Efficacy and long-term safety of alipogene tiparvovec (AAV1-LPLS447X) gene therapy for lipoprotein lipase deficiency: an open-label trial

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We describe the 2-year follow-up of an open-label trial (CT-AMT-011–01) of AAV1-LPLS447X gene therapy for lipoprotein lipase (LPL) deficiency (LPLD), an orphan disease associated with chylomicronemia, severe hypertriglyceridemia, metabolic complications and potentially life-threatening pancreatitis. The LPLS447X gene variant, in an adeno-associated viral vector of serotype 1 (alipogene tiparvovec), was administered to 14 adult LPLD patients with a prior history of pancreatitis. Primary objectives were to assess the long-term safety of alipogene tiparvovec and achieve a >40% reduction in fasting median plasma triglyceride (TG) at 3–12 weeks compared with baseline. Cohorts 1 (n = 2) and 2 (n = 4) received 3 × 1011 gc kg−1, and cohort 3 (n = 8) received 1 × 1012 gc kg−1. Cohorts 2 and 3 also received immunosuppressants from the time of alipogene tiparvovec administration and continued for 12 weeks. Alipogene tiparvovec was well tolerated, without emerging safety concerns for 2 years. Half of the patients demonstrated a >40% reduction in fasting TG between 3 and 12 weeks. TG subsequently returned to baseline, although sustained LDL5447X expression and long-term changes in TG-rich lipoprotein characteristics were noted independently of the effect on fasting plasma TG.

INTRODUCTION

Lipoprotein lipase (LPL) deficiency (LPLD) is a rare autosomal recessive disease with an estimated prevalence of 1–2 in 1 000 000 individuals. It is characterized by severe hypertriglyceridemia, chylomicronemia, and risk of recurrent and potentially fatal pancreatitis.1 Signs and symptoms include hepatosplenomegaly, lipemia retinalis, severe abdominal pain, peripheral neuropathy and an increased risk of cardiometabolic complications.1–6 Patients with recurrent episodes of acute pancreatitis may also develop chronic pancreatitis and signs of exocrine or endocrine pancreatic insufficiency, including diabetes mellitus.1,3,7

LPL is a central enzyme in the catabolism of triglyceride (TG)-rich lipoproteins, namely chylomicrons (CMs) and very-low-density lipoproteins.8,9 The adipose and skeletal muscle tissue are important sites of LPL production. Once produced, LPL is secreted and translocated to the luminal surface of endothelial cells to ensure direct contact with circulating TG-rich lipoproteins. When LPL is deficient, CM levels and pancreatitis risk increase dramatically.10 The pathophysiology underlying CM-related pancreatitis has not been completely elucidated. One hypothesis is that large CMs lodged in pancreatic capillaries expose them to pancreatic lipase, with the subsequent release of free fatty acids through the hydrolysis of CM-associated TGs. High concentrations of free fatty acids are thought to damage pancreatic cells leading to pancreatitis.11–15

Fasting plasma TG concentrations exceed normal values 10- to 100-fold in LPLD.5,16 Chylomicronemia is observed when fasting plasma TG concentration is >10.0 mmol l−1, and the risk of pancreatitis importantly increases with values above 20 mmol l−1 (approximately 2000 mg dl−1).1,7,16 Traditional disease management in LPLD aims to decrease fasting TGs to below or near 10 mmol l−1 by restricting the fat intake to 10–15% of the total daily calorie intake, requiring the use of medium-chain TG oil and excluding alcohol from the diet throughout life.1 This restrictive diet limits social freedom and adds to the burden of disease; it is not always sufficient to eliminate the risk of complications, even when patients are compliant. Currently available TG-lowering agents are not effective in controlling chylomicronemia in LPLD patients.16–18 Additional disease management approaches are therefore required. Enzyme supplementation therapy is not feasible because of the very short half-life of LPL in the circulation. Gene therapy, which aims to induce the expression of functional LPL in muscle, offers an attractive therapeutic approach.

