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To cite this version:

HAL Id: inserm-00726449
http://www.hal.inserm.fr/inserm-00726449
Submitted on 30 Aug 2012

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Altered Skeletal Muscle Lipase Expression and Activity Contribute to Insulin Resistance in Humans

Pierre-Marie Badin, Katie Louche, Aline Mairal, Gerhard Liebisch, Gerd Schmitz, Arild C. Rustan, Steven R. Smith, Dominique Langin, and Cedric Moro

OBJECTIVE—Insulin resistance is associated with elevated content of skeletal muscle lipids, including triacylglycerols (TAGs) and diacylglycerols (DAGs). DAGs are by-products of lipolysis consecutive to TAG hydrolysis by adipose triglyceride lipase (ATGL) and are subsequently hydrolyzed by hormone-sensitive lipase (HSL). We hypothesized that an imbalance of ATGL relative to HSL (expression or activity) may contribute to DAG accumulation and insulin resistance.

RESEARCH DESIGN AND METHODS—We first measured lipase expression in vastus lateralis biopsies of young lean (n = 9), young obese (n = 9), and obese-matched type 2 diabetic (n = 8) subjects. We next investigated in vitro in human primary myotubes the impact of altered lipase expression/activity on lipid content and insulin signaling.

RESULTS—Muscle ATGL protein was negatively associated with whole-body insulin sensitivity in our population (r = −0.55, P = 0.005), whereas muscle HSL protein was reduced in obese subjects. We next showed that adenovirus-mediated ATGL overexpression in human primary myotubes induced DAG and ceramide accumulation. ATGL overexpression reduced insulin-stimulated glycogen synthesis (−30%, P < 0.05) and disrupted insulin signaling at Ser1101 of the insulin receptor substrate-1 and downstream Akt activation at Ser473. These defects were fully rescued by nonselective protein kinase C inhibition or concomitant HSL overexpression to restore a proper lipolytic balance. We show that selective HSL inhibition induces DAG accumulation and insulin resistance.

CONCLUSIONS—Altogether, the data indicate that altered ATGL and HSL expression in skeletal muscle could promote DAG accumulation and disrupt insulin signaling and action. Targeting skeletal muscle lipases may constitute an interesting strategy to improve insulin sensitivity in obesity and type 2 diabetes.

Diabetes 60:1734–1742, 2011

Skeletal muscle insulin resistance is a strong risk factor of type 2 diabetes and cardiovascular diseases (1,2). Dysfunctional adipose tissue can lead to lipid oversupply and increased flux of free fatty acids (FFAs) into skeletal muscle and is associated with the accumulation of intramyocellular triacylglycerols (IMTGs) (3–5). This chronic lipid overload in tissues can evolve to a state of lipotoxicity leading to cell dysfunction (6). The term “lipotoxicity” defines more generally in skeletal muscle a state of lipid overload (increased concentrations of long-chain acyl-CoA, diacylglycerols [DAGs], and ceramide) causing insulin resistance (7–12). DAGs have been shown to activate novel protein kinase C (PKC) isoforms, such as the novel PKCθ leading to insulin receptor substrate-1 (IRS-1) serine phosphorylation and impaired downstream insulin signaling (10–13). DAGs can be formed through multiple pathways but are also formed as intermediates during triacylglycerol (TAG) synthesis and hydrolysis (14). The control of lipolysis in skeletal muscle has mainly been attributed to hormone-sensitive lipase (HSL), which exhibits a 10-fold higher specific activity for DAG than TAG (15). HSL null mice display normal TAG hydrolyase activity after an overnight fast and accumulate large amounts of DAG (16). Recently it was shown that adipose triglyceride lipase (ATGL) plays a major role in the regulation of cellular TAG stores in various tissues of the body, including heart and skeletal muscle (17,18). ATGL specifically drives the hydrolysis of TAG into DAG (18). ATGL-deficient mice are more insulin sensitive and glucose tolerant despite a threefold increase in TAG content in their skeletal muscle (17). The molecular mechanism underlying this phenotype remains unclear. In the current study, we hypothesized that an imbalance of ATGL relative to HSL could increase intracellular DAG concentrations and promote insulin resistance. To test this hypothesis, we first examined the relationship between muscle ATGL expression and whole-body insulin sensitivity in a wide range of subjects. We next manipulated the expression/activity of ATGL and HSL in vitro in cultured human primary skeletal muscle cells and evaluated its impact on lipid pools and insulin signaling.

