Lessons Learned from the Clinical Development and Market Authorization of Glybera

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Introduction

Development of the first commercially-approved gene therapy product in the West represents a remarkable medical achievement. The product Glybera, or alipogene tiparvovec, is based on an adeno-associated virus (AAV) vector to replace the gene responsible for the expression of lipoprotein lipase (LPL), which is deficient in some patients who have extremely elevated levels of serum triglycerides causing recurrent and life threatening pancreatitis. Glybera received marketing authorization for a subset of patients with LPL deficiency from the European Commission (EC) in November 2012. A summary of the early pre-clinical research forming the basis for the clinical development of this product was recently reviewed by the principal scientists in *Human Gene Therapy* (Kastelein et al., 2013).

The goal of this paper is to chronicle the clinical development of Glybera from proof-of-concept in animal models through review and eventual authorization by the EMA. As is the case with most pioneering efforts, the path to success was not easy and was associated with multiple twists and turns, rejections and iterations. Our review and critique of this process was made possible because of the incredible transparency that the scientists, physicians and regulators provided about the pre-clinical and clinical data and the deliberations of the EMA and its associated committees. The research teams published virtually every relevant pre-clinical and clinical experiment in peer-reviewed journals, and the EMA made available detailed summaries of its reviews. This paper provides background about the disease and the vector, followed by summaries and critiques about the pre-clinical and clinical studies and the multiple iterations of regulatory reviews.
The Disease

Familial lipoprotein lipase deficiency (LPLD) is a rare, autosomal recessive disorder affecting about 1 person per million (Brunzell 1999). The underlying pathophysiology of LPLD is a lack of functionally active lipoprotein lipase (LPL), which plays an important role in triglyceride (TG), chylomicron (CM), and very low density lipoprotein (VLDL) metabolism (Figure 1). LPL is produced primarily in skeletal muscle and adipose tissue, where it is secreted as a homodimer. This active homodimer is then transported across the vascular endothelium and immobilized on the luminal surface of blood vessels. In the vascular lumen, LPL interacts with circulating chylomicrons, the triglyceride-rich particles that carry lipids absorbed by the intestine. LPL converts TGs found in the core of chylomicrons into free fatty acids (FFA) that can be taken up and used by peripheral tissues. The chylomicron remnant is then endocytosed by hepatocytes via interaction with apolipoprotein E on the chylomicron surface. The chylomicron remnant is subsequently salvaged and repackaged into VLDL by the liver and secreted into the plasma to supply endogenously synthesized lipids to the peripheral tissues. LPL then participates in the conversion of VLDL to LDL in the plasma (Mead et al., 2002). Without LPL, chylomicrons cannot be depleted of their dietary fats, leading to severe hypertriglyceridemia and hyperchylomicronemia. These lipids then accumulate and lead to pathology in various tissues, although the exact relationship between lipid accumulation and disease presentation has not been established.

The first symptoms of LPLD present during childhood as severe abdominal pain or failure to thrive, and patients may exhibit eruptive xanthomas, lipemia retinalis, and hepatosplenomegaly. The most severe manifestation of LPLD is acute pancreatitis, which is the primary cause of morbidity and mortality in these patients. (Frossard et al., 2008). Recurrent
pancreatitis can lead to exocrine pancreatic insufficiency and diabetes. Despite the presence of these outward symptoms, the diagnosis of LPLD is dependent on molecular sequencing of the gene, as the phenotype cannot be clinically distinguished from other causes of hyperchylomicronemia. The currently accepted treatment for LPLD includes severe dietary restriction of fat intake to 10-15% of total daily calories and supplementation with medium-chain triglycerides, which are metabolized through a chylomicron-independent pathway. Notably, even with strict compliance to the diet and close monitoring by clinicians, TG levels rarely drop out of the range (greater than 1000-2000 mg/dL) that is considered to confer an elevated risk of pancreatitis (Gaudet et al., 2010; Yadav and Pitchumoni 2003). Therefore, there is a clear need for an effective treatment for LPLD.

Vector

A naturally occurring truncated human LPL variant, LPL<sup>S447X</sup>, was selected as a transgene due to its higher activity in vivo (Ross et al., 2005). To deliver the 1.9kb LPL<sup>S447X</sup> transgene (Gotoda et al., 1989), an adeno-associated viral vector (AAV) was selected. Adeno-associated viruses, first described in 1965 (Atchison et al., 1965), are nonpathogenic, single-stranded DNA viruses with genomes of approximately 4.7kb. To date, dozens of AAVs have been isolated from a variety of mammalian species (Asokan et al., 2012). Of these, AAV2, a common human isolate, has been the most utilized vector for research purposes. As a result, it is also the most characterized AAV serotype in regards to its safety profile as a vehicle for transgene delivery in human gene therapy studies.

The AAV genome is composed of two inverted terminal repeats (ITRs) which flank two viral genes, <i>rep</i> and <i>cap</i>. While the ITRs are required, cis-acting elements, the non-structural <i>rep-
encoded proteins and the structural cap-encoded proteins, responsible for viral replication and tropism, respectively, can be supplied in trans during virus production (Asokan et al., 2012). This not only allows for a large portion of the AAV genome to be replaced with a therapeutic transgene, but also the packaging of an AAV2-derived genome by CAP proteins from other serotypes, resulting in AAV2 with altered tropism and immunogenicity. The recombinant vector used for this study, AAV1-LPL$^{S447X}$, was produced in this way, using CAP proteins from an AAV serotype 1 and ITRs from AAV2 (Rip et al., 2005).