Pre-clinical gene therapy studies have shown that biologically active LPL can be produced in muscle by non-pathogenic, non-integrating,19,20 adeno-associated virus (AAV) type 1-mediated gene transfer21–23 without tissue-specific regulation of the transgene. AAV1-LPLS447X encodes a naturally occurring gain-of-function LPL variant associated with lower plasma TG and a lower rate of cardiovascular disease24 than wild-type LPL. Intramuscular (IM) administration of AAV1-LPLS447X has been shown to result in the resolution of chylomicronemia and a life-long reduction in plasma TG concentrations in LPL-deficient mice.25 In humans, the
first interventional clinical study was carried out in eight LPLD patients using AAV1-LPLS447X, produced using plasmid-based production in human embryonic kidney (HEK293) cells. Results of this study demonstrated statistically significant TG reductions in all patients up to 12 weeks. Follow-up after 18–31 months showed a return of TG to baseline levels, which at the time was hypothesized to be related to an immune response to AAV1-capsid proteins. AAV1-LPLS447X produced in HEK293 cells was not amenable to large-scale production; therefore, a switch was made to using baculovirus-based production in insect cells; AAV1-LPLS447X produced in this manner was termed alipogene tiparvovec. We describe herein results from the >2 years follow-up of the first clinical study conducted with alipogene tiparvovec (clinical study CT-AMT-011–01; ClinicalTrials.gov number: NCT01109498).

STUDY DESIGN
This open-label, dose-escalation clinical trial assessing the safety and efficacy of alipogene tiparvovec was conducted at the ECOCENE-21 Clinical Research Center, Chicoutimi, QC, Canada. A total of 22 adult LPLD patients with a history of pancreatitis participated in a prospective observational study (Preparation, PREP-02) of 4 months (range: 18–78 weeks) duration to determine baseline disease manifestations and the maximum effect of a controlled low-fat diet on chylomicronemia and fasting plasma TG levels (Supplementary Material S1). LPLD diagnosis was ascertained by genotyping and post-heparin measure of LPL activity. Among the participants to PREP-02, 14 subjects meeting eligibility criteria received alipogene tiparvovec while continuing the same low-fat diet in the interventional study (Supplementary Material S2). All participants kept a diary of food and beverage intake for the 3 days before each study visit and were thus instructed to carefully adhere to a low-fat diet in which fat intake represented no more than 20–25% of caloric intake (<55 g fat per day, assuming a 2000 calorie diet) during both the observational and interventional studies to minimize diet-induced fluctuation in plasma TG levels. These subjects were assigned to three cohorts as follows: Cohorts 1 (n = 2) and 2 (n = 4) received 3×10^11 gc kg^-1, and cohort 3 (n = 8) received 1×10^12 gc kg^-1 (Table 1). Cohorts 2 and 3 also received immunosuppressants from the time of alipogene tiparvovec administration and continued for 12 weeks. The immune suppression regimen consisted of cyclosporine A (3 mg kg^-1 per day) and mycophenolate mofetil (2 g per day) initiated at the time of alipogene tiparvovec administration and maintained for 12 weeks thereafter. A period of 12 weeks was considered sufficient for the prevention of potential capsid-related immunogenicity, based on observations in Rhesus macaques.

Primary objectives were to assess the long-term safety profile of alipogene tiparvovec and achieve a reduction in fasting median plasma TG of at least 40%, 3–12 weeks after therapy compared with baseline. Secondary objectives were to achieve a reduction in fasting TG to ≤10.0 mmol l^-1 within 12 weeks, to measure the biological activity and expression of LPLS447X in the muscle after 26 weeks, to evaluate potential immune responses against LPLS447X and AAV1 capsid proteins, and to assess biodistribution and shedding of AAV1-LPLS447X vector DNA. The study, conducted in accordance with Good Clinical Practices (CPMP/ICH/135/95) and the Declaration of Helsinki, was approved by Health Canada, and the Board of the Chicoutimi Hospital and its Ethics Committee. All subjects provided written informed consent. The Ethics Committee of the Ministry of Health (FRSQ), Quebec Province, provided long-term study supervision. Monitoring of safety data was carried out by the Academic Medical Center, Amsterdam, The Netherlands. The ECOCENE-21 clinical site was audited by the European Medicines Agency.

RESULTS
Adverse events
One serious adverse event (AE; acute pancreatitis) occurred during the observational (PREP-02) study, and another subject experienced acute pancreatitis between the PREP-02 and interventional study, resulting in an overall pancreatitis incidence of 0.20 event/subject/year during prospective observation (Supplementary Material S3). Following injection of alipogene tiparvovec, all subjects (n = 14) reported one or multiple mild-to-moderate AEs. These did not cluster to one organ system and showed no dose relationship. Twelve subjects reported injection site events, such as local and transient bruising, edema, sensitivity and/or pain lasting a few days/weeks. One subject (Cohort 3) developed a serious AE, considered probably related to drug administration, with fever (39.9 °C) 10 h after injection. This resolved spontaneously within 12 h. No clinically relevant changes in clinical laboratory assessments, vital signs, chest X-rays and physical examination occurred during the study. No treatment-related changes in creatine phosphokinase, high sensitivity C-reactive protein or lactate dehydrogenase were observed. Immunosuppression did not result in any untoward side effects and did not have an impact on biochemical/inflammatory markers. Twelve subjects experienced non-severe infections (mostly nasopharyngitis (59%)), but incidence was within the expected seasonal