RESEARCH DESIGN AND METHODS

Skeletal muscle cell culture. Satellite cells from vastus lateralis or rectus abdominis biopsies of lean, healthy, insulin-sensitive subjects were isolated by trypsin digestion, preplated on an uncoated petri dish for 1 h to remove fibroblasts, and subsequently transferred to T-25 collagen-coated flasks in Dulbecco’s modified Eagle’s medium (DMEM) low glucose (1 g/L) supplemented with 10% FBS and growth factors (human epidermal growth factor, BSA, dexamethasone, gentamicin, amphotericin B [Fungizone, Invitrogen, Grand Island, NY], and fetuin) as previously described (19). Cells from several donors were pooled and grown at 37°C in a humidified atmosphere of 5% CO2.

Differences of myoblasts into myotubes was initiated at approximately 90% confluence by switching to α-minimum essential medium with antibiotics, from INSERM, U1048, Obesity Research Laboratory, Institute of Metabolic and Cardiovascular Diseases (I2MC), Toulouse, France; the Paul Sabatier University, University of Toulouse, Toulouse, France; the Institute of Clinical Chemistry, University of Regensburg, Regensburg, Germany; the Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, Norway; the Translational Research Institute for Metabolism and Diabetes and the Burnham Institute, Florida Hospital, Winter Park, Florida; and the Centre Hospitalier Universitaire de Toulouse, Biochemistry Laboratory, Biology Institute of Purpan, Toulouse, France.

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Received 24 September 2010 and accepted 3 March 2011.

DOI: 10.2337/db10-1364

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-1364/-/DC1.

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Overexpression of lipases. Human ATGL and HSL cDNAs were cloned into the pcDNA3 vector (Invitrogen Corp., Carlsbad, CA). DNA sequencing was performed to check correct insertion of the cDNA using an ABI100 automatic sequencer (Applied Biosystems, Courtaboeuf, France). Adenoviruses expressing in tandem green fluorescent protein (GFP) and ATGL or HSL were constructed, purified, and titrated (Vector Biolabs, Philadelphia, PA). An adenovirus containing the GFP gene only was used as a control. Myotubes were infected with the control (GFP), ATGL, and HSL adenoviruses at day 4 of differentiation and remained exposed to the virus for 24 h in serum-free DMEM containing 150 μM/l of oleate complexed to BSA (ratio 4:1). Oleate was preferred to palmitate for lipid loading of the cells to favor TAG synthesis and to avoid the intrinsic lipotoxic effect of palmitate (20,21). No adenovirus-induced cellular toxicity was observed as determined by chemiluminescent quantification of adenylate kinase activity (ToxiLight, Lonza Group Ltd., Basel, Switzerland). For insulin signaling experiments, infected myotubes were incubated for 20 min in DMEM low glucose with or without 100 μM/l of insulin (Sigma-Aldrich, Lyon, France).

Determination of glycolysis synthesis. Cells were preincubated with a glucose- and serum-free α-minimum essential medium for 90 min and then exposed to DMEM supplemented with D[U-14C]glucose (1 μCi/mL; PerkinElmer, Boston, MA) in the presence or absence of 100 μM/l insulin for 3 h. After incubation, glycolysis synthesis was determined as described previously (22).

Pulse-chase studies of lipid metabolism. Cells were pulsed overnight (18 h) with a combination of [1-14C]oleate (1 μCi/mL; PerkinElmer) and unlabelled oleate (100 μM/l final concentration) to prelabel the endogenous TAG pool. After incubation, myotubes were chased for 3 h in nutrient-deficient DMEM containing 0.1 mM/l of glucose and 0.5% FFA-free BSA. At the end of incubation, total lipids were extracted in chloroform/methanol (2v/1v) and separated by thin-layer chromatography as previously described (23). Incorporation rates were normalized to total protein content measured in each well.

