**Background and aims:**

Somatotroph adenoma morbidity results mainly from excessive hormone secretion and mass effects. The most common germline mutation affects the gene encoding aryl hydrocarbon receptor interacting protein (*AIP*) with 17-20% of patients with familial isolated pituitary adenomas (FIPA) and 23% of childhood-onset with no family history.

A recent meta-analysis showed that only 55% of patients treated with first-generation somatostatin analogues (SSAs) for more than 3 months achieved IGF-1 normalisation, and approximately half the patients develop SSAs resistance. Patients with *AIP* mutations or low AIP protein expression have adenomas with reduced SSAs responsiveness, while good response is seen with 30-40% of sporadic somatotrophinomas harbouring somatic gain-of-function *GNAS* mutations.

First-generation SSAs (octreotide) bind selectively to SSTR2 and, to a lesser extent, SSTR5. A second-generation SSA, pasireotide, has higher and lower affinities for SSTR5 and SSTR2 respectively. Pasireotide has higher efficacy compared to octreotide in treatment-naïve patients and in patients with inadequate control on octreotide.

There are two hypotheses suggesting the link of SSTR2 analogues and AIP: (i) octreotide mediates its effect through AIP and ZAC1 with the suggested involvement of SHP1-PI3K-PDK1-AKT-GSK3 pathway. ZAC1 regulates an imprinted gene network including Igf2/H19/Cdkn1c/Dlk1. (ii) Reduced inhibitory G-alpha-protein-2 (Gαi-2) levels present in AIP deficient somatotroph cells can also mediate SSAs resistance. Targeting resistance can help patients avoiding repetitive surgeries and the risks associated with radiotherapies.

*Aims*:

1. To compare adenoma development, IGF-1, prolactin, and weights following administration of pasireotide, octreotide and vehicle in *Aip*-knockout and *Gnas*-mutated mice.

2. To identify differentially expressed genes via RNA sequencing in pasireotide and octreotide-treated *Aip*-knockout and *Gnas*-mutated mice.

3. To compare expression of Gαi-2, ZAC1 and members of the imprinted gene network at the mRNA and protein levels.

4. To compare expression of SSTR2 and SSTR5 at the mRNA and protein levels in *Aip*-knockout and *Gnas*-mutated mice.

5. We will confirm the key changes observed in the animal samples in human somatotrophinoma samples from patients with germline *AIP* mutations who were pre-treated with SSA before surgery. Although the availability of this tissue is extremely limited, we have currently 4 such samples.

**Results**:

Mice were bred to generate the pituitary-specific knockout animals, *Aip*Flox/Flox; *Hesx1Cre/+* (AIP-KO). We have now a large colony and during the limited time of the project we generated a large amount of data on these animals. Because of unforeseeable circumstances, Covid-19 pandemic, difficulties in re-deriving the animals, pinworm infection, and difficulties in breeding the GH-Cre animals which have unexpectedly 75% less pups per litter, the experiments have been performed only on the SSA-resistant mouse model, the pituitary-specific AIP-KO (*Aip*Flox/Flox; *Hesx1Cre/+*). We are currently generating the GNAS animals and performing the planned studies.

Ten AIP-KO animals per group were randomly assigned to the 3 treatments (vehicle, octreotide-LAR 30mg/kg or pasireotide-LAR 60mg/kg, monthly injections). Only males were used to reduce variability. Based on our previous data, AIP-KO animals start to develop pituitary hyperplasia at embryonic day 18.5, at 3 weeks of age they start to show elevated IGF-1 levels and starting at 3 months of age the animals show an increased body weight. By 15 months more than 85% of the animals will develop pituitary tumours, and consistently with the human phenotype, 85% of these tumours are secreting GH. Since by 3 months of age the animals developed hyperplasia, high IGF-1, and increased body weight, we decided to start the treatment with SSAs at this age (**Figure 1**).

As expected, the body weight in our AIP-KO steadily increased in the vehicle treated animals reaching a 40% increase by 7.5 months of age. When treated with octreotide, the increase is reduced to 23% providing a slight improvement. However, when treated with pasireotide the body weight decreased to levels similar to the wild-type animals with an increase of only 7.7% at 7.5 months of age (**Figure 2**).

We also measured the IGF-1 levels prior treatment (**Figure 3**, left) ensuring similar starting levels in all treatment groups, and after treatment with SSAs (**Figure 3**, right). Only a slight reduction was observed in the octreotide-treated group, 6.6%, while a significant reduction upon pasireotide treatment, 30%, was observed.

SST2 locates more on the membrane on the tumour cells (**Figure 4**), and SST5 mRNA is highly expressed in the tumour tissues (**Figure 5**). Surprisingly we did not appreciate a difference in ZAC1 protein levels, but we observed a more nuclear staining in the wild-type animals compared to the more cytoplasmatic staining in the Aip-KO (**Figure 6**).

We assessed the differences in RNA expression via qPCR following SSAs treatments of the somatostatin receptors and we observed a significant reduction of Sst2 and Sst5 only upon pasireotide treatment (**Figure 7**).

We have performed RNAseq analysis on AIP-KO animals upon treatment with SSAs to shed light on the resistance mechanisms, 27 animals in total. We have compared these data to our gene expression analyses in human AIPpos tumours and normal pituitaries, 5 samples per group (**Figure 8**). Unexpectedly, neither Gαi2 protein nor Zac1 were in the deregulated genes upon SSAs treatments, log fold change -0.007 and -0.4, respectively. Similarly, filamin A (FLNA), a known factor involved in SSAs resistance in human samples, was not altered, log fold change -0.26 (data not shown). However, FLNA in mainly regulated at its protein level and we decided to investigate the protein levels. Strikingly, we discover that FLNA was downregulated in the tumour samples compared to wild-type (**Figure 9**). FLNA is a cytoskeletal protein which is recruited by SST2. FLNA can inhibit SST2 degradation, maintaining stability after prolonged stimulation. The deregulation of FLNA in AIP-deficient cells could explain the difference in response between octreotide and pasireotide, as FLNA affect primarily SST2 and pasireotide binds more effectively to SST5.

Furthermore, among the 23 genes commonly dysregulated in the two groups (**Figure 8**), we are currently analysing multiple candidates. These could provide novel insight and possibly they will be useful either as markers or to design novel therapies.