Table 1. Characteristics and treatment regimen of the 14 subjects who received alipogene tiparvovec

| Subject ID | Genotype      | Gender | Age | Cohort | Dose alipogene tiparvovec (gc kg^-1) | Immuno-suppressant regimen |
|------------|---------------|--------|-----|--------|--------------------------------------|---------------------------|
| 01         | P207L/P207L   | F      | 60  | 2      | 3×10^11                              | CsA + MMF                 |
| 04         | P207L/P207L   | F      | 50  | 1      | 3×10^11                              | CsA + MMF                 |
| 06         | P207L/P207L   | M      | 51  | 1      | 3×10^11                              | CsA + MMF                 |
| 07         | P207L/P207L   | F      | 56  | 2      | 3×10^11                              | CsA + MMF                 |
| 08         | P207L/P207L   | M      | 28  | 3      | 1×10^12                              | CsA + MMF                 |
| 09         | P207L/G188E   | F      | 62  | 3      | 1×10^12                              | CsA + MMF                 |
| 10         | P207L/D9N     | M      | 42  | 2      | 3×10^11                              | CsA + MMF                 |
| 11         | P207L/P207L   | F      | 48  | 3      | 1×10^12                              | CsA + MMF                 |
| 13         | P207L/G188E   | F      | 40  | 3      | 1×10^12                              | CsA + MMF                 |
| 14         | P207L/P207L   | F      | 51  | 2      | 3×10^11                              | CsA + MMF                 |
| 15         | P207L/P207L   | F      | 50  | 3      | 1×10^12                              | CsA + MMF                 |
| 18         | P207L/P207L   | M      | 37  | 3      | 1×10^12                              | CsA + MMF                 |
| 19         | P207L/P207L   | F      | 28  | 3      | 1×10^12                              | CsA + MMF                 |
| 20         | P207L/P207L   | F      | 36  | 3      | 1×10^12                              | CsA + MMF                 |

Abbreviations: M, male; F, female; CsA, cyclosporine A (3 mg kg^-1 per day); MMF, mycophenolate mofetil (2 g per day).
range. Six serious AEs were reported during the long-term follow-up (LTFU), including one severely affected diabetic patient, with history of frequent recurrent pancreatitis and chronic renal failure before treatment, who had a cardiac arrest leading to death 2 years into the LTFU period. None of the serious AEs were related to alipogene tiparvovec administration.

Viral biodistribution and shedding
Peak levels of alipogene tiparvovec-derived vector DNA were detected 24h after administration in serum (max. $1.7 \times 10^9$ gc ml$^{-1}$), saliva (max. $8.0 \times 10^8$ gc ml$^{-1}$) and urine (max. $2.0 \times 10^9$ copies per ml). Levels in semen were first measured at Week 1 (four of five male subjects (max. $3.6 \times 10^9$ gc per µg DNA)). Vector DNA in most samples dropped to around/below the limit of detection ($1.0 \times 10^7$ gc per µg DNA) within 4–6 weeks, except in one subject (Figure 1). Vector DNA clearance from semen occurred after 6–10 weeks, although low levels, barely above the limit of detection, were later found once in two of the five male subjects.

Host immune response
Anti-LPLS447X antibodies were not observed in any subject. Anti-AAV1 antibodies were detected in approximately half ($n = 8$) of the subjects before alipogene tiparvovec administration, and all subjects, whether exhibiting pre-existing antibodies or not, showed a treatment-emergent increase in anti-AAV1 antibodies after administration, persisting at high titer through the post-treatment period (Table 2). Treatment-emergent anti-AAV antibody responses were not affected by immune suppression or the termination of the immunosuppressive regimen. On the basis of the data obtained from peripheral blood mononuclear cells of adequate quality, a moderate and non-persistent T-cell response was observed directed against the AAV1 capsid (and not against LPLS447X) in 9 out of the 14 subjects (Supplementary Material S4).