The first infectious clone of AAV1 was characterized in 1999 in Jim Wilson’s laboratory by post-doctoral fellow, Weidong Xiao, in an effort to develop AAV vectors with improved performance over that achieved with AAV2 (Xiao et al., 1999). The frequency of neutralizing antibody (NAB) to AAV1 in normal human subjects was less than that observed with AAV2. In addition to the lower sero-prevalence, AAV1 vectors transduce skeletal muscle with substantially higher efficiency than what is observed with AAV2. Given that skeletal muscle is a site for physiologic expression of LPL, the efficiency of transduction in this tissue made AAV1 an attractive vector for delivery of the LPL transgene (Rip et al., 2005).

**Preclinical**

**Preclinical Models**

Two LPL-deficient animal models were used for preclinical testing of AAV1- LPL$^{S447X}$: an LPL knockout mouse and a naturally-occurring feline model carrying a mutant allele encoding a catalytically inactive LPL protein. A summary of the salient features of these animal models is provided in Table 1. Development of the LPL knockout mouse model was complicated by the fact that complete LPLD is fatal in the neonatal period in this species. Rescue of LPL$^{−/−}$ mice at
birth with intramuscular delivery of an adenoviral vector carrying a human LPL$^{S447X}$ transgene prevented immediate lethality and allowed mice to survive to adulthood. Rescued adult mice developed an LPL-deficient phenotype, which included hypertriglyceridemia, high LDL cholesterol and low HDL cholesterol. These Ad-LPL$^{S447X}$ rescued mice were utilized for the initial studies of the efficacy of AAV-mediated delivery of the LPL$^{S447X}$ transgene. While the development of these mice provided a convenient model to study changes in disease-specific parameters in response to AAV1-LPL$^{S447X}$, interpretation of the results is compromised by residual expression of low levels of human LPL from the initial Ad-mediated rescue. This model is further complicated by the presence of significant local muscle pathology including fiber degeneration, lipid accumulation, and collagen deposition, which may have resulted from the initial vector delivery or from localized LPL overexpression.

The second preclinical model of LPLD studied was an LPL-deficient cat (Ross et al., 2006), (Table 1). These animals are naturally homozygous for an LPL$^{G412R}$ mutation that results in complete lack of catalytic function and vastly elevated serum triglycerides. Unlike the LPL$^{-/-}$ mice, the LPL-deficient cats do not require additional LPL rescue to survive to adulthood, which is more similar to the human disease state (Table 1). The LPLD cats also recapitulate the most serious clinical sequelae of LPLD—severe, recurrent pancreatitis—and lack the unusual muscle phenotype present in the murine model.

**Efficacy and stability in preclinical models**

In both the murine and feline preclinical studies of AAV1-LPL$^{S447X}$ gene therapy, transgene expression and activity were primarily evaluated indirectly via reductions in total serum triglycerides and resolution of the visible turbidity of the lipemic plasma that is characteristic of
LPLD. While LPL protein and activity were also directly measured in serum immediately following administration of heparin, which liberates the enzyme from its heparan sulfate scaffold on the vascular endothelium, reduction in serum triglycerides proved to be a more sensitive marker for the presence of LPL. Notably, chylomicron metabolism, the primary aberrant pathway associated with LPLD (Figure 1), was not characterized in animal models. A potential reduction in pancreatitis was also not evaluated, given that the duration of the feline studies was insufficient to effect notable changes in the progression of the pancreatic pathology observed in LPLD cats. However, given the known association between serum triglycerides and pancreatitis in humans, it was assumed that triglycerides would provide a useful surrogate marker for a reduction in pancreatitis risk.

In the first study of gene therapy in the LPLD mouse, neonates rescued with Ad-LPL were treated at 10-16 months of age with AAV1-LPLS447X [(Ross et al., 2004) and Table 2]. Two cohorts of animals were studied each receiving a different dose of vector (8x10^{11} GC/kg and 8x10^{12} GC/kg) injected intramuscularly. The low dose group exhibited a modest decrease in triglycerides without detectable serum LPL protein, while the high dose animals had detectable serum LPL protein, as well as a reduction in serum triglycerides to levels observed in heterozygous mice associated with resolution of visible plasma lipemia. Though post-heparin serum LPL protein gradually declined, correction of serum triglycerides persisted for the duration of the 52 week study. Treated mice demonstrated rapid clearance of an intravenous lipid bolus 8 months after vector administration, indicating significant functional LPL expression (Table 3). These results determined the minimally effective dose (MED) for AAV1-LPLS447 at which a significant decrease in serum TGs levels was observed in mice to be 8x10^{11} GC/kg.
A subsequent study was performed in the feline model of LPLD. Animals treated with doses as low as 1x10^{11} GC/kg exhibited a rapid correction of serum triglycerides to levels equivalent to those of LPL^{+/-} cats (Table 2). These results were complicated by the presence of a robust humoral immune response to the transgene product, which limited the duration of the effect to several weeks. The addition of a cyclophosphamide regimen only modestly attenuated this response; therefore, long-term evaluation of expression and efficacy was not carried out in this model. The use of a truncated human LPL transgene in the feline model complicated the interpretation of the humoral immune response which is a potential complication in human studies. However, the emergence of these responses in the cat model made it impossible to evaluate the longevity of the treatment and its long-term impact on efficacy and safety. Another noteworthy observation in the feline model is a minimally effective dose 8-fold lower than that identified in mice (Table 3). Possible reasons include species differences in LPL substrate binding affinity, variation in efficiency of transgene expression, changes in vector tropism, and variability in the half-life of LPL protein. The discrepancy could also be explained by other differences in the two models, including preexisting muscle damage in the mouse, the presence of baseline LPL^{S447X} in control and experimental mice, and differences in the routes of administration (Tables 1 and 2).

