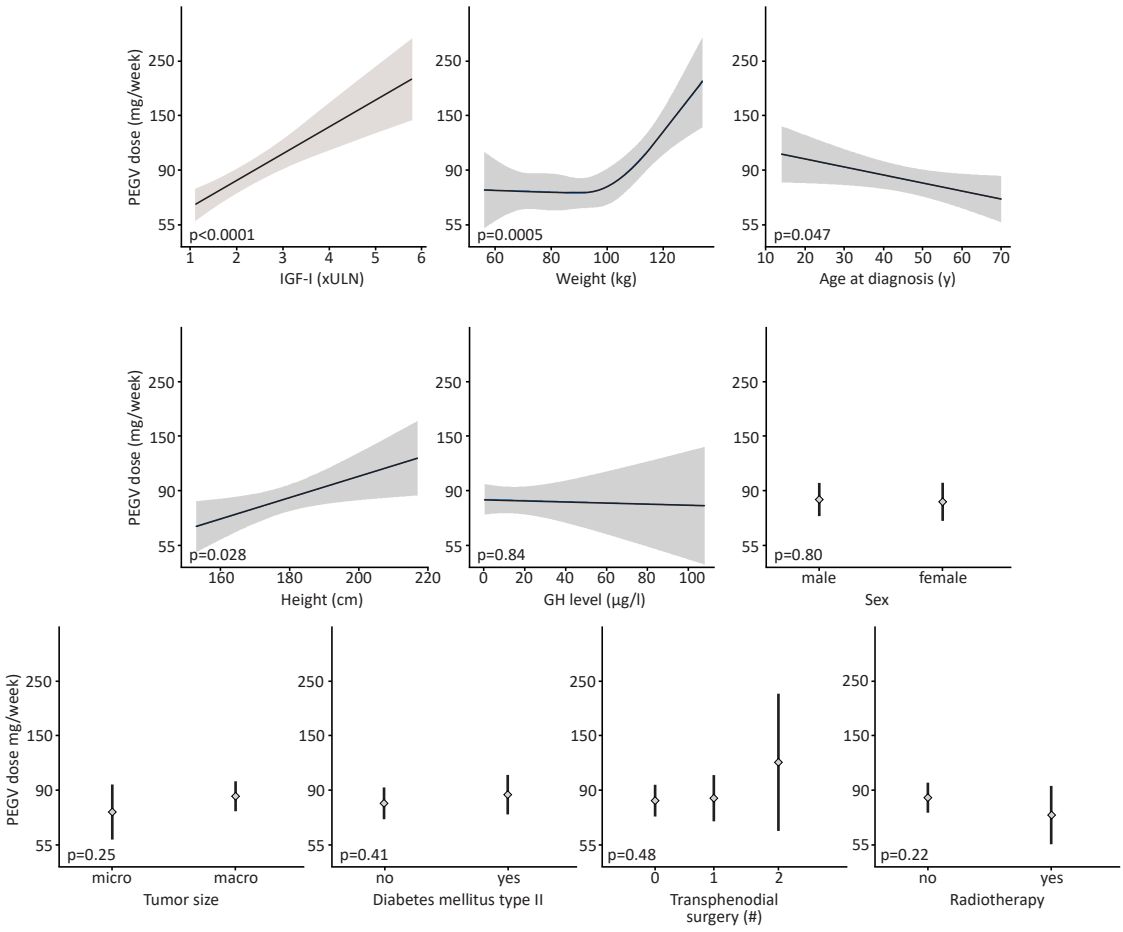


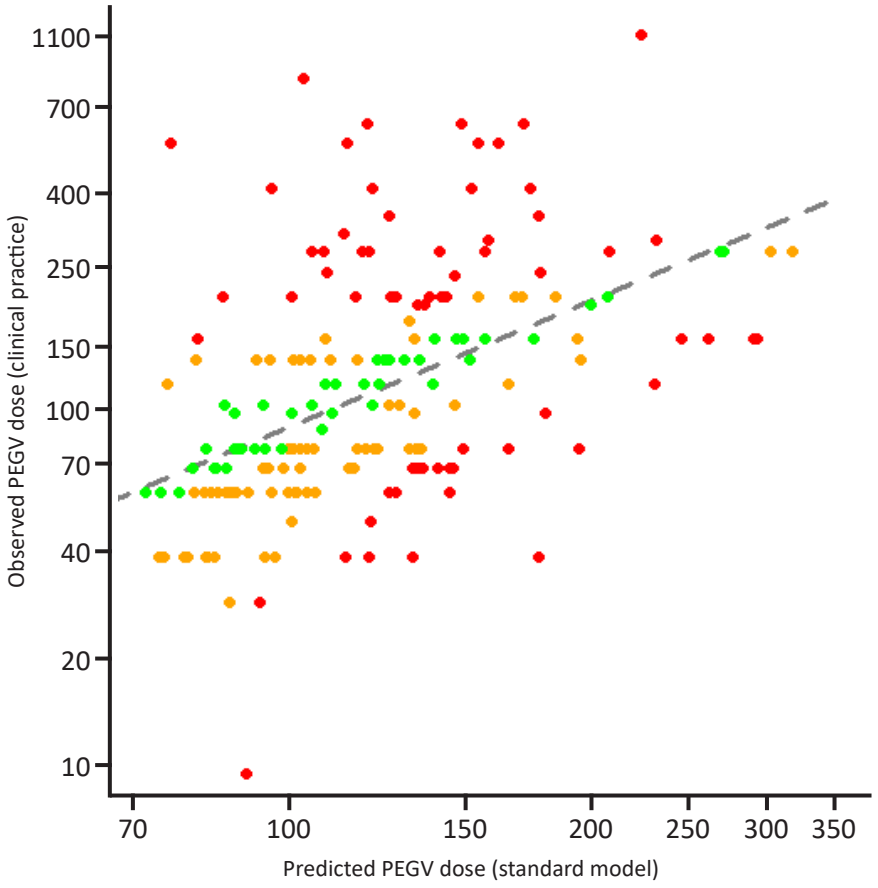
Figure 1. Identification of potential predictors during combination treatment

Univariate analyses of multiple determinants potential for the prediction of the PEGV dose needed to achieve normalization of IGF-I levels during combination treatment. IGF-I xULN, age at diagnosis, weight and height were significantly associated with PEGV dosing during PEGV treatment in combination with LA-SSA.

PEGV: pegvisomant, IGF-I: insulin-like growth factor I, ULN: upper limit of normal, GH: growth hormone, micro: microadenoma, macro: macroadenoma.

being added in the final model. Other potential predictors were not associated with the PEGV normalization dosage (Figure 1).

Figure 2 depicts the performance of the standard prediction model (x-axis) as compared to the true PEGV normalization dosage (y-axis) and the difference between the predicted and true normalization PEGV-dose for each individual (colored dots are corresponding to the table colors; Figure 2). The standard prediction formula for PEGV normalization dosage based on multivariable models (EXP^(5.5994 +



	Potential overtreatment		Correct treatment	Potential undertreatment	
Model type	**over 60** mg/week	**20 to 60** mg/week	**between 20 and -20** mg/week	**-20 to -60** mg/week	**below -60** mg/week
Conservative (decrease overtreatment)	6 (3.2%)	20 (10.6%)	64 (34.0%)	41 (21.8%)	57 (30.3%)
Standard	27 (14.4%)	63 (33.5%)	40 (21.3%)	16 (8.5%)	42 (22.3%)
Progressive (decrease undertreatment)	90 (47.9%)	41 (21.8%)	16 (8.5%)	14 (7.4%)	27 (14.4%)

Figure 2. Association of combined predictive values with the PEGV dose needed for IGF-I normalization

This Figure shows the association of the combined predictive values (X-axis, the model) with the PEGV dose needed for IGF-I normalization as obtained in clinical practice (Y-axis). The regression line is represented by the dashed line (grey). The individual data-points are colored according to the distance from the regression line (red: distance = 60 mg/week, orange 20-60 mg/week, green <20 mg/week). Data-points in the Figure depict the standard model. The conservative and progressive model were defined as the normal model minus or plus 40 mg/week, respectively. The table below depicts the n (%) of the different model groups and also display the potential shift between the models.

PEGV: pegvisomant, IGF-I: insulin-like growth factor I.

IGF-I ULN*0.2585 + weight*-0.0365 + weight^2*0.00025 + age*-0.0045)) (Table 2) predicted the final PEGV normalization dose correctly in 63.3% of all patients within a range of +/- 60 mg/week and in 21.3% of all patients within a range of +/- 20 mg/week (Figure 2). In addition, a more conservative model (standard prediction model minus 40 mg/week) correctly predicted the PEGV normalization dosage in 66.4% of all patients within a range of +/- 60 mg/week, and in 34.0% of all patients within a range of +/- 20 mg/week (Figure 2). For a more progressive model (standard model plus 40 mg/week), these numbers were 37.7 and 8.5%, respectively (Figure 2).

Table 2. Multivariable analysis of the final model to predict optimal PEGV dosing

Variable	Estimate	SE	p-value
Intercept	5.5994	0.9382	<0.0001
IGF-I – xULN*	0.2585	0.0459	<0.0001
Weight – kg*	-0.0365	0.0192	0.0830
Weight^2 – kg*	0.0002	0.0001	0.0038
Age at diagnosis – years	-0.0045	0.0033	0.1700

As the outcome is not normally distributed, the model should be calculated as: e(final model).
PEGV: pegvisomant, SE: standard error, IGF-I: insulin-like growth hormone I, ULN: upper limit of normal.
*before the addition of PEGV to LA-SSA.