Local injection site response
Variable local responses were observed in injected muscle tissue compared with non-injected muscle. These responses ranged from none or minor (subjects 01 and 13), to slight (06), moderate (04 and 10) and a more pronounced local response (09 and 11). A more pronounced local response was characterized by non-specific muscle-fiber degeneration and regeneration, neutral lipid accumulation within fibers and perivascular to endomysial infiltration by CD8+ T-cells, CD20+ B-cells and CD68+ macrophages (Figures 2 and 3b). Subsarcolemmal accumulations (positive in NADH-oxidoreductase, Periodic Acid-Shiff, and Gomori-Trichrome stains; negative in succinate dehydrogenase stain) were observed and identified by electron microscopy as tubular aggregates (Figure 3a). Positive staining for SERCA-1

### Table 2. Humoral immune response to AAV1

| Subject | Dose (gc kg$^{-1}$) | Antibodies against AAV1 |
|---------|---------------------|------------------------|
|         | Pre-administration  | Post-administration    |
| 01      | $3 \times 10^{11}$ + ISR | + + + +  |
| 04      | $3 \times 10^{11}$   | n.d. + + +  |
| 06      | $3 \times 10^{11}$   | - + +  +  |
| 07      | $3 \times 10^{11}$ + ISR | + + +  |
| 08      | $1 \times 10^{12}$ + ISR | - + +  |
| 09      | $1 \times 10^{12}$ + ISR | - + +  |
| 10      | $3 \times 10^{11}$ + ISR | + + +  |
| 11      | $1 \times 10^{12}$ + ISR | + + +  |
| 13      | $1 \times 10^{12}$ + ISR | + + +  |
| 14      | $3 \times 10^{11}$ + ISR | + + +  |
| 15      | $1 \times 10^{12}$ + ISR | + + +  |
| 16      | $1 \times 10^{12}$ + ISR | + + +  |
| 19      | $1 \times 10^{12}$ + ISR | - + +  |
| 20      | $1 \times 10^{12}$ + ISR | + + +  |

Abbreviation: ISR, immunosuppressants. The test results of the samples were scored by comparison with those of a negative control, (a serum sample from a healthy human control). To this end, algorithms were developed to convey the optical density results into a semi-quantitative scoring system. Based on the algorithms, samples were said to be strongly positive (+ + +), weakly positive (+ +) or negative (−) for AAV1 antibodies.

Figure 1. Presence of AMT-011 Vector DNA in body fluids: Results are depicted as median values per treatment group, per time point. Dashed lines indicate limit of detection (bottom line) and limit of quantification (top line) of the assay (a: shedding in serum; b: shedding in saliva; c: shedding in urine; d: shedding in semen).
and -2 suggests that these accumulations represent a proliferation of the sarcoplasmic reticulum. The expression of major histocompatibility complex class I/II surface receptors was upregulated in the sarcolemma of a few fibers close to the cellular infiltrates. In its more moderate form (04 and 10), fiber degeneration and regeneration was noted, but tubular aggregates were not always observed (04 only), and there was less lipid accumulation.

Fewer perivascular and endomysial infiltrates consisted mainly of CD8+ T-cells and CD68+ macrophages, with only a minor CD20+ component; there was no expression of major histocompatibility complex class I/II on fibers. Progressively less degenerative muscle-fiber responses, less lipid accumulation and less tissue infiltration were observed for biopsy samples from subjects 06, 13 and 01 (Table 3). There was a trend towards a more pronounced local response in samples showing more LPL expression (Tables 3 and 4). Apoptosis, excess fibrosis or widespread necrosis were not observed in injected muscle tissue, and there were no gross abnormalities; overall, the muscle structure and function was preserved.

AAV1 vector DNA sequence and LPLS447X expression in injected muscle

All seven biopsies showed AAV1 vector DNA 26 weeks after therapy (Table 4). Levels in corresponding non-injected samples were at or below the level of quantification, irrespective of dose. Muscle tissue homogenates tested positive for LPL protein and activity in three of seven injected muscle samples. Expression of LPL protein was confirmed in four of seven subjects by staining serial cross-sections of injected muscle using the antibody 5D2. Sections for five of seven subjects also showed an increase in intracellular lipids (Oil Red O stain for neutral lipid) consistent with increased local LPL activity (Figure 3a, Table 4). These findings agree with other studies that show persistent transgene expression.
Effect on fasting TG, TG-rich lipoproteins characteristics and clinical outcomes

As expected, fasting TG levels during the PREP-02 study remained >10.0 mmol l⁻¹ and comparable to historical values in all subjects. After 3–12 weeks alipogene tiparvovec administration, all but two subjects demonstrated reduced median TG compared with baseline (average reduction: 39.53%); 50% achieved the secondary endpoint of TG <40% reduction in TG. Four subjects achieved the secondary endpoint of TG <10.0 mmol l⁻¹. Two subjects showed no response on fasting TG levels. TG reductions during week 3–12 were statistically significant for the total group. Subjects showed no response on fasting TG levels. TG reductions at 12 and 52 weeks compared with baseline (Cohort 3; P = 0.0009).