Western blot analysis. Muscle tissues and cell extracts were homogenized in a buffer containing 50 mM/l HEPS, pH 7.4, 2 mM/l EDTA, 150 mM/l NaCl, 30 mM/l NaPO4, 10 mM/l NaF, 1% Triton X-100, 10 μM/l protease inhibitor (Sigma-Aldrich), 10 μl/ml phosphatase I inhibitor (Sigma-Aldrich), 10 μl/ml phosphatase II inhibitor (Sigma-Aldrich), and 1.5 mg/ml benzamidine. The homogenates were centrifuged for 25 min at 15,000g, and supernatants were stored at −80°C. Solubilized proteins from muscle tissue and myotubes were run on a 4–20% SDS-PAGE (Bio-Rad, Hercules, CA), transferred onto nitrocellulose membrane (Hybond ECL, GE Healthcare, Buckinghamshire, U.K.), and incubated with the primary antibodies (ATGL, Cell Signaling Technology Inc., Beverly, MA; Perilipin-A, Abcam Inc., Cambridge, MA). The HSL antibody was as previously described (24). Antibodies for insulin signaling proteins were: IRS-1, Akt, p-Akt, Akt, p-Ser473, Akt, p-Tyr612, IRS-1, p-IRS-1, p-Tyr612-IRS-1, IRS-1, and p-Ser660-IRS-1 were all from Cell Signaling Technology Inc. Subsequently, immunoreactive proteins were determined by enhanced chemiluminescence reagents (ECL, Amersham). The signal was measured using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Statistical analyses. All statistical analyses were performed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA). One-way ANOVA followed by Bonferroni post hoc tests were performed to determine differences between groups and treatments. Two-way ANOVA and Bonferroni post hoc tests were used when appropriate. Normal distribution of the data was tested using the Shapiro-Wilk normality test. The relationships between muscle ATGL protein and clinical variables were analyzed using Spearman rank correlations. All values in Figs. 1 to 7 and Table 1 are presented as mean ± SEM. Statistical significance was set at P < 0.05.

RESULTS

Relationship between muscle ATGL and insulin sensitivity. We first investigated the relationship between skeletal muscle ATGL protein content and insulin sensitivity...
in human vastus lateralis samples of young lean, young obese, and type 2 diabetic subjects. The characteristics of the subjects are described in Table 1. As expected, obese and type 2 diabetic subjects had lower insulin sensitivity and higher body fat, fasting glucose, and fasting insulin compared with lean subjects. Muscle ATGL protein content was significantly elevated in type 2 diabetic subjects when compared with lean and obese subjects (0.50 ± 0.04, 0.45 ± 0.05, and 0.65 ± 0.07 arbitrary units [AU] for lean, obese, and type 2 diabetic subjects, respectively) (Fig. 1A and E).

We also confirm a reduced HSL Ser660 phosphorylation (0.43 ± 0.08 vs. 0.27 ± 0.05 AU, −36%) (Fig. 1C and E) and HSL protein content (0.47 ± 0.09 vs. 0.25 ± 0.07 AU, −48%) (Fig. 1B and E) in skeletal muscle of obese compared with lean subjects. HSL Ser660 phosphorylation (0.65 ± 0.21 vs. 0.43 ± 0.08 AU) and total HSL (1.05 ± 0.43 vs. 0.47 ± 0.06 AU) tended to increase in type 2 diabetic subjects compared with lean subjects. The ratio of phosphorylated HSL Ser660 to total HSL was not significantly changed between groups (Fig. 1D). We noted a significant inverse relationship between muscle ATGL protein content and whole-body insulin sensitivity measured by euglycemic hyperinsulinemic clamp across individuals (Fig. 1F). Similarly, muscle ATGL protein was positively correlated with fasting glucose levels ($r = 0.56$, $P < 0.005$). Perilipin-A protein was not detectable in any of the muscle samples excluding significant contamination by infiltrated adipocytes (data not shown).