**Safety in preclinical models**

Formal assessments of safety were conducted in preparation for the first clinical trial under conditions of Good Laboratory Practices (GLP) (Rip 2005). Wild type mice in a C57BL/6 background were selected for an evaluation of maximally tolerated dose (MTD) and dose-limiting toxicities. Three dosages of vector were administered to male and female mice with the
highest dose of $1 \times 10^{13}$ GC/kg being 100-fold greater than the MED used in the feline efficacy study. Up to 90 days post-treatment, the authors reported no significant changes in the health of the mice or their eating patterns. While the overall health of the mice was not affected, minor inflammation was observed in muscle histology sections at injection sites and female mice were found to have a reduced body weight gain of about 30%, hypothesized to be due to increased energy expenditure after treatment. The investigators concluded that the treatment is safe and MTD was not achieved even at the highest dose that could be administered. To determine the bio-distribution of the vector, two doses, $1 \times 10^{11}$ and $1 \times 10^{13}$ GC/kg, were given to male and female mice. Tissue samples of the injected mice were analyzed for vector DNA at 7, 28, and 90 days following injection. At the 90 day time point, vector DNA was observed in the highest concentration at the injection site but also observed in the liver and inguinal lymph nodes. In addition, mice were also observed to have small, but detectible, dose-dependent levels of vector DNA in the gonads at the day 90 time point.

**Summary of Glybera Clinical Trial Data**

**AMT-010-01**

This first safety and efficacy trial for AAV1-LPL$^{S447X}$ was initiated by Amsterdam Molecular Therapeutics in July 2005 (Figure 2). Eight subjects met the eligibility criteria, which included 1) LPL activity $\leq$20 % of normal, 2) mutations in the gene coding for LPL, 3) an LPL mass $>5$% of normal, and 4) TG levels $>10$ mmol/L. Prior to vector delivery, subjects participated in an observational study (PREP-01) during which they maintained a low fat diet controlled by a dietician in order to assess baseline levels of TGs. These data showed that dietary control did not lead to significant reduction or stabilization of serum triglycerides (EMA 2012, p. 41). After
completion of the observational study, the subjects were split into two dose cohorts, with the low
dose cohort receiving $1 \times 10^{11}$ GC/kg AAV1-LPL$^{S447X}$ and the high dose cohort receiving $3 \times 10^{11}$
GC/kg. The vector was administered via multiple injection, either 40 or 60 injections, depending
on dose cohort, into the quadriceps muscles. The primary objective of the trial was to establish
efficacy and safety of AAV1-mediated delivery of LPL. The primary efficacy endpoint was
defined as a reduction in individual median fasting plasma TG to a level $\leq 10$ mmol/L or a
reduction in median fasting plasma TG greater than or equal to 40%. The subjects were
evaluated at 12 weeks and then again at 18 to 31 months after vector administration for a long-
term follow-up. No serious adverse events were reported over the evaluation period, and the
most common adverse event reported was general muscle pain at the injection site. At 12 weeks
after vector administration, 50% of subjects had reached the primary efficacy endpoint, which
was a median decrease in TG levels compared to baseline TGs. LPL protein and activity were
detected in muscle biopsies taken between 26 and 36 weeks after administration, but there was
no discussion of LPL detection in the blood. However, the long-term follow-up showed a loss in
efficacy with regards to TG levels that was attributed to an immune response to the AAV1
capsid, as CD8(+) T-cell responses against the AAV capsid have previously been reported to
affect transgene expression in a trial of AAV2 to liver of patients with hemophilia B (Mingozzi
et al., 2003). However, antibodies against the transgene were not detected. Conclusions reached
from AMT-010-01 included the need for a higher dose to boost transgene expression and the use
of an immunosuppressive regimen to curtail the immune response to the vector. Overall, the
effect of the therapy appeared to be transient based on serum triglycerides, which highlights one
of the chief concerns about this product being limited to a single administration (Stroes et al.,
2008).
AMT-011-01

In August of 2007, after completion of AMT-010-01, Amsterdam Molecular Therapeutics initiated a second trial, AMT-011-1 (enumerated differently to reflect a changed manufacturing process), to assess efficacy and long-term safety in a larger number of subjects [(2010) and Figure 2]. Importantly, the HEK293 system used to manufacture AAV1-LPL$^{S447X}$ for the first study was replaced by a baculovirus production system to increase vector yield, despite the fact that this slightly decreased the potency of the vector in vivo in mice and by up to four-fold in vitro (EMA 2012, p. 25). This final AAV1-LPL$^{S447X}$ therapeutic vector is referred to as alipogene tiparvovec. Similar to the first trial, participants were first enrolled in the PREP-02 observational study to be monitored for a minimum of 4 months prior to the clinical study. In addition to the prior eligibility criteria, participants were also required to have a history of pancreatitis in order to examine the effect of Glybera on clinical outcomes. Fourteen of the 22 subjects enrolled in the observational study received a single series of injections of Glybera. Six subjects received a dose of $3 \times 10^{11}$ GC/kg, four of whom were placed on an immunosuppressive regimen consisting of 3 mg/kg of cyclosporine A per day and 2 g per day of mycophenolate mofetil in an attempt to attenuate potential cellular immune responses against the vector or the LPL transgene. Eight subjects received a dose of $1 \times 10^{12}$ GC/kg and were also placed on immunosuppression, which was initiated at the time of injection and discontinued at 12 weeks. Seven subjects consented to a muscle biopsy at 26 weeks.