expression in man following AAV-mediated gene transfer to muscle.30–32

Table 3. Summary of immunohistochemical staining of cryosections of injected muscle biopsies isolated from seven LPLD subjects following IM administration of alipogene tiparvovec

| Patient | Dose (gc kg⁻¹) | ISR | CD3 | CD4 | CD8 | CD68 | CD20 | HLA-ABC (fibers/infl. cells) | HLA-DR fibers/infl. cells |
|---------|----------------|-----|-----|-----|-----|------|------|----------------------------|--------------------------|
| 01      | 3 × 10¹¹       | ISR |     |     |     |      |      |                            |                          |
| 04      | 3 × 10¹¹       |     | ISR |     |     |      |      |                            |                          |
| 06      | 3 × 10¹¹       |     | ISR |     |     |      |      |                            |                          |
| 09      | 1 × 10¹²       |     | ISR |     |     |      |      |                            |                          |
| 10      | 3 × 10¹¹       |     | ISR |     |     |      |      |                            |                          |
| 11      | 1 × 10¹²       |     | ISR |     |     |      |      |                            |                          |
| 13      | 1 × 10¹²       |     | ISR |     |     |      |      |                            |                          |

Abbreviations: LPLD, lipoprotein lipase deficiency; HLA, human leukocyte antigen; IM, intramuscular; ISR, immunosuppressants; Scoring reflects relative levels of infiltration: − none; 1+ rare; 2+ moderate; 3+ high number. *Single fiber affected. Scores provide a semi-quantitative and relative means of discriminating between patients. A score of 3+ represents the highest level of infiltration observed in this study.

Table 4. Alipogene tiparvovec derived DNA sequence and LPL expression in muscle of seven LPLD patients following IM administration of alipogene tiparvovec

| Subject | Dose (gc kg⁻¹) | Q-PCR (gc per μg gDNA) | LPL mass (ng ml⁻¹) | LPL activity (nmol min⁻¹ mg⁻¹) | LPL IHC | ORO |
|---------|----------------|------------------------|-------------------|-------------------------------|---------|-----|
| 01      | 3 × 10¹¹ + ISR | 900                    | 0                 | 0                             | 0       |     |
| 04      | 3 × 10¹¹       | 170000                 | 110               | 24.06                         | 5.91    |     |
| 06      | 3 × 10¹¹       | 22000                  | 30                | 0                             | 0       |     |
| 09      | 1 × 10¹² + ISR | 77000                  | 0                 | 85.75                         | 23.29   |     |
| 10      | 3 × 10¹¹ + ISR | 110000                 | 30                | 0                             | 0       |     |
| 11      | 1 × 10¹² + ISR | 630000                 | 0                 | 182.76                        | 77.52   |     |
| 13      | 1 × 10¹² + ISR | 130000                 | 30                | 0                             | 0       |     |

Abbreviations: I, injected muscle; C, non-injected muscle; ISR, immunosuppressants regimen; LPL, lipoprotein lipase; Q-PCR, quantitative PCR; IHC, immunohistochemistry; ORG, Oil red O; HLA, human leukocyte antigen. Q-PCR: alipogene tiparvovec derived DNA sequence (encoding LPLS447X) gene copy numbers as measured in biopsy tissue homogenate. LPL mass and LPL activity as measured in muscle tissue homogenates. LPL IHC: detection of LPL by immunohistochemistry on tissue sections; ORO: (intracellular) neural lipids as detected in tissue sections stained by Oil Red O. Stained tissue sections were examined by two trained observers, with respect to the presence or absence of various histopathological parameters and specific immunoreactivity for the different markers. Two representative frozen sections per individual and biopsy specimen were stained for CD3, CD4, CD8, CD68, HLA-DR and HLA-ABC and assessed by the two observers independently; a consensus score was obtained. The degree of staining was rated on a semi-quantitative three-point scale: 0, no positive cells; 1+: rare positive cells; 2+: moderate number of positive cells; 3+: high number of positive cells. A similar three-point scale was used to score the amount of lipid detected in the ORO-stained sections. It should be noted that this scoring was intended to discriminate between subjects, relating only to the relative amount of infiltration or lipid accumulation noted in the biopsy specimens, and does not represent an overall severity score.