**Adenovirus-mediated ATGL overexpression.** We used an adenovirus gene delivery method to overexpress ATGL in our cell culture model. ATGL protein expression was induced by $\sim 3.4 ± 0.2$-fold (Fig. 2A). ATGL overexpression increased TAGH activity twofold (Fig. 2B), but did not change DAGH activity as expected (Fig. 2C). We checked that the ATGL enzyme was operative in intact cells by directly measuring the rate of incorporation of radiolabeled oleate into different lipid pools. We could show that ATGL overexpression reduced the rate of incorporation of [1-14C] oleate into TAG (Fig. 2D), whereas the rate of incorporation was increased into DAG (Fig. 2E) and intracellular FFA (Fig. 2F).

**Elevated ATGL expression promotes DAG and ceramide accumulation.** We found that total TAG content was reduced in myotubes overexpressing ATGL (0.60 ± 0.07-fold, $P < 0.01$) (Fig. 3A). Conversely, consistent with an
imbalance of ATGL relative to HSL, total DAG content increased in myotubes overexpressing ATGL (1.5 ± 0.1-fold) (Fig. 3B). Ceramide content was also increased in myotubes overexpressing ATGL (2.9 ± 0.7-fold), possibly as a consequence of increased intracellular FFA flux and de novo ceramide synthesis (Fig. 3C).

**Elevated ATGL expression impairs insulin signaling and action.** We first showed that glycogen synthesis under insulin stimulation was impaired by 30% (P < 0.05) in myotubes overexpressing ATGL (Fig. 4A). Insulin-stimulated glycogen synthesis was significantly reduced in myotubes overexpressing ATGL (0.72 ± 0.07-fold, P = 0.01). We further showed that ATGL overexpression increased Ser1101–IRS-1 phosphorylation by twofold at baseline and under insulin stimulation (P < 0.001) (Fig. 4B and D). IRS-1 phosphorylation at Ser1101 inhibits IRS-1 tyrosine phosphorylation and function and is a primary target for PKC (13), suggesting a possible link between ATGL and PKC activation. Downstream insulin activation of Akt on the residue Ser473 was also impaired (change insulin minus baseline −31%, P < 0.05) during ATGL overexpression. Ser473 Akt phosphorylation was increased at baseline (P < 0.05) and reduced under insulin stimulation in ATGL-overexpressing myotubes, whereas total Akt protein content was not different across treatments (Fig. 4C and D).

**ATGL-mediated insulin resistance involves DAG and PKC activation.** Because several isoforms of novel PKC have been linked to insulin resistance (33), we used a broad range nonselective PKC inhibitor in our experiments. Calphostin C treatment greatly enhanced insulin-stimulated Akt activation in ATGL overexpressing myotubes (+163%, P < 0.01 compared with GFP with calphostin C)
PKC inhibition by the nonselective inhibitor calphostin C rescued insulin-stimulated glycogen synthesis (Fig. 5A) and glycogen phosphorylase activity (Fig. 5B) in myotubes overexpressing ATGL. (100 nmol/L) was sufficient to fully rescue ATGL-mediated insulin resistance. ATGL-mediated inhibition of glycogen synthesis and Akt phosphorylation was not prevented by the de novo ceramide synthesis inhibitor myriocin (10 μmol/L) (Supplementary Fig. 1).

**HSL overexpression is sufficient to rescue ATGL-mediated insulin resistance.** We tested the hypothesis that restoring a proper cellular lipolytic balance could reverse ATGL-mediated insulin resistance. To do so, we concomitantly overexpressed ATGL and HSL in differentiated myotubes and examined insulin signaling and action. HSL overexpression by itself did not produce lipotoxicity and insulin resistance (Supplementary Fig. 2). The concomitant overexpression of HSL and ATGL increased both HSL (3.4 ± 0.8-fold, \( P < 0.05 \)) and ATGL (3.2 ± 0.6-fold, \( P < 0.05 \)) protein content, TAGH activity (4.4 ± 2.0-fold, \( P < 0.01 \)), and DAGH activity (5.1 ± 1.8-fold, \( P < 0.01 \)). When coexpressed with ATGL, HSL was sufficient to completely rescue ATGL-mediated insulin resistance. This was evidenced by a full restoration of insulin-mediated Akt activation (Fig. 6A and B) and glycogen synthesis (Fig. 6C) to GFP control levels in myotubes coexpressing ATGL and HSL compared with ATGL alone.