Predictors of PEGV dosing required for disease control during PEGV monotherapy

A positive linear association was observed between weight and the PEGV dosage required for disease control (p≤0.001; Figure 3 and Figure 4). None of the other potential predictors were associated with the PEGV normalization dosage (Figure 3). Figure 4 depicts the performance of weight (x-axis) as a predictor for PEGV normalization dosage as compared to the true normalization dosage (y-axis) and the difference between the predicted and true normalization dosage for each individual (colored dots are corresponding to the table colors; Figure 4). The standard prediction formula for PEGV normalization dosage based on weight (EXP^(4.092 + weight*0.00868)) predicted the final PEGV normalization dose correctly in 77.1% of all patients within a range of +/- 60 mg/week and in 31.3% of all patients within a range of +/- 20 mg/week (Figure 4). In addition, a more conservative model correctly predicted the PEGV normalization dosage in 67.4% of all patients within a range +/- 60 mg/week, and in 32.5% of all patients within a range of +/- 20 mg/week. For a more progressive model, these numbers were 56.6 and 14.5%, respectively.

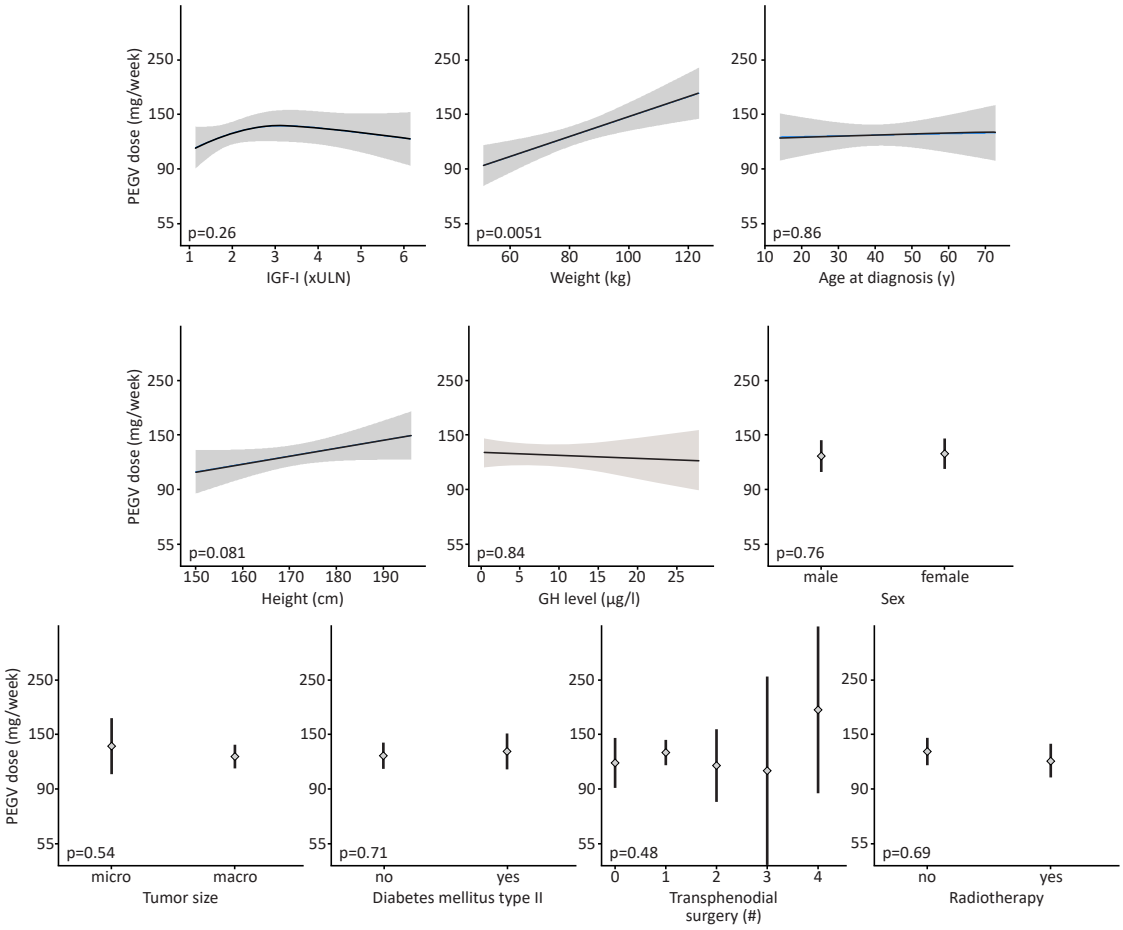
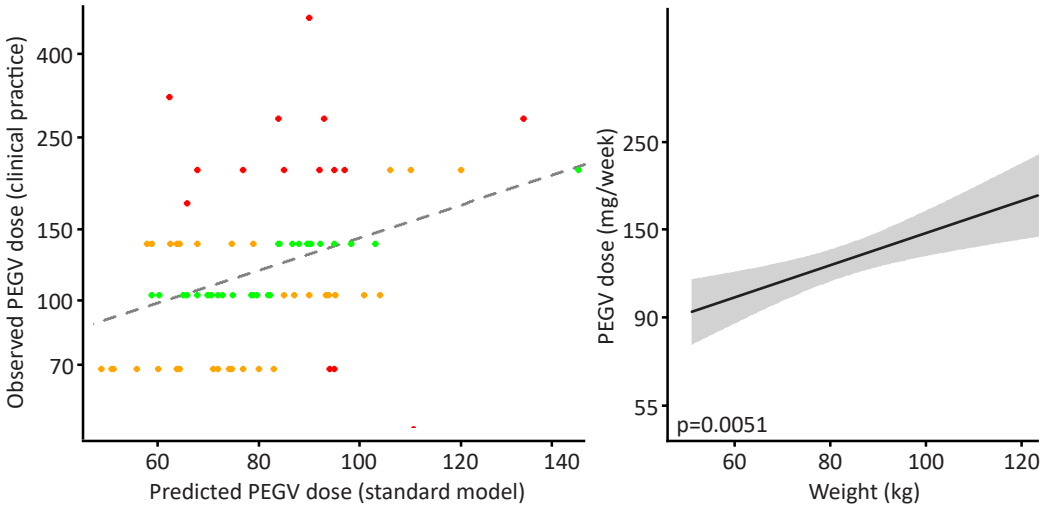


Figure 3. Identification of potential predictors during PEGV monotherapy

Univariate analyses of multiple determinants potential for the prediction of the PEGV dose needed to achieve normalization of IGF-I levels during PEGV monotherapy. Only weight was significantly associated with PEGV dosing during PEGV monotherapy.

PEGV: pegvisomant, IGF-I: insulin-like growth factor I, ULN: upper limit of normal, GH: growth hormone, micro: microadenoma, macro: macroadenoma.



	Potential overtreatment		Correct treatment	Potential undertreatment	
Model type	**over 60** mg/week	**20 to 60** mg/week	**between 20 and -20** mg/week	**-20 to -60** mg/week	**below -60** mg/week
Conservative (decrease overtreatment)	1 (1.2%)	3 (3.6%)	27 (32.5%)	26 (31.3%)	26 (31.3%)
Standard	4 (4.8%)	27 (32.5%)	26 (31.3%)	11 (13.3%)	15 (18.1%)
Progressive (decrease undertreatment)	30 (36.1%)	26 (31.3%)	12 (14.5%)	9 (10.8%)	6 (7.2%)

Figure 4. Association of weight with the PEGV dose needed for IGF-I normalization
This Figure shows the association of the patient's weight (X-axis) with the PEGV dose needed for disease control during PEGV monotherapy as obtained in clinical practice (Y-axis). The regression line is represented by the dashed line (grey). The individual data-points are colored according to the distance from the regression line (red: distance=60 mg/week, orange 20-60 mg/week, green <20 mg/week). Data-points in the Figure depict the standard model. The conservative and progressive model were defined as the normal model minus or plus 40 mg/week, respectively. The table below depicts the n (%) of the different model groups and also displays the potential shift between the models.
PEGV: pegvisomant, IGF-I: insulin-like growth factor I.

DISCUSSION

The PEGV dose required for normalization of IGF-I levels in acromegaly is highly variable and a wide inter-individual variation in PEGV serum levels is observed despite identical PEGV dosage (28,29). Previous studies suggest that this variability depends on disease activity and individual response to the drug (8,16). Therefore, PEGV titration is a process that requires a tailored approach for each individual. This is the first study that focuses on identifying predictors for PEGV dosing and

developing a multivariable model in order to predict the required PEGV dose to achieve normalization of IGF-I levels in acromegaly patients. The main findings of this study are; 1) IGF-I, weight, height and age at diagnosis are associated with the PEGV dose required for normalization of IGF-I levels in patients treated with LA-SSA combined with PEGV and; 2) that weight is associated with the PEGV dose required for normalization of IGF-I levels in patients treated with PEGV monotherapy.