Seven subjects (half of the total) met the primary endpoint for this study, a reduction in median plasma TG of at least 40% between 3 and 12 weeks. Four of those subjects also met the secondary efficacy endpoint of a median fasting plasma TG level $\leq 10$ mmol/L between 3 and 12
weeks. TG reductions were statistically significant in the study group as a whole as well as for the high dose group; however, by 26 weeks, plasma TG in all subjects returned to baseline, was interpreted to mean the immune suppression did not increase the duration of efficacy of Glybera.

All subjects experienced a mild to moderate injection site reaction, and muscle biopsies at 26 weeks demonstrated lipid accumulation and immune infiltrates at the injection site. The authors noted that severity of the injection site reaction appeared to be correlated with the level of LPL expression in the muscle tissue, but no quantitative relationship was identified. There were several adverse events reported during two years of follow-up, but none were attributed to vector administration (See section: Glybera Approval). In addition, shedding of the virus in saliva, urine, and semen were minimal and dropped below the level of detection by week 10; the conclusion was that alipogene tiparvovec is considered safe.

Because of the transient effect on plasma TG, the authors also examined post-treatment chylomicron and VLDL lipoprotein fractions in order to determine whether Glybera may have a prolonged effect on another aspect of lipoprotein metabolism. In the high dose cohort, VLDL was significantly increased at 12 weeks post-injection. In addition, total cholesterol and TG content in the VLDL fractions were increased at 12 and 52 weeks along with corresponding decreases in total cholesterol and TG content of the chylomicron fraction. Because LPL is an essential enzyme in the conversion of chylomicrons to VLDL, these data suggest that there was a sustained increase in plasma LPL activity despite the lack of long-term effect on total plasma TG.

This study was not designed to evaluate overall clinical benefit with regards to specific disease symptoms; however, the authors did record incidence of pancreatitis and self-reported symptoms by the subjects enrolled. One episode of pancreatitis occurred during the PREP study.
and two episodes occurred during 2 years of follow-up. The authors stated that this constitutes a five-fold decrease in pancreatitis incidence after administration of Glybera, as compared to patient history prior to treatment although the numbers are too low to reach statistical significance. Moreover, subjects reported improvement in clinical signs of the disease such as ‘capacity to eat more food’ and ‘increased energy levels’. However, these claims must be interpreted with caution as they are based on a small sample size and subjective reports. Altogether, this clinical trial demonstrated that Glybera is well-tolerated and associated with a transient decrease in total plasma TG and sustained alterations in chylomicron metabolism (Gaudet et al., 2013).

**AMT-011-02**

In February 2009, five LPLD subjects were enrolled in a third trial to measure the effect of Glybera on chylomicron metabolism and to validate the use of postprandial chylomicrons (pp-CM) as a replacement for total plasma TG as a primary efficacy endpoint [(NIH 2009) and Figure 2]. All subjects received a one-time series of injections at a dose of $1 \times 10^{12}$ GC/kg. In addition to the immunosuppression regimen used in AMT-011-01, a single dose of IV methylprednisolone was administered 30 minutes before the vector. The enrollment criteria for the study remained the same as in AMT-011-01.

To assess the effect of Glybera on chylomicron metabolism, the authors performed a challenge study to measure postprandial plasma lipids and metabolites before treatment and 14 weeks after receiving Glybera. Each subject ingested a study meal containing $^{3}$H-Palmitic acid, and hourly blood samples were drawn to analyze tracer in plasma lipoprotein fractions. Data from individuals with LPLD were compared to data from healthy controls for the first six time
points. Prior to dosing, LPL-deficient subjects displayed markedly-elevated postprandial plasma TG levels and increased TG content of the chylomicron fraction. At 14 weeks post-treatment, postprandial chylomicron clearance and triglyceride content of the chylomicron lipoprotein fraction were similar to normal subjects. This study strongly suggests that Glybera results in functional LPL expression 14 weeks after treatment and that this LPL is able to exert an effect on lipid metabolism, despite plasma TG levels typically returning to baseline by this time point.

Three subjects were challenged again at 52 weeks, and again demonstrated accelerated chylomicron clearance, although the effect was less pronounced than at the 14 week timepoint (EMA 2012, p. 56). The results of this study established a new efficacy endpoint and suggest that LPL expression persists despite the transient decrease in plasma TG [(Carpentier et al., 2012); EMA 2012, p. 87].

**AMT-011-03**

Evidence of metabolic efficacy in conjunction with the preliminary evidence that Glybera may reduce the incidence of pancreatitis in LPLD prompted further investigation of the effect of Glybera on pancreatitis. In a retrospective study including 17 of the 22 subjects from all prior clinical trials, the authors reported a decrease in the number of episodes of pancreatitis after treatment when evaluating events during a 3-year period before and after Glybera delivery. When comparing 3 years before and after treatment, the hazard ratio was estimated to be between 0.41 and 0.49. However, the significance of this decrease is dependent on the length of time used for the analysis. This retrospective analysis is further complicated by the variability in the incidence of pancreatitis events, both before and after treatment. While some of the subjects had only single events in the past, 3 subjects accounted for up to 11 of the 41 pre-treatment
events. Several (8 of the 17) subjects were pancreatitis-free after treatment but also had no incidents of pancreatitis in the equivalent time period before treatment (EMA 2012, p. 60). Meanwhile, a majority of the episodes in the follow-up are in 3 subjects. While these data remain unpublished, the EMA review documents include this analysis, stating that it is “insufficient to provide evidence of efficacy for a reduction in the rate of pancreatitis” but that it suggests a potential benefit (EMA 2012). A longer period of follow-up will be necessary to evaluate the effect on acute pancreatitis.