**Selective HSL inhibition impairs insulin signaling.** We next examined the effect of HSL-selective inhibition by the BAY compound on DAG levels and insulin action. BAY has been shown to be a highly selective HSL inhibitor in previous studies (24). We observed a moderate increase in total DAG levels (+29% \( P = 0.068 \)) in myotubes treated for 24 h with 1 μmol/L of BAY (Fig. 7A). BAY treatment did not increase ceramide content (0.57 ± 0.07 vs. 0.57 ± 0.06 nmol/mg for control and BAY, respectively). In the same treatment condition, BAY reduced Tyr612–IRS-1 phosphorylation on insulin treatment (−30%, \( P < 0.05 \)) (Fig. 7B) and coexpression of ATGL with HSL increased IRS-1 activity (+30%, \( P = 0.03 \)) (Fig. 7B and D). We did not observe additive effects of ATGL overexpression combined with HSL inhibition compared with ATGL overexpression alone on insulin-stimulated Akt Ser473 phosphorylation (Supplementary Fig. 3).

**DISCUSSION.** Ectopic fat deposition in non-adipose tissues such as skeletal muscle is a common feature of insulin resistance in obesity and type 2 diabetes (3–5). The mechanisms underlying IMTG accumulation and elevated lipotoxicity in insulin-resistant states are not yet fully understood. We show for the first time a novel mechanism by which an altered lipolytic balance in skeletal muscle might contribute to lipotoxicity and insulin resistance in humans. An imbalance of ATGL relative to HSL promotes DAG accumulation and induces insulin resistance at least in part through a DAG/PKC pathway.

We first showed that ATGL protein expression in vastus lateralis samples obtained from a wide range of subjects was negatively associated with whole-body insulin sensitivity measured by euglycemic hyperinsulinemic clamp. It is interesting to note that the glucose disposal rate during this clamp is mostly accounted by skeletal muscle and thus mostly reflects skeletal muscle insulin sensitivity in lean subjects. Higher doses of insulin are required to fully suppress hepatic glucose production in obese and type 2 diabetic subjects as previously shown (34). Thus, the relationship between muscle ATGL and insulin sensitivity is stronger in lean subjects than in obese and type 2 diabetic
subjects. When the data were examined by group, only obese type 2 diabetic subjects displayed increased muscle ATGL protein content. This finding is slightly in contrast with recent data showing an increased skeletal muscle ATGL protein expression in nondiabetic obese versus age-matched lean individuals (35). The discrepancy could be explained by an effect of aging because the type 2 diabetic subjects were older than the lean and obese subjects in our study. Thus, age-related changes in skeletal muscle lipases with respect to insulin sensitivity should be further explored. Elevated muscle ATGL protein in obese type 2 diabetic subjects is in agreement with a higher muscle DAG

![FIG. 5. ATGL-mediated insulin resistance involves PKC activation. A: Representative blots of Ser473 pAkt, total Akt, and GAPDH in the presence (+) or absence (−) of insulin and the nonselective PKC inhibitor calphostin C (1 μmol/L) in control myotubes (GFP) and myotubes overexpressing ATGL. B: Quantitative bar graph of insulin-stimulated Ser473 Akt phosphorylation (n = 3); insulin-stimulated Akt phosphorylation was calculated as the Δ between basal and insulin stimulation in each condition. *P < 0.05 vs. GFP; #P < 0.01 vs. GFP calphostin C. C: Insulin-stimulated glycogen synthesis in myotubes expressing GFP and ATGL in the absence (control) or presence of calphostin C. Glycogen synthesis was expressed as the Δ change between glycogen synthesis under insulin stimulation and glycogen synthesis at baseline. *P < 0.05 vs. GFP (n = 4).](diabetes.diabetesjournals.org)

![FIG. 6. Rescue of ATGL-mediated insulin resistance by HSL. A: Representative blots of Ser473 pAkt, total Akt, and GAPDH in the presence (+) or absence (−) of insulin in control myotubes (GFP) and myotubes overexpressing ATGL alone (Ad-ATGL) or in combination with HSL (Ad-ATGL+Ad-HSL). B: Quantitative bar graph of insulin-stimulated Ser473 Akt phosphorylation (n = 4); insulin-stimulated Akt phosphorylation was calculated as the Δ between basal and insulin stimulation in each condition. *P < 0.05 vs. GFP. C: Insulin-stimulated glycogen synthesis was measured in control myotubes (GFP) and myotubes overexpressing ATGL alone (Ad-ATGL) or in combination with HSL (Ad-ATGL+Ad-HSL). *P < 0.05 vs. GFP (n = 6).](diabetes.diabetesjournals.org)
content previously reported in these subjects (36). We next evaluated the causal relationship between elevated ATGL expression and insulin resistance in primary culture of skeletal muscle cells.