To the best of our knowledge, only one previous study has investigated determinants of the PEGV dose needed for IGF-I normalization. Parkinson *et al.* observed that GH and IGF-I levels, sex, weight and previous radiotherapy were associated with the PEGV dose required for disease control in patients treated with PEGV monotherapy (n=118) (20). In our study, IGF-I xULN was the best predictor for PEGV dosing, yet GH levels were not associated with the required PEGV dose. The most likely explanation for this difference is the variability of the GH-assays. The study by Parkinson *et al.* used a single assay for the measurement of all GH levels, while GH levels in our study were measured in several local hospitals and thereby consequently measured by different GH-assays. This can lead to measurement errors and a bias. Moreover, single GH has a limited clinical usefulness as it has a short half-life and is pulsatile excreted into the bloodstream. Therefore, random single measurements of GH are less suitable as a biochemical marker for acromegaly in clinical practice. These aspects are less prominent for IGF-I measurements, as they are expressed as the upper limit of normal and are less sensitive to daily variations as compared to GH. Despite, the limitations of GH-measurement, we chose to include and analyze these GH levels, because of its biological plausibility as a candidate predictor and the intension that our prediction model is going to be used in multiple hospitals and consequently GH-measurements will be performed with several different assays.

The best predictor during combination treatment, besides IGF-I, is the patients weight before the start of PEGV. Patients with a higher bodyweight, require a higher PEGV dosage, which is a logical and expected phenomenon. However in our study a positive non-linear association was observed, suggesting a threshold effect from approximately 100 kg body weight which remained similar after correction for sex, age and IGF-I levels. A possible explanation for this effect threshold could be that these patients have different disease activity and therefore have a different body composition, possibly more fat mass. Former studies already reported an association between weight and PEGV dose titration (19,20,30). Future studies should investigate whether a clinical assessment of body composition (ratios of lean vs. fat mass percentages) may improve the prediction of the PEGV dose required for biochemical normalization.

Female gender is reported to have a better PEGV response with similar PEGV doses during PEGV monotherapy, however this gender-difference was not statically

significant anymore when PEGV doses were expressed per kg body weight (19). Another study did observe that women needed a higher average PEGV dose of 0.04 mg/kg/day during PEGV monotherapy (20). It has been speculated that sex differences in PEGV pharmacokinetics may influence absorption, distribution and/or clearance of the drug as well as the modulation of GH sensitivity by estrogens and fat (31-33). However, regardless of weight differences, we could not confirm a sex difference in relation to the PEGV normalization dose during our study both in patients treated with PEGV monotherapy and in combination with LA-SSA.

Opposite to patients treated with the combination therapy, we found that IGF-I was not a predictor of PEGV dosing during PEGV monotherapy, despite its biological plausibility. This may be explained by differences in the disease severity of patients in the combination versus monotherapy groups, given that the LAS combination cohort requires a median PEGV dose of 210 mg/week on top of the maximum LA-SSA dosage, while the LAS cohort treated with PEGV monotherapy required a median dose of 105 mg/week. According to the literature, to achieve efficacy rates of more than 90% during PEGV monotherapy, the average expected weekly dose is above 120-130 mg (12,34). Studies about the combination treatment reported PEGV doses that range between 60-140 mg/week in addition to LA-SSA [normalization rates range between 67-97%] (8,10,35). These data show that the LAS-monotherapy group contains less severe acromegaly patients, while the LAS-combination treatment group contains more severe acromegaly patients relative to data from the literature, presumed that the PEGV dose represents disease severity. On the other hand, LA-SSA has a direct and an indirect effect, which results in GH-independent decrease of IGF-I secretion (36,37). A Danish group observed that PEGV serum levels increase by 20% when combined with LA-SSA (38). Besides dosing difference, it may be expected that the use of two drug modalities is naturally more given to patients with more disease severity. Additionally, IGF-I (xULN) levels before the addition of PEGV in both LAS cohorts treated with monotherapy and combination treatment are higher. On the other hand, it should take into account the differences between the various IGF-I-assays which were used in the different cohorts.

The PEGV doses of the LAS cohort required for IGF-I normalization were strikingly high compared to the Rotterdam cohort. The distribution of normalization PEGV dosage were right skewed as opposed to the normally distributed Rotterdam cohort. This most likely reflects the fact that the LAS cohort represents the more severe cases in Europe, while the experience with PEGV in Rotterdam has led to a relatively low threshold for prescribing PEGV in addition to LA-SSA. This may not directly be linked to a difference in IGF-I levels before the addition of PEGV in our study, however, LAS patients are younger and are having more diabetes mellitus type II, which are characteristics of more severe acromegaly. Another

possible explanation could be the interest of the research group in Liège for genetic disorders causing acromegaly, taking into account that the possible prevalence of a mutation in the aryl hydrocarbon receptor interacting protein (AIP) gene, X-linked acrogigantism (X-LAG) and/or familial isolated pituitary adenoma (FIPA) patients could be higher in this cohort. Despite these differences, we found that a meta-analysis of both cohorts (as a form of external replication) performed well and also the separate analyses per cohort showed the same effect directions. By combining both cohorts, the results of this study are widely generalizable as this approach has led to a study population that reflects a wide range of acromegaly patient that is eligible to start PEGV treatment.

This study was potentially limited by the retrospective design, which consequently led to missing data. In order to cope with both differentially and randomly missing data, we used multiple imputation. This study was also limited by the relative small sample size. However, this is expected given the low prevalence of acromegaly as well as the fact that only a subset of acromegaly patients is treated with LA-SSA in combination with PEGV. The Rotterdam cohort harbored exclusively patients that were normalized by LA-SSA in combination with PEGV, as PEGV doses were up-titrated until normalization of IGF-I levels were achieved. The exclusion of patients from the LAS cohort not normalized by LA-SSA in combination with PEGV (n=16, 8.5%) or PEGV alone (n=6, 7.2%) has remained limited. In order to overcome these limitations and to replicate our results, prospective studies utilizing a multicenter set-up are required.

This model is designed for patients who are about to start PEGV treatment after failure of LA-SSA monotherapy. Furthermore, this study is not designed to predict PEGV overdosing, since PEGV doses were increased until IGF-I levels were normalized. But this prediction model should be considered as a useful clinical tool during PEGV dose titration, which can be time consuming over multiple outpatient clinic visits, especially when a high PEGV dose is needed to control the disease.

CONCLUSION

This is the first study that focuses on identifying predictors for the PEGV dose required for disease control in acromegaly and the development of a multivariate prediction model for the required PEGV dose. The model is designed for patients who are about to start PEGV after failure of LA-SSA monotherapy and could be used as a clinical guidance tool during the start of PEGV dose titration. In this study, the PEGV dose needed for normalization of IGF-I levels in addition to LA-SSA is associated with IGF-I levels, weight and age in a multivariate prediction model and predicted the final PEGV normalization dose correctly in 63.3% of all patients within a range of $^{+}$/$^{-}$ 60 mg/week [21.3% within a range of $^{+}$/$^{-}$ 20 mg/week]. The required PEGV dose

during monotherapy was associated with the patient's weight and predicted the final PEGV normalization dose correctly in 77.1% of all patients within a range of +/- 60 mg/week [31.3% within a range of +/- 20 mg/week]. For an acromegaly patient of 60 years old, weight of 80 kilograms, height of 1.75 meters, and a IGF-I level of 1.6x the ULN using the maximum dose of LA-SSA, the standard model will calculate 83.3 mg PEGV weekly. In this case, we will recommend to start with 80 mg weekly and titrate up or down guided by the IGF-I level (target 1.0x the ULN).

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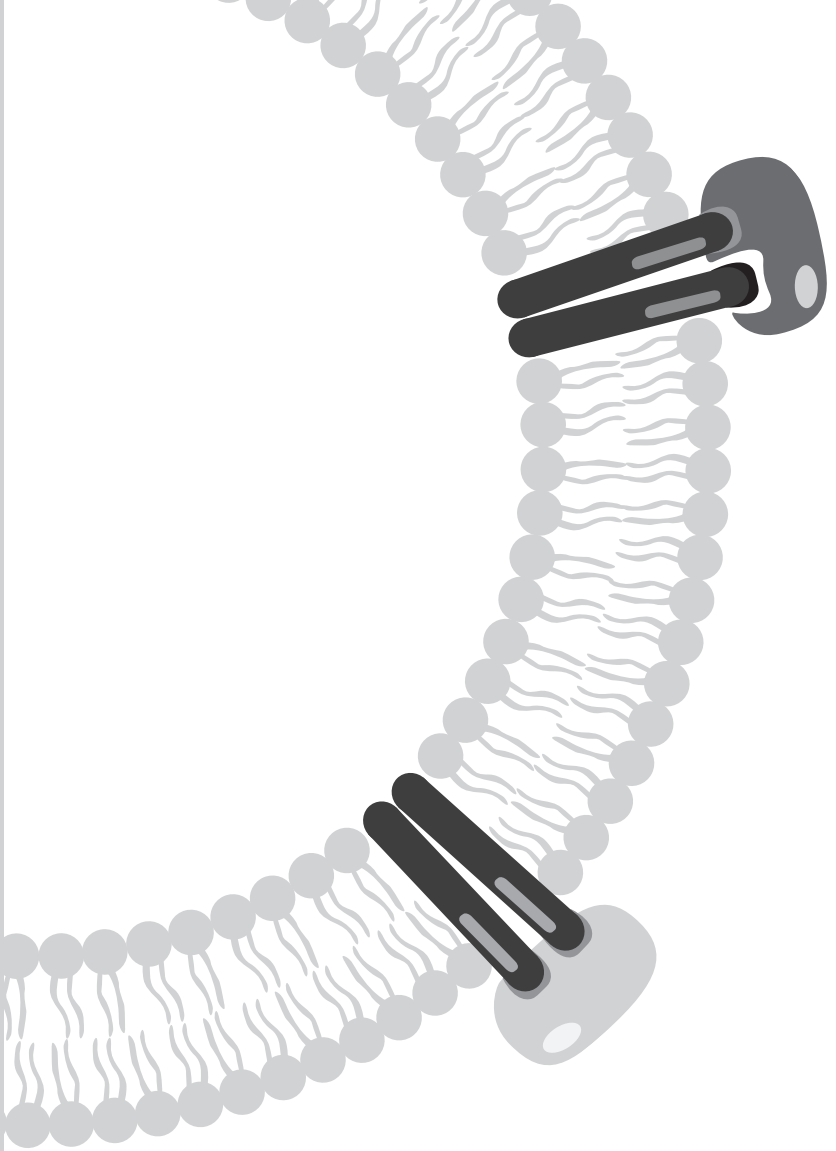