**Approval**

Although multiple pathways for drug approval exist in the European Union, novel biologic agents are required to follow a centralized review process overseen by the European Medicines Agency (EMA; Figure 3). Approval through this pathway leads to a recommendation to the European Commission (EC), which has the authority to grant marketing authorization in all EU member states. Within the EMA, drugs for human use are evaluated by the Committee for Human Medicinal Products (CHMP), which carries out the scientific review and makes final recommendations to the EC for marketing approval. For Glybera, the primary responsibility for review fell on a relatively new subdivision of the EMA, the Committee for Advanced Therapies (CAT), which was formed in 2009 specifically to provide expert evaluation of gene and cell therapies. The CAT is charged with carrying out the initial review of products that fall within its scope and submitting a draft recommendation to the CHMP for (or against) approval. The CHMP provides oversight and input during CAT deliberations and has the authority to adopt or reject a CAT draft opinion. The deliberations of both the CAT and the CHMP are publicly available and have been published (EMA 2012)
In December 2009, AMT submitted the marketing authorization application for Glybera to the EMA (Figure 2). Given that a gene therapy had never been approved in the West, it is noteworthy that two of the most likely sources of concern for this type of product—safety issues related to viral vector-mediated gene transfer, and the process of manufacturing and characterizing a viral vector—did not prove to be major obstacles to approval. The initial review brought up several of these expected theoretical concerns, including insertional mutagenesis, immune toxicity, tumorigenicity, germline transmission of vector sequences, and manufacturing issues such as contaminating DNA sequences and *in vitro* assessment of potency (EMA 2012, p. 30). After some discussion with the applicant and submission of additional data, all of these issues were addressed to the satisfaction of both the CAT and CHMP.

In the examination of the cumulative clinical trial data, safety proved to be of limited concern; most subjects experienced only local discomfort after treatment, and muscle biopsy findings of localized inflammation and changes in fiber architecture were not felt to be clinically significant. However, it was noted that functional tests on injected muscle should have been performed to rule out any lasting effect. One subject was diagnosed with suspected deep venous thrombosis and pulmonary embolism after treatment, which was thought to be potentially related to receiving a large number of intramuscular injections. Another subject experienced transient polyarthritis and myositis at the time of discontinuation of immunosuppression, but this was deemed to have most likely been caused by a preexisting condition. The 12 week course of immunosuppression was identified as a potential source of serious risk, particularly problematic because it had not prevented the observed local inflammatory infiltrate, indicating that its inclusion was unjustified. Still, it became clear through the review process that both the CAT and CHMP would deem the safety risks acceptable so long as efficacy could be demonstrated.
Demonstrating efficacy, however, proved to be the primary hurdle for Glybera’s approval. Based on data from animal models and expert opinion suggesting that serum triglycerides are a useful surrogate marker of pancreatitis risk, reduction in triglycerides was selected as the primary efficacy endpoint in the clinical trials. Problems with this approach became apparent even in the observational studies. In Prep-01 in particular, the CAT noted a high variability of baseline fasting triglyceride levels in these subjects despite their following a strict diet (EMA 2012, p. 41). In the interventional studies, 50% of subjects transiently met the 40% reduction in serum fasting TG level endpoint; however, triglycerides were highly variable throughout the study and improvements were not sustained in any subject. Based upon these data, the CAT stated that “the plasma levels of triglycerides (TGs) are a poor predictor of efficacy”, with which the CHMP “agreed that TG plasma levels are not the appropriate biomarker for efficacy” (EMA 2012 pg. 88). The CAT accepted a change in primary endpoint to functional measures of postprandial chylomicron (pp-CM) metabolism, recognizing the “evolving scientific knowledge” of this rare disease. However, when this endpoint was examined in AMT-011-02, observations were extended to 52 weeks in only 3 subjects, which was not sufficient to establish long-term efficacy (EMA 2012, p. 56). While analysis of the retrospective data by blinded, independent experts based upon Revised Atlanta criteria suggested a reduction in incidence of pancreatitis (AMT-011-03), the CAT and CHMP brought attention to the fact that the majority of pancreatic events occurred in 3 of the 17 subjects they evaluated; therefore, the number of events in the other subjects was too limited to make any robust conclusions (EMA 2012, p. 60). Prior to the initiation of the clinical studies, the EMA had suggested that protocols be put in place to objectively evaluate all potentially relevant clinical parameters (such as abdominal pain and organomegaly). Instead, subjects self-reported improvement in these...
categories, and the fact that AMT relied on these subjective reports was considered to be a “major deficiency” in their application (EMA 2012, p. 52). Since decreases in triglycerides in this trial were transient, and no long term efficacy was seen or reported in an objective fashion, the CAT suggested that it would likely be unacceptable to promote this drug as a life-long cure. In June 2011, based upon the results of the preclinical and clinical trials, the CAT declared that the risk-benefit balance was negative due to the potential risks of multiple muscle injections along with immunosuppressants as well as the lack of accompanying efficacy data. The CHMP agreed, saying that AMT did not sufficiently demonstrate safety and sustained efficacy. They recommended refusal of the Glybera application, citing three particular causes for concern: the lack of evidence for a long-lasting effect on TG or pp-CM levels, insufficient evidence of reduction in incidence of pancreatitis, and significant risks associated with the inclusion of immune suppression, especially with insufficient justification of its inclusion (EMA 2012, p. 85).

