**The common c.-32-13T>G splicing variant of GAA gene: from functional characterization to the identification of new therapeutic targets for Pompe disease**
January 23, 2020 webinar presented by Andrea Dardis, PhD, Regional Coordinator, Regional Centre for Rare Diseases, Academic Hospital of Udine, Italy

Dr. Dardis thanked the webinar organizers, expressing regrets for missing the AMDA/IPA International Pompe Patient and Scientific Conference in October 2019, for which she prepared this presentation. She also noted that her presentation reflects collaboration between the International Centre for Genetic Engineering and Biotechnology and Regional Coordinator Centre for Rare Diseases, Academic Hospital Santa Maria della Misericordia, Italy.

Dr. Dardis briefly reviewed the role of GAA gene deficiency in Pompe disease (Glycogen Storage Disease II or GSDII) leading to deficient activity of the GAA enzyme causing accumulation of glycogen in lysosomes and cellular damage. The GAA gene is located on human chromosome 17q25.2-25.3 and contains 20 exons (an exon is any part of the gene that will encode part of the final mature RNA produced by the gene after introns are removed by RNA splicing). There are more than 500 identified mutations (http://www.hgmd.cf.ac.uk), most of which are private, involving small numbers of families. However, the c.-32-13T>G (IVS1) mutation is present in 40-70% of the alleles (alternate form of gene due to mutation) of patients with late-onset GSDII. Because of its frequency, this mutation, described 20 years ago as a splice mutation, is a subject of great interest. For a gene to be able to synthesize a protein, it must be transcribed by Messenger RNA (mRNA), a single-stranded RNA molecule that is complementary to one of the DNA strands of a gene. The mRNA is an RNA version of the gene that leaves the cell nucleus and moves to the cytoplasm where proteins are made. RNA splicing is critical to get the correct transcription. A genetic change can lead to wrong splicing in RNA. With the c.-32-13T>G (IVS1) mutation in the GAA gene, the splicing mutation in intron 1 of the GAA gene prevents efficient recognition of exon 2 by the spliceosome. The overall GAA exon 2 splicing efficiency is affected. Almost all late-onset GSD II patients have this mutation in at least one allele. Some patients express up to 25% of normal GAA activity and just a little increase in exon inclusion might be enough to achieve a beneficial effect. The question then is how to increase the efficiency of splicing?

Dr. Dardis discussed several approaches involving the use of antisense oligonucleotides (ASO) to increase the efficiency of splicing (Goina E, Peruzzo P, Bembi B, Dardis A, & Buratti E. Glycogen reduction in myotubes of late-onset Pompe disease patients using antisense technology. *Molecular Therapy*. 6 September 2017; 25(9):2117-2128). In searching for silencer elements within exon 2 of GAA for ASO development, she and other researchers looked for regulatory splicing elements by creating a series of seven overlapping deletion constructs. They found that the deletions of two regions of exon 2 resulted in the increase of exon inclusion, thus mapping the splicing silencers of GAA exon 2. Using a minigene approach, they increased inclusion of exon 2 in the mRNA and GAA enzyme production by targeting a specific silencer with a combination of antisense morpholino oligonucleotides (AMOs) they developed to block the silencer sequence regions to establish equilibrium of positive and negative factors and rescue normal splicing in the presence of the c.-32-13T > G mutation. The combination of AMO 1, 2, & 3 resulted in a 2.5-fold increase in exon 2 inclusion. They tested this combination on patient-derived fibroblasts (cells most commonly found in the body's connective tissue) resulting in successful rescue of normally spliced GAA mRNA. Dr. Dardis noted that they were able to increase expression of normal mRNA, about 30% of total GAA mRNA, and a 50% increase in GAA enzyme activity. A similar effect on exon 2 inclusion was observed in patient-derived myotubes (developmental stage of muscle fibers).

They were able to partially restore the correctly spliced variant of GAA mRNA so that, again, 30% of total GAA mRNA was represented by normal spliced GAA mRNA, leading to a 40% increase in GAA enzyme activity in these myotubes and, more importantly, a significant reduction of glycogen storage. Dr. Dardis commented that this strategy worked very well in vitro.

Dr. Dardis discussed another approach to improving efficiency of splicing using small molecules that can increase GAA expression activity. She described designing a high throughput in vitro system to screen for small molecules, given there are hundreds of thousands of these molecules. They cloned the exon 2 of GAA including the mutation using a fluorescence process so exon 2 was excluded from mRNA. The aim was to identify molecules that decrease fluorescence. Accessing libraries of small molecules, including 1280 FDA-approved small molecules (Preswick library), multiple rounds of screening were conducted. Ultimately, one small molecule, Defe, modulated by iron availability, was identified that increased exon 2 inclusion and GAA expression activity. Dr. Dardis described validation of Defe in fibroblasts with the c.-32-13T>G mutation with results indicating a 50% increase in enzymatic activity. However, the effect of Defe is reverted by iron overload in the body (iron is a vital component of hemoglobin, the substance in red blood cells that transport oxygen in the body). This raised the question as to whether the effect of Defe on exon 2 inclusion is mediated by Hypoxia Inducible Factor 1-alpha (HIF1-a), a protein-coding gene associated with oxygen delivery and metabolic adaptation to hypoxia. While HIF1-a does not mediate the effect of Defe on exon 2 inclusion, silencing of this gene partially reverts the effect of Defe on GAA activity while induction of it increases efficiency of splicing mRNA and GAA enzymatic activity. The mechanism for how HIF1-a induction increases residual GAA enzymatic activity remains to be determined. Dr. Dardis described identifying other molecules in their phytochemical and fragment libraries, five in each, that also decrease fluorescence, work that is currently being validated. Future steps in research include: (1) validation of phytochemical and fragment library hits; (2) better characterization of the mechanism of action of Defe on GAA; (3) identification of new therapeutic targets; and (4) validation of the effect of Defe in muscle cells with the aim of increasing inclusion and at least partially decreasing glycogen accumulation.

Dr. Dardis acknowledged her colleagues at the Regional Center for Rare Diseases and the Molecular Pathology Laboratory, in particular, Dr. Emanuele Buratti, as well as her sponsors, including AMDA. She opened the webinar to questions:

Question: Is it possible to correlate the amount of correctly spliced ISV1 with patient function?
Answer: Yes, in theory, although the amount of splicing could be different, in, for example, fibroblasts vs. muscle cells. There are other factors to consider.

Question: What is the potential clinical relevance of iron overload in patients who might receive treatment with Defe? Would therapy be most effective in patients not receiving iron supplementation for example? Answer: We don't know and need to better understand the mechanism.

Question: Have you considered evaluating Defe in the Pompe mouse model? Are there any existing mouse models that may work? Answer: The mouse model is a problem because it is a knock-out mouse that doesn't have this specific mutation. We would have to develop a mouse model.