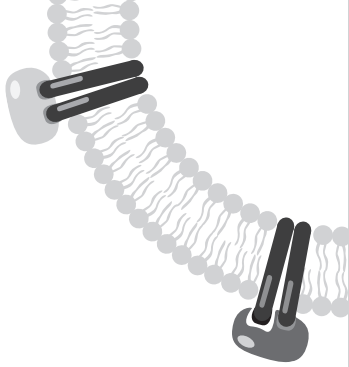

Chapter 7

General discussion and future perspectives

PARTLY BASED ON:
Combined Treatment of Somatostatin Analogues
with Pegvisomant in Acromegaly
S.E. Franck, A. Muhammad, A.J. van der Lely, S.J.C.M.M. Neggers
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INTRODUCTION

More than a decade ago, pegvisomant (PEGV) became available as a treatment modality for acromegaly patients. Particularly, valuable for patients who were not successfully cured after transsphenoidal surgery, radiotherapy and/or achieved disease control after (adjuvant) long-acting somatostatin analogues (LA-SSA) treatment. In the beginning, PEGV was used as a single drug agent. Concerns were raised whether PEGV might induce growth of the pituitary adenoma. Most of the available experience was based on the treatment with LA-SSA and its success in tumor volume reduction. One of the advantages of the combination of PEGV with LA-SSA is that tumor size control or even tumor shrinkage can be expected, besides lowering the required PEGV dose and thereby induce possible cost reduction. Recently, one of the acromegaly consensus groups has recommended switching to combination treatment in patients with partial response to LA-SSAs (1).

This chapter will focus on the main discussion points of the previous chapters; efficacy and safety issues of the combination treatment in the Rotterdam cohort between 2004 and 2013 (chapter 2), somatostatin receptor (SSTR) expression during the combination treatment (chapter 3), the role of the polymorphism deletion 3 of the growth hormone receptor (d3-GHR) in PEGV-treated acromegaly (chapter 4 and 5) and the prediction of the required PEGV dose in order to normalize insulin-like growth factor I (IGF-I) levels during monotherapy and in combination with LA-SSA (chapter 6). The conclusion of this thesis and the future perspectives will be discussed at the end of this chapter.

1. COMBINATION TREATMENT IN THE ROTTERDAM COHORT

Long-term data of the combination treatment is needed to confirm that efficacy rates and its safety profile retain over time. Chapter 2 of this thesis reports these aspects in currently the largest single-center cohort (Rotterdam cohort) using combination treatment from 2004 and 2013 (2). Here we discuss these efficacy rates, changes in tumor volume and other safety aspects of LA-SSA-treatment combined with PEGV.

1.1 Efficacy and dose reduction

PEGV monotherapy or combined with LA-SSA is highly effective in treating acromegaly, as pharmacology dictates that in principle in virtually every patient with acromegaly control of the disease should be possible. The appropriate PEGV dose varies among acromegaly patients, the Rotterdam cohort (n=112) reported that patients using the highest dose of LA-SSA needed a median weekly PEGV dose of 80 mg [range: 30 – 300] to achieve normal IGF-I levels in 97% of the patients (2). Monotherapy of PEGV requires a higher cumulative weekly dose of around 130-

140 mg to achieve a similar normalization rate of 89-97% (3,4). In contrast to these reports, an Italian observational study reported no difference in median required PEGV dose to normalize IGF-I Levels in patients using PEGV monotherapy compared to patients treated with the combination therapy (5). However, these groups were not similar according to severity of the disease. The PEGV monotherapy group had significant lower growth hormone (GH) and IGF-I levels at baseline compared to the combination group in this Italian observational study (5). Large observational registries such as the ACROSTUDY™ and German pegvisomant observational Study (GPOS) (6,7), in which patients using PEGV are included regardless their concomitant medication, observed lower efficacy rates around 60%, compared with the clinical trials of PEGV monotherapy and combination treatment (3,4,8,9). In the ACROSTUDY™ PEGV was combined with LA-SSA in 23%, with a dopamine agonist in 6%, and a combination of the three agents in 4% of patients. After five years the mean weekly dose was 106 mg in patients with a normal IGF-I, and 113 mg in those with an elevated IGF-I (9). However, these studies were not designed to evaluate efficacy and dose titration, but aimed for the evaluation of safety aspects, such as rare side effects. The lower efficacy rates in the observational registration studies might be explained by the relative lower dose of PEGV. To achieve efficacy rates of more than 90% during PEGV monotherapy, the average expected weekly dose is probably above 130 mg (3,4).

Escape, defined as the need to increase the dose of PEGV because of an increase in IGF-I levels during PEGV treatment, was reported in 34% of a Spanish cohort (10). The majority of patients was easily controlled with either an increase in PEGV dose, additional medical treatment or both. Whether this increase in the necessary dose of PEGV should be called an escape is questionable, as most patients were easily controlled and remained controlled on a higher dose of PEGV. A significant number of these patients escaped from PEGV within the first six months after discontinuation of LA-SSA. Presumably during this period these patients were actually still receiving combination treatment, due to the long half-life of LA-SSAs. The Rotterdam cohort could not observe this phenomenon during combination treatment in acromegaly patients, except for one patient which case is described in chapter 2 (2).

1.2 Tumor volume

While LA-SSA is successful in reducing tumor volume, previous concerns were raised whether PEGV might induce growth of the pituitary adenoma. Despite the fact that in a few cases an increase in tumor size during PEGV therapy was reported, there are no data suggesting that PEGV directly promotes tumor growth (11-13). In the GPOS study, changes in tumor size were systematically monitored in 307 patients, from

which 28 patients were treated with PEGV in combination with LA-SSA, however predominantly treated with PEGV monotherapy (14). In eight out of 307 patients (2.7%) an initial increase in tumor size was reported, while none of them were treated with PEGV in combination with LA-SSA. In only three of these eight patients a real, but minor, clinically irrelevant increase in tumor size after PEGV treatment was observed (14). In two of these patients a detectable rebound increase in tumor size after discontinuation of LA-SSA therapy was the probable reason for this increase in adenoma size. In the three remaining patients a steady increase of the tumor was already ongoing before the start of PEGV. In a Spanish study in 75 patients, five (6.7%) acromegaly patients were identified with an increase in pituitary tumor size (15). All of these patients were pre-treated with LA-SSA and then switched to PEGV monotherapy. Noteworthy in this study is that the reference magnetic resonance imaging (MRI) was made just after LA-SSA was discontinued (15). Therefore, the reported tumor size increases in this study may also be explained by the rebound phenomenon after cessation of LA-SSA treatment.

In the Rotterdam cohort of acromegaly patients (n=141) treated with combination therapy, growth of the adenoma has been reported in only one patient, while this growth was already ongoing before the addition of PEGV (2). Moreover, during combined treatment tumor size shrinkage of more than 20% of the largest diameter before and during combination treatment was observed in 17% of the patients and the vast majority had a stable tumor volume. In previous studies of our group similar data was observed. We, therefore, conclude that PEGV apparently does not influence the natural course of tumor growth, and the observed tumor shrinkage is most likely effectuated by the continuation of the LA-SSAs, also in combination with PEGV. However, ongoing alertness is required to monitor tumor size by repetitive pituitary imaging.

1.3 Safety aspects

The large observational studies, ACROSTUDY™ and GPOS, designed to evaluate side effects of PEGV, reported lipohypertrophy in 2.2 and 7.4% respectively (6,7). In the Rotterdam cohort four patients (2.8%) developed lipohypertrophy which appeared to be reversible by a more frequent rotation of the injection-site (2). However, in one of these patients during combined treatment cosmetic surgery was necessary in order to reduce the significant lipohypertrophy.