In October 2011, AMT requested a reexamination of their application and included rebuttals to the three primary concerns. They acknowledged that TG levels may not be the ideal endpoint for efficacy, but contended that the switch to pp-CM metabolism should resolve this issue. In addition, they committed to a post-approval study of all previously-dosed subjects in order to confirm appropriateness of this endpoint and argued that this should be sufficient for approval. To address the validity of the pancreatitis endpoint, AMT put together a panel of experts in order to more objectively evaluate the pancreatitis data to increase its relevance despite the small number of patients. Clinicians were also consulted to evaluate the relative risk of immunosuppression, which was felt to pose minimal risk given the limited duration of treatment. After consideration of this response, the CAT accepted the relevance of the new pp-CM endpoint and efficacy regarding incidence of pancreatitis in the smaller subset of patients
with recurrent pancreatitis. They therefore gave a positive opinion for Glybera for the treatment of this more restricted group of LPLD patients (EMA 2012, p. 103). The CHMP, however, determined that there were not enough data points to sufficiently show efficacy for either pp-CM or pancreatitis and subsequently recommended refusal of the application. Interestingly, they did agree that concerns associated with immunosuppression would have been considered resolved if sufficient efficacy had been demonstrated.

In January 2012, the European Commission requested that the CHMP reassess their position on the risk-benefit balance of Glybera in patients with recurrent pancreatitis. The CHMP requested additional clinical data, such as rates of diabetes and hospitalization, but did not change its opinion that Glybera was not suitable for authorization. Following this third refusal in April 2012, the EMA reported that the CHMP had breached the review protocol by issuing its response to the EC request without first allowing the CAT to adopt a draft opinion. The prior decision was therefore considered void, and the review process was initiated for a fourth time. The CAT and CHMP had open dialogue sessions with representatives from AMT (now uniQure) to discuss their residual concerns. The CAT maintained their opinion that while there was a need to add to efficacy data post-marketing, approval under exceptional circumstances is justified for the restricted patient population (EMA 2012, p. 122). The CHMP then simply stated agreement with the CAT opinion that “Glybera is indicated for adult patients diagnosed with familial lipoprotein lipase deficiency and suffering from severe or multiple pancreatitis attacks despite dietary fat restrictions.” However, a large proportion of the members of the CHMP had dissenting opinions, arguing that the agreed-upon post-marketing evaluations of Glybera’s effect on pp-CM should have been performed prior to EMA approval. Following the CHMP recommendation, the European Commission approved Glybera for marketing in the EU in
November of 2012. Provisions of the approval included the enactment of a pharmacovigilance program, a risk management plan, biannual periodic safety update reports, and the formation of a registry of every patient dosed with Glybera.

**Summary**

The pursuit of successful gene therapy in 2013 as defined by products that treat, cure or prevent diseases in human beings is indeed a “venture into the unknown.” The story of Glybera as depicted in this review highlights the challenges of pioneering efforts in the field of novel human biopharmaceuticals. To be frank, the Glybera story is not elegant or tidy; the product does not cure the disease, and some may say its clinical effectiveness is still in question. However, this research clearly broke new ground and set the stage for the next wave of gene therapy products.

Our detailed accounting of events associated with the development and regulatory review of this product afforded the opportunity to reflect on lessons learned which may inform future efforts. We have done so as third parties not directly involved in the clinical development of the product although one of us (JMW) invented the AAV vector used in the final product.

The selection of LPLD as an early gene therapy product makes sense. Identification of the transgene is obvious since this is gene replacement for a recessive disease. The investigators, however, used a variant of LPL associated with hyperactivity (LPL$^{S447X}$) rather than a gene that encodes the normal version of the protein, which is a reasonable strategy to improve efficacy. Small and large animal models for LPLD are available and were used in developing the preclinical data to support the first human study. The metabolic consequences of LPL expression, i.e., a drop in serum triglycerides, could be used as a biomarker for efficacy and for stability of transgene expression and potentially as a surrogate for predicting clinical efficacy (i.e., reduced
risk of pancreatitis). There are no effective treatments for LPLD, and it leads to severe and often life threatening pancreatitis indicating substantial unmet need.

The investigators performed extensive studies in two animal models of LPLD. As is often the case, the mouse model did not completely reproduce the phenotype of the human in that a complete deficiency led to neonatal lethality in the mouse. Injection of adenovirus expressing human LPLS447X to newborn pups was a clever way to temporarily rescue the mice prior to administration of the AAV vector. However, the immunogenic nature of adenovirus vectors likely confounded the interpretation of AAV gene transfer by eliciting transgene specific immune responses and possibly inducing inflammation in muscle prior to intramuscular AAV delivery to the muscle. The feline model of LPLD is more authentic than the mouse in terms of phenotype and proved to be a useful way to establish activity of the vector in terms of the MED. The cats did develop antibodies against the human transgene that interfered with the assessment of durability of expression, which turned out to be a key question in the human trials. It is unclear if the outcome in the feline experiments would have been different if the vector expressed a feline version of the LPL gene. It is interesting that the MED in the larger animal model is lower than that observed in the mouse model which is different than what has been observed in pre-clinical studies for other AAV gene therapies such as hemophilia B where the MED was much higher in the canine model in comparison to the mouse model (Manno et al., 2006). It is difficult to correlate the MED in LPL deficient cats with that observed in human since the end points for efficacy used in the human dose escalation study (i.e., serum triglycerides) were flawed; studies at the low dose were not uninformative. However, the dose of vector tested in the human efficacy trials (i.e., $10^{12}$ GC/kg) was informative since it significantly
improved clearance of post prandial chylomicrons. This dose was 10-fold higher than the MED in cats.