Table 5. Primary efficacy outcome in individual subjects (fasting plasma TG (mmol l⁻¹)) before and 3–12 weeks after administration of alipogene tiparvovec

| Subject | Median pre-therapy TG<sup>b</sup> | Median post-therapy TG (W3–W12) | % Reduction W3–W12 vs pre-therapy |
|---------|----------------------------------|----------------------------------|----------------------------------|
| 1       | 22.55                            | 21.39                            | 5.15                             |
| 4       | 49.10                            | 30.41                            | 38.07                            |
| 6       | 15.88                            | 4.37                             | 72.50                            |
| 7       | 23.30                            | 6.73                             | 71.11                            |
| 8       | 22.90                            | 18.72                            | 18.25                            |
| 9       | 23.81                            | 5.38                             | 77.42                            |
| 10      | 21.87                            | 29.01                            | 32.68                            |
| 11      | 28.38                            | 24.44                            | 13.89                            |
| 13      | 34.21                            | 10.17                            | 70.29                            |
| 14      | 22.38                            | 14.76                            | 34.07                            |
| 15      | 65.48                            | 25.17                            | 61.56                            |
| 16      | 16.52                            | 9.56                             | 42.16                            |
| 19      | 13.02                            | 13.95                            | 7.15                             |
| 20      | 21.39                            | 10.99                            | 48.62                            |

Abbreviation: TG, triglycerides. TG values generally had reverted back to around baseline values by the Week 26 visit. None of the participants had historical plasma TG values <10 mmol/l. *Normal fasting whole plasma TG levels in unaffected individuals range between 1 and 2.3 mmol l⁻¹.

<sup>b</sup>Median of last five values from PREP-02 and of Week-3 baseline visit from CT-AMT-011-01.
DISCUSSION

Alipogene tiparvovec was generally well tolerated for up to 2 years and was associated with signs of clinical benefits and persistent LPL expression in muscle, independently of the effect on plasma TG. Signs of long-term transgene expression are consistent with results of previous studies having shown multi-year expression following a single IM administration of AAV.30–31 AAV is a non-pathogenic, non-integrating viral,19,20 and AAV genomes persist as extrachromosomal monomers or concatamers that support long-term expression.

Alipogene tiparvovec-derived vector DNA was present transiently in serum, urine, saliva and, at extremely low levels, in semen. Other clinical studies have indicated the absence of the vector sequence in semen following IM AAV2 or AAV1 administration.30,31 However, the sensitivity of the PCR-based assay used in our study was 5- to 10-fold higher than those used in previous studies. Overall, data indicate minimal risk, if any, of germ-line transmission. The risk to the environment or other individuals associated with shedding such low amounts of AAV is limited, as further spread of replication-deficient AAV vectors is highly unlikely.

All subjects showed a robust antibody response, and more than half (9 out of 14) demonstrated a moderate and non-persistent T-cell response to AAV1 capsid proteins. Muscle biopsy assessments suggest a relationship between the extent of the local injection site response and the level of local LPL activity (and LPL protein and activity, accumulation of intracellular lipids) rather than with observed anti-AAV immune responses.17 The safety evaluation demonstrated that with the exception of one serious AE, drug-related AEs were mild or moderate, did not cluster to one organ system and showed no dose relationship. One subject suffered an episode of pancreatitis following a high-fat meal within 1 week of alipogene tiparvovec administration, when transgene expression is not expected to be optimal. Preclinical observations have shown that LPL is unlikely to be expressed before 3 weeks after alipogene tiparvovec administration leading to the CT-AMT-011–01 protocol criteria that measure changes in TGs from 3 weeks onwards.

TG values during the observational PREP-02 study confirmed previous findings that the prescription of a severe dietary fat restriction does not reduce fasting TG to a level at which the risk of pancreatitis and other chylomicronemia symptoms may be eliminated.1,7,12,17 In LPLD, the extent of chylomicronemia, and hence plasma TG levels, are affected by diet. Alipogene tiparvovec in addition to a low-fat diet was effective in lowering fasting plasma TG levels 3–12 weeks after administration in all but two patients. The fact that TG reverted to baseline after 19–26 weeks was initially interpreted as a sign of transient efficacy.25 However, several signs of clinical efficacy independent of plasma TG were noticed up to 2 years after the LPL gene transfection and raised the possibility that TG-rich lipoprotein characteristics, particularly the size, lipid content and kinetics of CMs, rather than plasma TG concentration per se, are the best surrogate markers of pancreatitis risk in LPLD. Signs of efficacy beyond week 12 included: (a) sustained modification in TG-rich lipoprotein characteristics independent of the effect on total TG; (b) persistent vector DNA, transgene expression and biological activity of LPLS447X in injected muscle after 26 weeks; (c) self-reported signs suggestive of improvement of the quality of life; (d) reduction in overall pancreatitis incidence and/or intensity of the crisis up to 2 years post-alipogene tiparvovec injection. These results and observations have led to the conception and execution of two additional studies specifically designed to evaluate the effect of alipogene tiparvovec on CM metabolism, kinetics and clearance (CT-AMT-011–02) and on pancreatitis and abdominal pain (CT-AMT-011–03). The later is designed as a case-review study to retrospectively and prospectively assess the prevalence, severity
and incidence of abdominal pain crises and acute pancreatitis in LPLD patients, including those having received alipogene tiparvovec or participated in the PREP-study.