PEGV-induced elevations in hepatocellular enzymes are usually mild and self-limiting, both during monotherapy PEGV as in combination with LA-SSA (5,16,17). These transient elevated transaminases (TET) of more than three times the upper limit of normal (>3 xULN) seem to occur more frequently during the combination treatment (7,9). Previous studies observed TET >3 xULN in 11.1 -15.3% of the patients

using LA-SSA combined with PEGV (5,9,17). One Italian study compared long-term treatment of PEGV alone with PEGV in combination with LA-SSA regarding to TET (5). Incidence of TET during monotherapy of PEGV was reported in 14.3% of the patients, while this incidence was 11.1% in the combined group. In the Rotterdam cohort (n=19/141, 13.5%) all cases were transient without PEGV dose adaptation or discontinuation of the drug, except for one patient (2). The development of TET was not PEGV dose-dependent (2). The ACROSTUDY™ and GPOS reported lower incidence rates of TET >3x ULN, 2.5 and 5.2% respectively. More frequent outpatient clinic visits and, thereby, more frequent assessments of ALT and AST might explain the observed differences in the incidence of TET, as the elevations in transaminases are transient and will pass unnoticed when follow-up intervals are wider.

A Spanish study (n=36) previously described an association between TET and Gilbert's polymorphism (UGT1A1*28) (18). This is an autosomal recessive inherited benign condition and causes mild unconjugated hyperbilirubinemia, which is associated with a higher risk of hepatotoxicity during treatment with drugs that are metabolized by the liver. We did not confirm this association between TET and this polymorphism in the Rotterdam cohort. A Danish randomized controlled trial (n=18) demonstrated that during co-treatment of LA-SSA and PEGV, the intrahepatic lipid content of the liver increased significantly compared to the group treated with LA-SSA alone (19). Moreover, the intrahepatic lipid content was positively correlated with the PEGV dose, which is in contrast to the finding in the Rotterdam cohort. The authors of the Danish study stated that the clinical impact of this phenomenon remains unclear, however the increase in intrahepatic fat might causally be linked to TET in PEGV-treated acromegaly. More studies are needed to confirm or disprove this possible explanation for the development of TET.

2. SOMATOSTATIN RECEPTOR EXPRESSION

One of the most common and likely explanations for the need of the combination treatment in patients for which LA-SSA alone was not sufficient enough to normalize IGF-I levels, is less expression of somatostatin receptor subtype 2 (SSTR2) on the cell surface of GH-secreting pituitary adenomas. A previous study reported that full-responders to LA-SSA showed significant higher SSTR2 expression compared to partial-LA-SSA-responders (20). This study was solely performed in patients using LA-SSA monotherapy, we were the first to assess the expression of SSTR2 and SSTR5 in patients that needed the addition of PEGV to LA-SSA in order to control disease activity, chapter 3 of this thesis (21). The Rotterdam cohort, therefore, most likely represents severe acromegaly patients, who were referred to our tertiary referral center. A previous Norwegian study, also performed in a tertiary referral university hospital, used comparable techniques and scoring systems for the SSTR

expression (22). In this study treatment naive GH adenomas seems to have a lower SSTR2 expression (median immunoreactivity score (IRS) 9) compared to the SSTR2 expression of our treatment-naive group (median IRS 6), suggesting that the receptor pattern in the Rotterdam cohort is already relatively low at baseline (21,22). Since all patients included in the Rotterdam cohort received PEGV in addition to LA-SSA, we were able to perform a correlation analysis. The SSTR2 IRS was inversely correlated with the required PEGV dose in treatment naive adenoma tissues.

The other underlying hypothesis of chapter 3 was whether various medical pre-treatment modalities differently affect the SSTR2 and SSTR5 expression on GH-secreting pituitary adenomas. Therefore, we assessed the SSTR2 and SSTR5 expression on GH adenomas in three pre-treatment groups: drug-naive, treated with LA-SSA monotherapy, or LA-SSA and PEGV combination therapy before surgery. We observed that the SSTR2 expression on GH adenomas is lower in patients treated with LA-SSAs in combination with PEGV, compared to drug-naive patients. Which could be explained by the presence of more severe acromegaly in this group as stated in the previous paragraph, however, down-regulation of cell surface SSTR2 by LA-SSA treatment should not be neglected (22-24). The previously described Norwegian study demonstrated down-regulation of SSTR2 by LA-SSA treatment in a randomized subset of acromegaly patients (n=13 mono LA-SSA, n=13 direct surgery) (22), possibly through ligand-induced receptor internalization. In the Rotterdam cohort, the median duration of LA-SSA before surgery was 20 months in the group of patients that was pre-treated with LA-SSA and PEGV.

Some limitations of the study in chapter 3 have to be taken into account during data interpretation, which are; 1) a rather small sample size; 2) the retrospective design of the study, including missing data; 3) the majority of the cohort represents severe acromegaly patients, as PEGV was needed to control IGF-I levels. However, these limitations are unavoidable as acromegaly is a rare disease and the combination of LA-SSA and PEGV is not the first-line treatment modality.

3. EFFECT OF THE EXON 3-DELETED GH-RECEPTOR

Transfection studies have shown that the lack of exon 3 in the GHR enhances GH signal transduction in vitro (25), and clinical data in the field of GH-deficiency suggested that this polymorphism confers a better response to recombinant GH replacement in GH deficient children (26). However, genome-wide association studies in the general population did not report any association with the GHR locus in healthy individuals concerning GH-targets such as final height and metabolic state (27-29). Studies evaluating the influence of d3-GHR on clinical and biochemical severity of acromegaly, produced contradictory evidence (30,31). Since GH-deficient children carrying d3-GHR have benefitted more from their recombinant GH treatment, this

pharmacogenetic phenomenon could be of importance in PEGV-treated acromegaly. The results in chapter 4 and 5, reflecting the Rotterdam cohort and a meta-analysis of several European cohorts, both did not show a significant effect on the response of PEGV treatment nor on the required PEGV dose between fl/fl-GHR and d3-GHR carriers (32,33).

The first two published studies reporting the effect of d3-GHR genotype on PEGV-pharmacodynamics in acromegaly observed beneficial effects regarding d3-GHR carriers, which was performed in a cohort of 19 and 44 patients (34,35). Hereafter, two larger acromegaly cohorts (n=104 and n=127) were published regarding this topic, which could not confirm these previous findings (33,36). These later findings were additionally confirmed, as the PEGV dose and PEGV serum levels in the circulation were not significantly different between carriers of the d3-GHR genotype and the fl/fl-GHR genotype in the Rotterdam cohort (33). It could be argued that the effect of the first two studies have been founded by coincidence as the sample size is limited. Moreover, publication bias can be present in the beginning of this publication series about the effect of d3-GHR in PEGV-treated acromegaly. On the other hand, the sample sizes of the two cohorts in the last negative studies, though with reasonably more patients, could also be too small to observe an effect. However, these phenomena always lurk whenever investigating a rare disease such as acromegaly.

To strengthen the evidence, we conducted a meta-analysis in which these acromegaly cohorts were pooled together. The results of this study demonstrated that the response of PEGV treatment and the required PEGV dose, when tested in 324 acromegaly patients, was not affected by the d3-GHR genotype. A recent Brazilian study confirmed these findings in a large multiethnic cohort (n=121) addressing several aspects; clinical/biochemical data and treatment outcomes (37). No significant differences were observed between patients harboring at least one d3-GHR allele and those harboring the fl/fl-GHR genotype regarding medical treatment response (LA-SSA monotherapy, LA-SSA combined with cabergoline and PEGV monotherapy). More specific, the median IGF-I reduction during PEGV in this study (n=14, not included in the meta-analysis of chapter 5) was not significantly different between the two genotypes (p=0.829). Another possibility is that the effect of the d3-GHR isoforms are very slight, and cannot be objectified. We, therefore, state that the effect of d3-GHR is at least not clinically relevant during PEGV-dosing.

4. PREDICTION OF THE REQUIRED PEGV DOSE COMBINED WITH LA-SSA

The required dose of PEGV to achieve disease control is highly variable between individual acromegaly patients, likewise is the variation in inter-individual PEGV

serum levels despite identical PEGV doses (38,39). A tailored approach for each individual is therefore required during PEGV dose titration. As previously described in chapter 4 and 5, the common polymorphism d3-GHR did not have an influence on PEGV response and dosing. Chapter 6 of this thesis did identify predictors for PEGV dosing, which we used to develop a multivariable model in order to predict the required PEGV dose to achieve disease control in acromegaly patients. IGF-I, weight, height and age at diagnosis are associated with the PEGV dose required for normalization of IGF-I levels in patients treated with LA-SSA combined with PEGV. In patients treated with PEGV monotherapy only weight is associated with the PEGV dose required for normalization of IGF-I levels.

GH levels reported in chapter 6 were not associated with the required PEGV dose, despite their biological plausibility as a candidate predictor. This is most likely caused by the variability of the GH-assays included in our study. GH measurements included in the study were performed in several local hospitals and therefore by different GH-assays, which can lead to measurement errors and a bias during the analysis. Besides, GH has a short half-life and is excreted by the pituitary in a pulsatile manner, which makes it as a biomarker less suitable in clinical practice. Analyses can be improved with GH measurements performed with one single assay and a blood sample that is withdrawn on a standard time point in the morning or, even more reliable an average of several standardized measurements during one day.