The design and interpretation of the human clinical trials turned out to be the most substantial challenge. One significant limitation in designing efficacy trials in familial LPLD is the limited number of patients available for participation. Familial LPLD is characterized as an ultra-orphan disease with a prevalence of only 1 per million (Brunzell 1999). The investigators further limited participation in the trial in several ways including the requirement that subjects have substantial levels of circulating endogenous (but defective) LPL enzyme as a way to avoid transgene-specific B and T cell responses. The scarcity of potential subjects severely makes it difficult to enroll numbers required to show statistical significance. In addition, it is very difficult to conduct placebo controlled randomized studies because of the low subject numbers as well as ethical concerns regarding the risks of sham injections or injections with empty vector. One solution to this problem is solid natural history data to allow for comparisons to historical controls and/or comparative measures of efficacy before and after treatment. Unfortunately, natural history data were limited for this disease; the team had to generate these data prior to initiating their clinical programs. The primary efficacy endpoint of a stable reduction of serum triglycerides was not consistently achieved and, when it was observed, it was not durable. Ongoing analysis of the natural history data concluded that serum triglycerides were simply too variable in these patients to be a useful endpoint for assessing transgene engraftment requiring that the investigators re-evaluate the design of their ongoing clinical development program. This led to the proposal to measure post-prandial kinetics of serum chylomicrons before and after gene therapy which made biological sense. The data were quite compelling in the few subjects in which it was measured. This, coupled with the demonstration of gene transfer by PCR and LPL
protein expression by immunohistochemistry in biopsied muscle after gene transfer, confirmed without question that immediately following vector injection, transgene derived \( \text{LPL}^{S447X} \) was expressed and impacted positively on chylomicron metabolism. The problem is that post-prandial chylomicron accumulation is not a validated end point; i.e., there are no existing data that correlate these measurements with improved clinical outcomes. A retrospective analysis of patients using the revised Atlanta classification of acute pancreatitis to evaluate the impact of gene therapy on the incidence of pancreatitis was conducted in an attempt to generate clinical efficacy data. The original trials were not powered to achieve statistical significance for this variable manifestation of the disease and the data collection regarding pancreatitis had flaws, both of which limited the usefulness of the exercise.

A number of important lessons emerged from the clinical development of Glybera including the value of extensive natural history data as a prelude to the design of clinical trials. Many orphan disease advocacy groups have proactively established patient registries to address this need. The experience with Glybera also illustrates the importance of selecting the best primary efficacy endpoint and the difficulty in progressing clinical development when this endpoint is not achieved.

Much has been written about the potential toxicity of AAV gene therapy with a focus on adverse immune responses. It is encouraging that EMA did not view immune toxicity as a barrier to registration. What was ironic is that the most substantial safety concern emanated from the investigators’ attempt to mitigate potential immune toxicity with concurrent immune suppressive therapy. They initiated the phase 1 trial without the intent to immune suppress. A constellation of findings emerged from this study that suggested immune responses may be extinguishing expression (and therefore efficacy) based on the eventual return of triglycerides to pre-treatment
levels concurrent with the presence of inflammation in muscle biopsies. The appearance of T cells to the vector capsid based on ELISPOT assays of peripheral blood implicated their role in these findings. A course of immune suppression with cyclophosphamide and MMF was included in the subsequent trial with a high dose injection of steroids added in the third trial. EMA concluded that the value of including immune suppression was not supported by data and that it added significant addition risk. In retrospect, this conclusion seems justified. One flaw in the rationale for introducing immune suppression in the second trial was the conclusion from the phase I trial that transgene expression was lost based on a return of triglycerides to background levels, which ultimately was determined to be an unreliable biomarker of LPL expression. In fact, in the phase I trial, late biopsy specimens confirmed ongoing transgene expression. The introduction of immune suppression in subsequent trials made little difference in terms of the appearance of capsid T cells, the kinetics of triglyceride changes or inflammation in muscle biopsies. Incorporating immune suppression into AAV clinical trials should be based on solid pre-clinical and clinical data. The prophylactic treatment of AAV gene therapy research subjects with immune suppressing drugs without strong justification should be discouraged.

One aspect of the Glybera review that was closely watched by the gene therapy community was the response of the EMA to issues related to manufacturing of the vector which fall under the umbrella of what is called chemistry, manufacturing and controls (CMC). A necessary consequence of being the first to market is that there is limited information as to how the product should be manufactured and tested for identity, potency and purity. Validated assays with associated reference standards don’t exist for many of these parameters. Furthermore the regulatory agencies have not established a complete set of criteria for “releasing” the product for use in humans. Analysis of the clinical data was furthermore confounded by the change in
manufacturing method from a standard transfection approach for the phase 1 trial to the more scalable baculovirus approach for the additional trials and the commercial product. A change in manufacturing process during clinical development is not uncommon and will be the norm in gene therapy since large scale commercial manufacturing processes are still in development. EMA worked collaboratively with the sponsor to evaluate comparability of products made by the different processes without the need for substantial additional studies. It is very encouraging that CMC concerns did not get in the way of market authorization although this will be a constantly evolving landscape with the rigor of product characterization being raised as we learn more and the indications move into less lethal diseases.