CMs are responsible for the transportation and delivery of fat following a meal. LPLD is thus, by its very nature, a postprandial disease. It would appear that there is no simple correlation between fasting whole-plasma TG and pancreatitis risk after therapy. The pathogenesis of pancreatitis associated with chylomicronemia is not completely understood, and factors other than total fasting plasma TG may be involved.3 The lipoprotein fractionation studies showed an apparent sustained shift of TG and cholesterol from the buoyant CM fraction (S₁₀ < 400) to a less buoyant fraction corresponding to the very-low-density lipoprotein density (S₁₀ 20–400) without changes in the number and composition of LDL and HDL particles. The conditions for ultracentrifugation used here preclude a complete separation of CM from very-low-density lipoproteins and other lipoproteins. The fixed spin time is likely to allow separation of only the more buoyant CM. After therapy, on average, more TG, cholesteryl and apolipoprotein B were recovered from the S₁₀ 20–400 fraction, suggesting that TG-rich particles became less buoyant. We hypothesize that such modifications in CM characteristics contribute to decreased pancreatitis and clinical benefits. This is supported by recent data from the CT-AMT-011–02 study, suggesting that CM clearance and TG-rich lipoprotein characteristics, rather than fasting TG, might be a more appropriate indicator of alipogene tiparvovec efficacy.3,34 The clinical significance of CM composition and its possible utility as a surrogate marker of alipogene tiparvovec efficacy is the subject of ongoing studies. Although involving a small number of patients and events, the five-fold reduction in the 2-year incidence of pancreatitis is compatible with a long-term expression of LPLS447X.

CONCLUSION
A single (one-time) IM administration of alipogene tiparvovec, in addition to a low-fat diet, was well tolerated. Alipogene tiparvovec was associated with a transient effect on fasting TG, persistent gene expression, sustained alterations in TG-rich lipoprotein distribution profiles and signs of clinical improvement, including a clinically meaningful decrease in pancreatitis incidence and characteristics. This is the first demonstration of a single gene therapy intervention leading to persistent transgene expression and sustained clinical benefit in a systemic metabolic disorder.

MATERIALS AND METHODS

Study drug
Alipogene tiparvovec is a recombinant AAV vector of serotype 1. The vector contains the coding sequence for the human gene variant LPLS447X. Transcription is driven by the cytomegalovirus promoter and terminated by a bovine growth hormone polyadenylation sequence. Alipogene tiparvovec was produced using insect cells and baculoviruses by the Amsterdam Molecular Therapeutics B.V. (Amsterdam, The Netherlands), in accordance with Good Manufacturing Practice guidelines (Supplementary Material S5).

Drug administration
Subjects, under spinal anesthesia, received alipogene tiparvovec by multiple IM injections divided equally between the muscleus vastus lateralis and muscleus vastus medialis of both the left and right muscleus femoralis. The calf muscles were also injected if the number of injections exceeded 40 injection sites (Supplementary Material S6). Follow-up evaluations took place at 1 day and 1, 2, 3, 4, 6, 8, 10 and 12 weeks (main study), and at 19, 26, 39, 52 weeks and 2 years after alipogene tiparvovec administration (LTFU) (Supplementary Material S6).

Adverse events
The coding of AEs was performed using MedDRA (International Federation of Pharmaceutical Manufacturers and Associations, Chantilly, VA, USA) version 9.1. Routine assessments included physical examination, vital signs and weight, chest X-ray electrocardiogram, blood pressure, urine analysis, serum biochemistry, hematology assessments and serology (Supplementary Materials S7 and S8). In addition, systemic humoral and cell-based immune responses to AAV-1 and LPLS447X were assessed, and biodistribution and shedding of AAV1-LPLS447X were monitored. Studies to examine local tolerance to alipogene tiparvovec and the injection procedure were carried out in muscle biopsy samples from injected and non-injected muscles taken 26 weeks after administration.

Any untoward clinical signs and symptoms, including injection site reactions and pancreatitis events, were recorded and graded. Pancreatitis events during observation, the main 12-week study phase and LTFU were determined by the attending physician according to the 2004 guideline of the Society for the Surgery of the Alimentary Tract on the treatment of acute pancreatitis.35 The frequency of pancreatitis events was calculated by dividing the total number of events observed in all patients by the total number of years of follow-up.