We overexpressed ATGL in human primary myotubes using an adenovirus and assessed the consequences on lipids and insulin action. Elevated expression of ATGL reduced TAG content and simultaneously increased DAG and ceramide content. Ceramides are potentially produced de novo through the action of serine palmitoyl-transferase I as previously shown (8). ATGL-mediated lipotoxicity was paralleled by impairment in insulin-stimulated glycogen synthesis and insulin signaling possibly due to PKC-mediated Ser1101-IRS-1 phosphorylation and downstream inhibition of Akt Ser473 phosphorylation. Of note, increased ATGL expression induced baseline Akt Ser473 phosphorylation independently of insulin. This suggests that ATGL may activate potential regulators of Ser473 Akt, such as mTORC2, by yet unknown mechanisms (37). Future studies will be required to dissect the precise mechanism by which ATGL elicits insulin resistance in primary culture of skeletal muscle cells.

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expression/activity in skeletal muscle, we next evaluated the consequences of inhibiting HSL activity on lipid pools and insulin sensitivity in myotubes. HSL activity is regulated by phosphorylation on serine residues in response to muscle contraction and catecholamines in vivo (48). Jocken et al. (46) found that reduced HSL phosphorylation at Ser563, Ser565, and Ser659 was entirely due to lower HSL protein content and associated with reduced resting glycerol release from the forearm muscle of obese subjects. We report that selective inhibition of HSL increases specifically total DAG levels and consequently disrupts insulin receptor signaling and action. This observation is somehow consistent with data on HSL knockout mice that are insulin-resistant at the level of the skeletal and cardiac muscles when fed a chow diet (49,50) and accumulate DAG in their muscles (16). Further studies will be required to unravel the precise mechanism by which reduced HSL expression/activity induces insulin resistance in skeletal muscle.

In conclusion, the current study highlights a new mechanism by which an altered lipolytic balance between ATGL and HSL induces DAG and insulin resistance in skeletal muscle. The molecular mechanism involves at least in part DAG-mediated PKC activation. Future studies should explore the cause-effect relationship between altered lipase expression and insulin resistance in vivo in animal models with targeted modulations of lipase expression in skeletal muscle. Targeting skeletal muscle lipases might be of potential therapeutic interest for improving insulin resistance in obesity and type 2 diabetes.

ACKNOWLEDGMENTS

This work was supported by grants from the National Research Agency (ANR-09-JCJC-0019-01) and the European Foundation for the Study of Diabetes/Novo Nordisk (to C.M.); the Commission of the European Communities (Integrated Project HEPADIP; http://www.hepadip.org/). Contract No. LSHM-CT-2005-018734 (to D.L.); and the National Institutes of Health grants US-1P30-DK-072476 (Pennington Biomedical Research Center/Nutrition Obesity Research Center) and R01-AG-030226 (to S.R.S.). The Hormone Assay and Analytical Services Core, Vanderbilt Diabetes Research and Training Center, supported by National Institutes of Health Grant DK-20593, performed TAG and DAG analyses.

No potential conflicts of interest relevant to this article were reported.

P.-M.B., K.L., A.M., G.L., and G.S. researched data and reviewed and edited the article. A.C.R. reviewed and edited the article. S.R.S. and D.L. contributed to discussion and reviewed and edited the article. C.M. researched data and wrote the article.

The authors thank Diana Albarado (Pennington Biomedical Research Center, Baton Rouge, LA), Shantele Thomas (Burnham Institute, Winter Park, FL), and Maarten Coonen (Maastricht University, the Netherlands) for excellent technical assistance.

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