In summary, the first market authorization of a gene therapy product in the West signifies a true milestone in the field of gene therapy representing a victory for the community of scientists and physicians who have helped shape this field over the last 3 decades. The team of scientists, physicians and business leaders who spearheaded this effort should be applauded for their persistence and resilience while the EMA should be recognized for their patience, flexibility and transparency. The fact is that this is only the beginning and a modest beginning at that. The “approval” of Glybera was associated with substantial post marketing requirements that allows commercial sale of the product concurrent with an ongoing evaluation of its safety and efficacy.
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Author Disclosure Statement

J.M.W. is a consultant to ReGenX Holdings, and is a founder of, holds equity in, and receives a grant from affiliates of ReGenX Holdings; in addition, he is an inventor on patents licensed to various biopharmaceutical companies, including affiliates of ReGenX Holdings.
References


Figure Legends
Figure 1. Production and Function of Lipoprotein Lipase. A. LPL is synthesized primarily in adipocytes, but also in skeletal muscle, heart, lung, liver and other organs to a smaller degree (1). LPL forms homodimers in the cell before being secreted (2) and translocated to the vascular endothelial surface. LPL is then anchored to heparan sulfate proteoglycans that line the vascular endothelial cell surface (3). B. Dietary fats entering the intestine are packaged into chylomicrons in enterocytes (1). The chylomicrons are then secreted into the lymphatic space. After finding their way back into the blood, chylomicrons encounter LPL anchored to the vascular endothelial surface. ApoCII on the surface of the chylomicron serves as a cofactor for LPL (2), allowing LPL to hydrolyze the chylomicron’s TG core to produce free fatty acids (FFA). These FFA can then be taken up by peripheral tissues. The chylomicron remnant, depleted of TG, is taken up by the liver via Low-Density Lipoprotein-Receptor (LDLR) binding to ApoE (3) and then
transformed into Very Low Density Lipoprotein (VLDL). At this point, ApoB-48 is exchanged for ApoB-100. VLDL is then secreted into the plasma where it can contact LPL again. Upon this interaction, LPL hydrolyzes the VLDL TG core and produces FFA (4), an essential step in the conversion to Intermediate Density Lipoprotein (IDL). IDL can then be further converted into Low Density Lipoprotein (LDL) which can be taken up by peripheral cells via LDLR for use by the tissues (5).
**Figure 2. Timeline of events related to Glybera’s approval.** *Preclinical Studies listed by Date of Publication.*

**Figure 3. Centralized approval process in the European Union.** The scientific review of human therapeutics is carried out by the committee for human medicinal products (CHMP), a division of the European Medicines Agency (EMA). For gene and cell therapies, a primary review is performed by the committee for advanced therapies (CAT), an independent specialist committee composed of 5 CHMP members and representatives selected by EU member states, as
well as European Commission (EC) appointed patient and clinician representatives. While the CAT adopts a draft opinion on marketing applications, the CHMP is ultimately responsible for making a recommendation to the EC, which has the final authority to grant marketing approval.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mouse</th>
<th>Cat LPL&lt;sup&gt;G412R&lt;/sup&gt; (catalytically inactive mutant)</th>
<th>Human</th>
</tr>
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<tr>
<td>Ad rescue at birth</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
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<tr>
<td>Immune response to hLPL</td>
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<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
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<tr>
<td>Muscle damage</td>
<td>Yes</td>
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**Table 1. Summary of disease parameters in preclinical models**

The murine LPLD model exhibits several characteristics distinct from the disease in humans, including a requirement for adenovirus-mediated rescue of LPL expression at birth, preexisting muscle damage and lipid accumulation, and absence of pancreatitis. The feline model more faithfully recapitulates the features of the human disease.
Table 2. Comparison of experimental design between preclinical and clinical studies

LPL transgene delivery was tested in murine and feline models of LPLD. The total vector dose and number of injections varied considerably between the preclinical studies using AAV1. Immunosuppression was initially introduced in the feline studies in order to attenuate the humoral immune response to the human LPL protein. Immunosuppression was carried forward into human trials in order to block suspected cytotoxic T cell responses to AAV1 capsid that were blamed for preventing long-term efficacy in AMT-010-01.
Table 3: Summary of results from preclinical and clinical studies

The minimally effective vector dose was reasonably predicted by the feline LPLD model, although the magnitude and duration of serum TG reduction were not predicted by preclinical studies. Importantly, the antibody response against human LPL observed in the feline studies was not replicated in human trials. Establishing the MED in humans is complicated because no subjects who received the lower dose were analyzed for post-prandial chylomicrons which is the only reliable metabolic marker of efficacy.

<table>
<thead>
<tr>
<th>MED</th>
<th>Mouse</th>
<th>Cat(^{\wedge})</th>
<th>Human(^{\wedge})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune response</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Duration of measurable LPL activity in plasma</td>
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<td>4 weeks</td>
<td>N/R</td>
</tr>
<tr>
<td>Maximum duration of LPL+/− range triglyceride levels</td>
<td>&gt; 52 weeks(^*)</td>
<td>10 weeks(^*)</td>
<td>Varied(^{\dagger})</td>
</tr>
</tbody>
</table>

\(^{\wedge}\) immunosuppression regimen

\(^*\)Duration of study

\(^{\dagger}\)Did not compare to LPL heterozygotes