Humoral immune responses to AAV-1 and LPLS447X were determined by measuring serum antibodies against the AAV1 capsid proteins and LPLS447X protein by enzyme-linked immunosorbent assay (general antibody titer, non-discriminatory for immunoglobulin subclasses; Xendo Drug Development B.V. (Groningen, The Netherlands), now part of QPS Holding LLC). Serum samples were collected at baseline, at weeks 2, 3, 4, 6, 8, 10 and 12, and at all time points during LTFU. Peripheral blood mononuclear cells, employed to measure cell-mediated immune responses, were used in an enzyme-linked immunospot assay for the detection of interferon-γ secretion in response to antigen stimulation; peripheral blood mononuclear cells were incubated with whole AAV1 capsid particles, with pools of peptides derived from the AAV1 capsid (VP1 protein sequence) or with recombinant LPLS447X protein, for 20–24 h, and cytokine secretion was detected with an anti-human interferon-γ antibody. Positive and negative controls were also included in the assay; assays were performed by Mingozzi et al.36 (CHOP, Philadelphia PA, USA). Peripheral blood mononuclear cells were collected from whole blood collected at baseline, at weeks 2, 4, 6, 8, 10, 12, and at all time points during LTFU. Biodistribution and shedding of AAV1-LPLS447X to evaluate the potential risk of transmission to third parties or the environment was assessed by detection of vector DNA in serum, saliva, urine and semen, using quantitative PCR. Primers and probe (Taqmam, Applied Biosystems, Carlsbad, CA, USA) used in the assay were specific for the boundary sequence of the vector DNA and LPL expression studies) were optimized, and spiking experiments were performed to determine the sensitivity of the method. Ten copies of the vector DNA sequence could be detected.

Muscle biopsies
Biopsies used in both safety (to assess local tolerance to the study drug) and efficacy assessments were obtained from injected and non-injected muscle of 7 of 14 subjects enrolled in the trial (two from Cohort 1, two from Cohort 2 and three from Cohort 3; the remaining subjects did not consent to the biopsy procedure). Biopsies were taken ~26 weeks after vector administration.

Each muscle biopsy was divided into several parts; these separate parts were used for the following: quantitative PCR (to measure vector DNA levels in muscle homogenate), transgene expression (LPL protein mass and activity in muscle homogenate) and histology (basic histology including immunohistochemical staining to characterize muscle fibers and inflammatory cells). A separate section was fixed in Karnovsky fixative for electron microscopy. Muscles biology was performed by Dr Eleonora Aronica of the Department of Neuro-Pathology of the Academic Medical Centre (AMC, Amsterdam, The Netherlands). Paraﬁn sections of formalin-ﬁxed material were prepared for general histology. Cryosections of 6 μm thick were routinely stained for ATPase pre-incubated at pH4.3, NADH–Oxidoreductase, succinate dehydrogenase, cytochrome oxidase/succinate dehydrogenase, Periodic Acid-Shiff, Oil Red O, Hematoxylin and Eosin, Acid

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Phosphatase, Congo Red, Non-specific Esterase and Gomori-Trichrome stains. Immunohistochemistry was performed for CD4, CD8, CD20, CD68 and human leukocyte antigens HLA-DR and HLA-ABC. Additional immunohistochemistry was performed for sarco(endo)plasmic reticulum Ca2 + ATPases (SERCA1 and SERCA2). myosin heavy chain and membrane attack complex (or C5b-9).

Statistical analysis
Because of intra-subject variability in TG levels, multiple data points were used to derive pre- and post-therapy values. The median of the six most recent measurements before the day of alipogene tiparvovec administration was used for pre-therapy values. All TG data from week 3 until and including week 12 were used for the main study post-administration TG response assessment. A linear mixed model was used to estimate the average reduction in individual TG after alipogene tiparvovec administration and whether there was a statistically significant reduction in TG calculated using median and mean values. Individual pre-therapy and post-therapy TG values until week 12 and 26 were compared using the non-parametric Wilcoxon test. A score of 0 or 1 was assigned to the subject’s TG levels to indicate success or failure (TG < 100 mg/dl or > 100 mg/dl, respectively). Using a χ2-statistics, a mixed model repeated measures and Wilcoxon signed rank test, it was determined whether alipogene tiparvovec, or a specific dose, lowers TG significantly. All hypotheses were tested with an overall two-sided significance level of 0.05.

CONFLICT OF INTEREST
The funding body (AMT) was involved in all aspects of the study (study design, data collection and analysis, and data interpretation in collaboration with the CRD and principal investigator). All authors (LWW, LT, SVD, NvdB, and VS-F) are employees of AMT. The remaining authors declare no conflict of interest. The principal investigator of the study (DG) has no financial interest in AMT and made all final editorial decisions regarding the manuscript.

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