Disease severity differs between acromegaly patients using the combination treatment in our study. The cohort contains patients from the Rotterdam cohort (n=112) and the Liège Acromegaly Survey cohort (LAS, n=76). The LAS cohort is created by a software tool which enables hospitals throughout Europe to include data reporting patient characteristics and treatment modalities. PEGV doses in combination with LA-SSA required for IGF-I normalization of the LAS cohort are strikingly higher (median 210 mg/week) compared to the Rotterdam cohort (median 80 mg/week). Additionally, IGF-I levels expressed as ULN before the addition of PEGV are significantly higher in the LAS patients compared to the Rotterdam cohort. Although, differences between the various IGF-I-assays which were used in the different cohorts should be taken into account. The LAS cohort probably reflects the presence of the more severe cases of Europe, while in Rotterdam the threshold for prescribing PEGV in addition to LA-SSA is relatively lower and thereby the Rotterdam cohort contains less severe acromegaly patients. However, we believe that by combining these two acromegaly cohorts treated with LA-SSA and PEGV a wide range of acromegaly patients is represented in this study, and that the results therefore are widely generalizable in clinical practice during the start of PEGV treatment in addition to LA-SSA.

5. CONCLUSION

In this thesis we have confirmed that LA-SSA in combination with PEGV as a treatment modality for acromegaly appears to be highly effective after experience for almost a decade in the Rotterdam cohort, provided that the required PEGV dose is used in order to control IGF-I levels. Side effects as lipohypertrophy and elevated transaminases were mild and transient. Tumor size control or even tumor shrinkage is observed in a vast majority of patients.

Normalization of IGF-I levels in acromegaly patients is associated with the expression of SSTR2 on somatotroph adenomas. In the Rotterdam cohort, the SSTR2 expression was lower in patients pre-treated with LA-SSA and PEGV compared to drug-naive acromegaly patients after transsphenoidal surgery. Moreover, a higher required PEGV dose in combination with LA-SSA was needed in patients with a lower SSTR2 expression on drug-naive somatotroph adenomas to achieve normalized IGF-I levels. (Partial) resistance for LA-SSA alone could be one of the reasons why these patients with a lower SSTR2 expression necessitate LA-SSA in combination with PEGV.

The common polymorphism d3-GHR is associated with disease severity and it has been reported to be more responsive to PEGV treatment in acromegaly patients. Clinical data of the Rotterdam cohort do not support a role for GHR genotype in treatment response or PEGV dosing in patients treated with LA-SSA in combination with PEGV. A meta-analysis obtained from four separated study cohorts including the Rotterdam cohort (n=324), confirmed that the presence of the d3-GHR in acromegaly patients has no impact on clinical practice.

Finally, the last study of this thesis did identify predictors for PEGV dosing. IGF-I levels, weight, height and age are associated with the optimal PEGV dose in order to normalize IGF-I levels in addition to LA-SSA. The IGF-I normalization dosage during PEGV monotherapy is only associated with the patient's weight. A multivariate prediction model which can be used as a clinical guidance tool for PEGV dosing in addition to LA-SSA can be found in chapter 6.

6. FUTURE PERSPECTIVES

Long-term data of the combination treatment in acromegaly presented in this thesis, confirm the high efficacy rates and generally well tolerated side effects reported by the first clinical PEGV-trials, more than a decade ago (3,4). However, acromegaly progresses slowly, therefore, long periods of follow-up data remain mandatory to assess more accurately the long-term outcome of its efficacy and safety profile but also data on morbidity and mortality, as we do already know from LA-SSAs alone which are on the market since 1988. A challenge for the future is to optimize the

quality of data. The studies performed in this thesis contain two main limitation; 1) the retrospective study design and; 2) the small sample sizes. However, these limitations are not unexpected and are a common issue during the investigation of a rare disease such as acromegaly, especially as only a subset of the patients is treated with a combination of LA-SSA and PEGV. Although, we here present the largest single center cohort to date that uses PEGV in combination with LA-SSA. In order to overcome these limitations and to replicate our results, prospective studies utilizing a multicenter set-up are required, which is particularly the case for the next study focusing on the SSTR expression during combination treatment.

Physicians tend to be mainly focused on biochemical parameters as GH and IGF-I levels during treatment of acromegaly. These parameters are definitely linked to a better outcome and a lower risk of morbidity and mortality (40,41). However, normalized serum GH and IGF-I levels do not necessarily result in complete resolution of signs and symptoms (42,43). A prospective, double blind, placebo controlled, crossover study demonstrated improved quality of life (QoL) in patients using LA-SSA combined with low-dose PEGV (43). Improved QoL was observed without significant changes in IGF-I levels after addition of PEGV to LA-SSA therapy in patients with normalized IGF-I levels during monotherapy of LA-SSA. Two things can be derived from this study; 1) new parameters are needed in the future for monitoring patients with acromegaly and; 2) less severe acromegaly patients can also benefit from the addition of pegvisomant to LA-SSA, not only the patients with partial response to LA-SSAs. Especially, since side effects remain mild and transient over time, as reported in chapter 2.

New medical treatment modalities for clinical practice are being examined. A phase III study recently demonstrated that oral octreotide, designed for absorption in the gut, is able to decrease GH and IGF-I levels with a favorable safety profile in patients previously controlled by LA-SSA injections. This is particularly appropriate for patients with injection site reactions besides its practical comfort, given that injects are unnecessary. (44). For the nearby future, when this drug is on the market, studies can be done to investigate a combination of oral octreotide and PEGV. Another agent which might be suitable for combining with PEGV is pasireotide, a multireceptor ligand with high affinity for SSTR5. Striking in chapter 3 of this thesis, is the overall high expression profile of SSTR5 in the patients selected from the Rotterdam cohort after surgery. Other studies reported that the drug provides biochemical control in naive patients as well as patients that were controlled by LA-SSA and were switched to pasireotide (45,46). However, the drug is associated with high rates of hyperglycemia, which limits its usefulness as this is already one of the problems to overcome in acromegaly patients (47). An attractive option in the future for patients that do not tolerate the diabetic effects of pasireotide might be DG3173 (somatoprim). This is a ligand with a pharmacokinetic profile similar to

LA-SSA but highly selective for GH suppression and has less inhibitory effects on insulin secretion (48). However, it is still unknown how the mechanism behind this pancreatic protection works. Currently, DG3173 is being tested in a phase II trial in acromegaly patients (49). The above sentences present alternative agents that directly target the GH-secreting adenoma, but not an alternative for PEGV, which acts on the peripheral tissues. Another approach to reduce peripheral GH-activity is via ATL1103, an antisense oligonucleotide which targets the GHR synthesis (50). This agent knocks down the gene in the liver that encodes the GHR, and thereby reduces GHR expression on the hepatocyte surface (51). This agent is still in an early stage of development, as a phase II study recently showed decreased levels of IGF-I after ALT1103 injection treatment (52). The future will show whether DG3173 and ATL1103 are suitable for clinical practice after later stage clinical trials.

PEGV dose prediction remains a challenge in the future, although chapter 6 of this thesis presents a real step forward by describing a clinical guidance tool for the start of PEGV dose titration. This clinical tool can be improved by the inclusion of more acromegaly patients in a multi-center prospective study design. Although, I personally believe that a real breakthrough will rise when the mode of PEGV-clearance is understood. Nowadays, we still do not know whether the drug is completely metabolized in the liver or if the kidney is playing a role during this process. One study demonstrated that PEGV together with the GHR, is internalized into GHR-expressing cells (53). A possibility is that during trafficking of this ligand, degradation occurs with loss of PEGylated moieties, which might be a clue for the final answer, which hopefully will be found in the nearby future.

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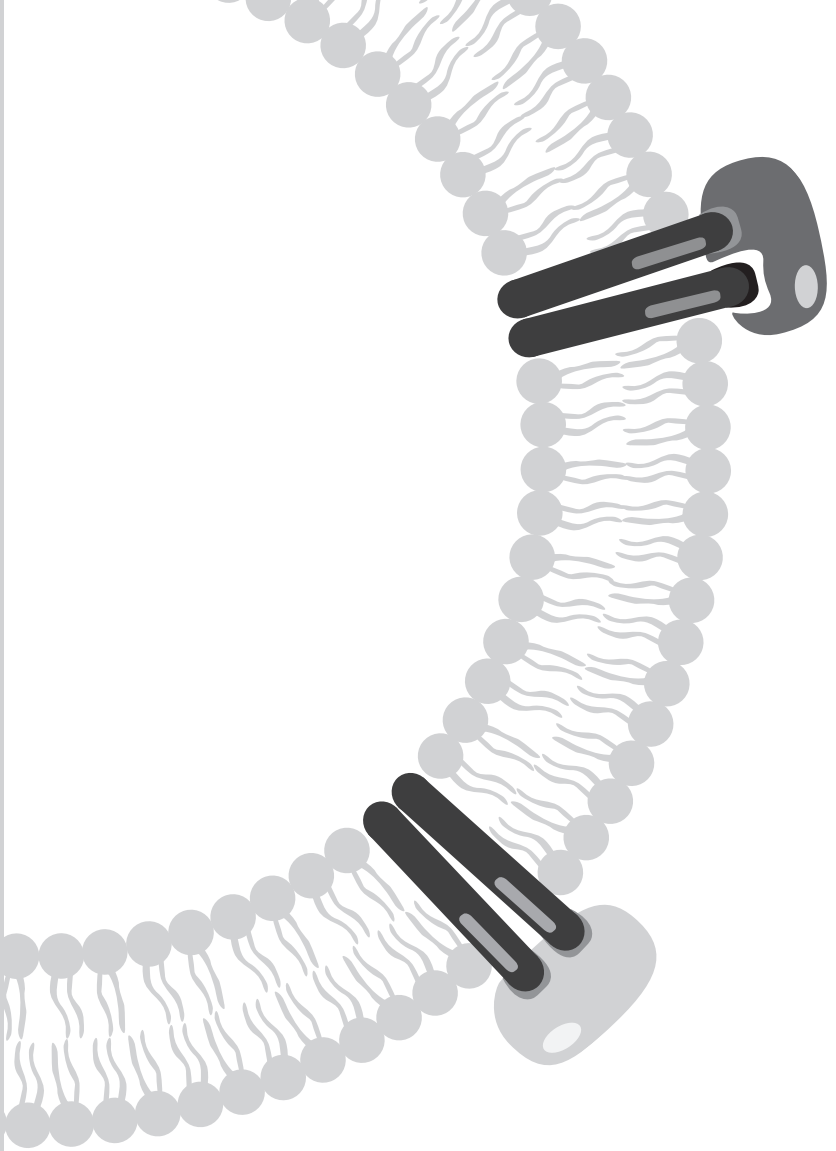

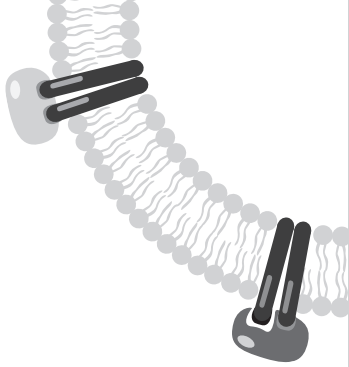

LIST OF ABBREVIATIONS

AcroQoL	Acromegaly quality of life questionnaire
AE	Adverse event
AGT1A1*28	Gilberts polymorphism
AIP	Aryl hydrocarbon receptor-interacting protein
Alk Phos	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BBB	Blood brain barrier
Bili	Total bilirubin
CAB	Cabergoline
CON	Control
D3-GHR	Growth hormone receptor polymorphism lacking exon 3
DA	Dopamine agonist
DM	Diabetes mellitus type II
FIPA	Familial isolated pituitary adenoma
Fl-GHR	Full-length growth hormone receptor
GH	Growth hormone
GHrH	Growth hormone releasing hormone
GHR	Growth hormone receptor
GPOS	German pegvisomant observational study
HE	Haematoxylin eosin
HPRT	Hypoxanthine phosporibosyltransferase
HWE	Hardy-Weinberg equilibrium
IGF-I	Insulin-like growth factor I
IQR	Interquartile range
IRS	Immunoreactivity score
JAK2	Janus kinase 2
LAS	Liège acromegaly survey
LA-SSA	Long-acting somatostatin analogue
MONO	Monotherapy
MRCP	Magnetic resonance cholangiopancreatography
MRI	Magnetic resonance imaging
NA	Not applicable
ND	Not determined
OCT	Octreotide
PASQ	Patient-assessed acromegaly symptom questionnaire
PCR	Polymerase chain reaction
PEGV	Pegvisomant

PRL	Prolactin
QoL	Quality of life
Q-PCR	Quantitative polymerase chain reaction
rhGH	Recombinant growth hormone
SDS	Standard deviation score
SE	Standard error
SRIF	Somatotropin releasing-inhibiting factor
SSTR	Somatostatin receptor
STAT	Signal transducer and activator of transcription
TET	Transient elevated transaminases
TSS	Transsphenoidal surgery
ULN	Upper limit of normal
X-LAG	X-linked acrogigantism
γ-GT	γ-glutamyl transpeptidase

SUMMARY

In **chapter 2** of this thesis we have confirmed that LA-SSA in combination with PEGV as a treatment modality for acromegaly still appears to be highly effective, after almost a decade of experience in the Rotterdam cohort (n=112). Normalization of IGF-I levels occurred in 97% of the patients, provided that the required PEGV dose was used. The median PEGV dose to achieve this efficacy rate was 80 mg/week [interquartile range: 60 – 120 mg]. Side effects such as lipohypertrophy (2.8%) and elevated transaminases of more than three times the upper limit of normal (13.5%) were mild and transient (n=141). Tumor size control and even tumor shrinkage is observed in a vast majority of patients. Pituitary tumor size increase was observed in one patient.

Normalization of IGF-I levels in acromegaly patients is associated with the expression of SSTR2 on somatotroph adenomas. In the Rotterdam cohort (n=39), the SSTR2 expression was lower in patients pre-treated with LA-SSA and PEGV compared to drug-naive acromegaly patients after transsphenoidal surgery, which is described in **chapter 3**. Moreover, a higher required PEGV dose in combination with LA-SSA was needed in patients with a lower SSTR2 expression on drug-naive somatotroph adenomas to achieve normalized IGF-I levels. (Partial) resistance for LA-SSA alone could be one of the reasons why these patients with a lower SSTR2 expression necessitate LA-SSA in combination with PEGV.

Chapter 4 and 5 focus on the common growth hormone polymorphism lacking exon 3, which is associated with disease severity and has been reported to be more responsive to PEGV treatment in acromegaly patients. Clinical data from the Rotterdam cohort (n=112) does not support a role for GHR genotype in treatment response or PEGV dosing nor PEGV serum levels in patients treated with LA-SSA in combination with PEGV. A meta-analysis obtained from four separate study cohorts including the Rotterdam cohort (n=324), confirmed that the presence of the d3-GHR in acromegaly patients has no impact on clinical practice. The polymorphism was not of added value for either the determination of the required PEGV dose or the prediction of PEGV responsiveness.

Finally, the last study of this thesis did identify predictors for PEGV dosing. IGF-I levels, weight, height and age are associated with the required PEGV dose in order to normalize IGF-I levels in addition to LA-SSA. The IGF-I normalization dosage during PEGV monotherapy is only associated with patients weight. A multivariate prediction model which can be used as a clinical guidance tool for PEGV dosing in addition to LA-SSA can be found in **chapter 6.**

SAMENVATTING

In **hoofdstuk 2** van dit proefschrift hebben we aangetoond dat somatostatine analoga met gereguleerde afgifte in combinatie met pegvisomant een zeer effectieve behandeling is voor acromegalie patiënten na bijna tien jaar ervaring binnen het Rotterdam cohort (n=112). In 97% van de patiënten normaliseerden de IGF-I waarden, mits zij de juiste dosering pegvisomant kregen toegediend. De mediane pegvisomant dosering om het IGF-I te normaliseren is 80 mg per week. Bijwerkingen als lipohypertrofie (2.8%) en verhoogde transaminasen van meer dan drie keer de bovengrens van normaal (13.5%) bleken mild en van voorbijgaande aard te zijn (n=141). In de overgrote meerderheid van de patiënten is het volume van de tumor niet toegenomen, in een gedeelte van de patiënten is de tumor zelfs afgenomen. Bij slechts een patiënt was het volume van de tumor toegenomen tijdens de combinatie-therapie.

Normalisatie van IGF-I waarden in acromegalie patiënten is geassocieerd met de expressie van de somatostatine receptor subtype 2 (SSTR2) op het oppervlak van groeihormoon producerende adenomen. Binnen het Rotterdam cohort was de SSTR2 expressie lager in patiënten die waren voorbehandeld met somatostatine analoga in combinatie met pegvisomant dan bij patiënten die nog geen medicatie hadden gekregen na transsfenoïdale chirurgie, zoals beschreven staat in **hoofdstuk 3** van dit proefschrift. Bovendien hadden patiënten met een lagere SSTR2 expressie een hogere dosering pegvisomant nodig in combinatie met een somatostatine analoog. (Partiële) resistentie voor monotherapie met een somatostatine analoog kan een van de redenen zijn waarom patiënten met een lager SSTR2 expressie een combinatie van somatotatine en pegvisomant nodig hebben om de ziekte onder controle te krijgen.

Hoofdstuk 4 en 5 gaan over een veel voorkomend groeihormoonreceptor polymorfisme waarbij exon 3 ontbreekt (d3-GHR), dat geassocieerd is met de ernst van de ziekte en wordt beschreven als effectiever reagerend op pegvisomant in acromegalie patiënten. Klinische data van het Rotterdam cohort (n=112) wijzen uit dat de pegvisomant dosering en de spiegel pegvisomant in het bloed niet worden beïnvloed door d3-GHR in patiënten die behandeld worden met pegvisomant in combinatie met somatostatine analoga. Een meta-analyse, verkregen door vier aparte cohorten inclusief het Rotterdam cohort (n=324), samen te voegen, bevestigde dat het voorkomen van d3-GHR in acromegalie patiënten geen invloed heeft op de klinische praktijk. Het polymorfisme had geen invloed op de pegvisomant dosering noch, op het kunnen voorspellen van de effectiviteit van pegvisomant.

De laatste studie die is opgenomen in dit proefschrift identificeerde factoren om de pegvisomant dosering te voorspellen. IGF-I waarden, gewicht, lengte en leeftijd waren geassocieerd met de uiteindelijke dosering pegvisomant die nodig was om de IGF-I waarden te normaliseren, in combinatie met een somatostatine analoog. De dosering die nodig was voor het onderdrukken van de ziekte tijdens monotherapie pegvisomant was alleen geassocieerd met het gewicht. Een multivariaat predictie model dat gebruikt kan worden als een hulpmiddel tijdens het doseren van pegvisomant in combinatie met een somatostatine analoog, is te vinden in **hoofdstuk 6.**

CURRICULUM VITAE

Sanne Franck werd op 22 november 1986 geboren te Utrecht en groeide op in Zoetermeer, waar zij haar eindexamen VWO behaalde aan het Erasmus college in 2005. Hetzelfde jaar startte zij de studie geneeskunde, nadat zij via de decentrale selectie was toegelaten. In het derde jaar van de opleiding heeft zij haar keuze-onderwijs gedaan in een verloskunde kliniek in het noorden van Ghana. De reguliere coschappen heeft zij volledig gelopen in het Elisabeth ziekenhuis te Tilburg, om daarna terug te keren naar het Erasmus Medisch Centrum. Onder leiding van Prof. dr. Peeters ronde zij haar afstudeeronderzoek af op het gebied van gedifferentieerd schildkliercarcinoom. Na het oudste coschap binnen de afdeling endocrinologie, behaalde zij haar artsexamen in augustus 2012.

Zij begon daarna onder leiding van Prof. dr. van der Lely en Dr. Neggers aan dit proefschrift getiteld 'Novel molecular insights into the combination treatment of acromegaly'. Tijdens haar promotie onderzoek heeft zij meerdere presentaties gegeven op nationale en internationale congressen onder andere op The Endocrine Society, ENEA Workshop, Dutch Endocrine Meeting, The EYES (European Young Endocrine Scientists) and The European Congress of Endocrinology, waarvoor haar enkele reisbeurzen en prijzen werden toegekend. In 2013 organiseerde zij de eerste EYES meeting in Rotterdam. Daarnaast runde zij ruim een jaar zelfstandig de polikliniek voor de "Lange Termijn Registratie na overleving van kinderkanker" of te wel de LATER-polikliniek en kreeg zij ruimschoots de kans om ervaring op te doen met patiëntenzorg op de Clinical Research Unit voor acromegalie patiënten.

In mei 2016 begon zij als arts-assistent niet in opleiding binnen de interne geneeskunde in het Reinier de Graaf gasthuis te Delft. Per januari 2017 is zij gestart met de opleiding interne geneeskunde in ditzelfde ziekenhuis.

PHD PORTFOLIO

Name PhD student:	S.E. (Sanne) Franck
Erasmus MC department:	Internal Medicine-Endocrinology section
Research School:	Netherlands Institute of Health Sciences
PhD period:	September 2012-April 2016
Promotor:	Prof. dr. A.J. van der Lely
Co-promotor:	Dr. S.J.C.M.M. Neggers

	Year	ECTS
General academic courses		
BROK (Basiscursus Regelgeving Klinisch Onderzoek) (clinical research course)	2014	2.0
Research Integrity Course	2014	0.3
English Biomedical Writing and Communication Course	2014	2
Research skills		
NIHES: Bio statistical methods I: basic principles	2013	5.7
Molmed: Basic introduction course on SPSS	2012	1
(Inter)national conferences-oral presentations		
Somatostatin Receptor Expression in GH-Secreting Pituitary Adenomas Treated with Long-Acting Somatostatin Analogues in Combination with Pegvisomant 4th ENEA Workshop: Acromegaly, Marseille, France	2015	1
Pegvisomant in Combination with Long-Acting Somatostatin Analogues in Acromegaly: the Role of the Growth Hormone Receptor Deletion of Exon 3 5th Dutch Endocrine Meeting, Noordwijkerhout, the Netherlands	2015	1
1ste JNVE congress, Amsterdam, the Netherlands	2014	1
2nd international EYES congress, Belgrado, Serbia	2014	1
ENDO retreat, Chicago, USA	2014	1

	Year	ECTS
Long-Term Efficacy and Safety of Pegvisomant in Combination with Long-Acting Somatostatin Analogues in Acromegaly		
4th Dutch Endocrine Meeting, Noordwijkerhout, the Netherlands, two oral presentations	2014	1
95th Meeting of the Endocrine Society, San Francisco, USA	2013	1
(Inter)national conferences-poster presentations		
The Effect of the Exon 3-Deleted GH-Receptor in Pegvisomant-Treated Acromegaly: a Systematic Review and Meta-Analysis		
Internal Medicine Science Days, Antwerp, Belgium	2016	1
Somatostatin Receptor Expression in GH-Secreting Pituitary Adenomas Treated with Long-Acting Somatostatin Analogues in Combination with Pegvisomant		
97th Meeting of the Endocrine Society, San Diego, USA	2015	1
Internal Medicine Science Days, Antwerp, Belgium	2015	1
Pegvisomant in Combination with Long-Acting Somatostatin Analogues in Acromegaly: the Role of the Growth Hormone Receptor Deletion of Exon 3		
96th Meeting of the Endocrine Society, Chicago, USA	2014	1
Internal Medicine Science Days, Antwerp, Belgium	2014	1
Long-Term Efficacy and Safety of Pegvisomant in Combination with Long-Acting Somatostatin Analogues in Acromegaly		
95th Meeting of the Endocrine Society, San Francisco, USA Two poster presentations	2013	1
15th European Congress of Endocrinology, Copenhagen, Denmark	2013	1
Internal Medicine Science Days, Antwerp, Belgium	2013	1
Clinical meetings/projects/participation		
Weekly grand round endocrinology department	2012-2015	6

	Year	ECTS
Weekly scientific meeting/work discussion endocrinology department	2013-2016	1
Outpatient clinic for Long-Term effects after childhood cancer	2013-2014	6
Outpatient clinic for acromegaly patients (Clinical Research Unit)	2014-2015	6

Symposia, seminars & workshops		
European multidisciplinary course on pituitary tumors, Annecy, France	2015	1
International Erasmus neuro-endocrinology course on pituitary diseases, Rotterdam, the Netherlands	2013-2014	1
PAN European Acromegaly Meeting, Rome, Italy	2013	1
11th Dutch Neuro-endocrinology symposium, Utrecht, the Netherlands Pathophysiology and treatment of pituitary adenomas	2013	1
Bi-annual regional endocrinology meeting, Rotterdam, the Netherlands	2012-2015	3

Teaching activities		
Teaching skills training 1st year medical students *Subject: Hypercortisolism and Diabetes Mellitus type II* Erasmus Medical University Center, Rotterdam, the Netherlands	2015	1
Teaching skills training 1st year medical students *Subject: Thyroid* Erasmus Medical University Center, Rotterdam, the Netherlands	2013-2014	2
Teaching basic course endocrinology for nurses *Subject: Hypothalamus and pituitary* Radboud care academy, Nijmegen, the Netherlands	2013-2015	3

Congress organization		
1st International EYES congress, Rotterdam, the Netherlands	2013	6

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2. **Franck SE**, van der Lely AJ, Neggers SJ. Extra-hepatic acromegaly. US endocrinology 2013; 9(1)

3. **Franck SE***, Neggers SJ*, de Rooij FW, Dallenga AH, Poublon RM, Feelders RA, Janssen JA, Buchfelder M, Hofland LJ, Jorgensen JO, van der Lely AJ. Long-Term Efficacy and Safety of Pegvisomant in Combination with Long-Acting Somatostatin Analogues in Acromegaly. The Journal of clinical endocrinology and metabolism 2014; 99:3644-3652

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5. **Franck SE***, Muhammad A*, van der Lely AJ, Neggers SJ. Combination Treatment of Long Acting Somatostatin Analogues and Pegvisomant in Acromegaly. Endocrine 2016; 52(2): 206-13

6. **Franck SE***, Gatto F*, van der Lely AJ, Hofland LJ, Neggers SJ. Efficacy of Medical Treatment in Relation to the Expression of Somatostatin Receptors in GH-Secreting Adenomas. Neuroendocrinology 2016; [Epub ahead of print]

7. **Franck SE**, Broer L, van der Lely AJ, Kamenický P, Bernabeu I, Malchiodi E, Delhanty PJ, Rivadeneira F, Neggers SJ. The Effect of the Growth Hormone Receptor Deletion of Exon 3 in Acromegaly Treated with Pegvisomant: a Systematic Review and Meta-Analysis. Neuroendocrinology 2016; [Epub ahead of print]

8. **Franck SE**, Korevaar TI, Petrossians P, Daly AF, Chanson P, Jaffrain-Réa ML, Brue T, Stalla GK, Carvalho D, Colao A, Hána V Jr., Delemer B, Fajardo C, van der Lely AJ, Beckers A**, Neggers SJ**. A Multivariable Prediction Model for Pegvisomant Dosing: Monotherapy and in Combination with Long-Acting Somatostatin Analogues. European Journal of Endocrinology 2016; [Epub ahead of print]

9. Gatto F, Feelders RA, **Franck SE**, van Koetsveld PM, Dogan F, Kros JM, Neggers SJ, van der Lely AJ, Lamberts SW, Ferone D, Hofland LJ. An *in vitro* Head-to-Head Comparison Between Octreotide and Pasireotide in GH-Secreting Pituitary Adenomas and the Role of Somatostatin Receptor Expression. Submitted

* Shared first authorship
** Shared last authorship

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