Effect of birth weight and 12 weeks of exercise training on exercise-induced AMPK signaling in human skeletal muscle

Brynjulf Mortensen,1,2 Janne R. Hingst,2 Nicklas Frederiksen,2 Rikke W. W. Hansen,2
Caroline S. Christiansen,2 Ninna Iversen,4,5 Martin Friedrichsen,2,3 Jesper B. Birk,2 Henriette Pilegaard,4,5
Ylva Hellsten,6 Allan Vaag,1,3 and Jørgen F. P. Wojtaszewski2,3

1Steno Diabetes Center, Gentofte, Copenhagen, Denmark; 2Molecular Physiology Group, The August Krogh Centre,
Department of Nutrition, Exercise and Sports, University of Copenhagen, Copenhagen, Denmark; 3Rigshospitalet, Department
of Endocrinology, University of Copenhagen, Copenhagen, Denmark; 4Centre of Inflammation and Metabolism, University of
Copenhagen, Copenhagen Denmark; 5Department of Biology, The August Krogh Centre, University of Copenhagen,
Copenhagen, Denmark; 6Integrated Physiology Group, The August Krogh Centre, Department of Nutrition, Exercise and
Sports, University of Copenhagen, Copenhagen, Denmark

Submitted 12 June 2012; accepted in final form 18 April 2013

Mortensen B, Hingst JR, Frederiksen N, Hansen RW, Christiansen CS, Iversen N, Friedrichsen M, Birk JB, Pilegaard H, Hellsten Y, Vaag A, Wojtaszewski JF. Effect of birth weight and 12 weeks of exercise training on exercise-induced AMPK signaling in human skeletal muscle. Am J Physiol Endocrinol Metab 304: E1379–E1390, 2013. First published April 23, 2013; doi:10.1152/ajpendo.00295.2012.—Subjects with a low birth weight (LBW) display increased risk of developing type 2 diabetes (T2D). We hypothesized that this is associated with defects in muscle adaptations following acute and regular physical activity, evident by impairments in the exercise-induced activation of AMPK signaling. We investigated 21 LBW and 21 normal birth weight (NBW) subjects during 1 h of acute exercise performed at the same relative workload before and after 12 wk of exercise training. Multiple skeletal muscle biopsies were obtained before and after exercise. Protein levels and phosphorylation status were determined by Western blotting. AMPK activities were measured using activity assays. Protein levels of AMPKα1 and -γ were significantly increased, whereas AMPKγ3 levels decreased with training independently of group. The LBW group had higher exercise-induced AMPK Thr172 phosphorylation before training and higher exercise-induced ACC2 Ser221 phosphorylation both before and after training compared with NBW. Despite exercise being performed at the same relative intensity (65% of V̇O₂peak), the acute exercise response on AMPK Thr172, ACC2 Ser221, AMPKα2β2γ1, and AMPKα2β2γ3 activities, GS activity, and adenine nucleotides as well as hexokinase II mRNA levels were all reduced after exercise training. Increased exercise-induced muscle AMPK activation and ACC2 Ser221 phosphorylation in LBW subjects may indicate a more sensitive AMPK system in this population. Long-term exercise training may reduce the need for AMPK to control energy turnover during exercise. Thus, the remaining γ3-associated AMPK activation by acute exercise after exercise training might be sufficient to maintain cellular energy balance.

Exercise training; fetal programming; AMPK

TYPE 2 DIABETES (T2D) is a complex, multiorgan metabolic disease influenced by both genetic and nongenetic factors, of which physical activity level and intrauterine environment are considered important environmental contributors (12).

Large-scale intervention studies have been successful in reducing, or at least postponing, the incidence of T2D by increasing the level of daily physical activity (41), and as such, exercise has proven an important nonpharmacological tool in T2D prevention and treatment. However, the molecular mechanisms responsible for these health benefits are not well established. During the last decade, accumulating evidence from studies of both human and rodent skeletal muscle indicates that AMP-activated protein kinase (AMPK) plays an important role in orchestrating several metabolic adaptations during/following exercise. AMPK is a heterotrimeric complex of which the different isoforms of each subunit potentially can combine into 12 different complexes with diverse functions (40). AMPK activation is suggested to mediate, at least in part, the increase in fat oxidation normally observed during exercise via Ser221 phosphorylation, and thereby inhibition of its downstream target acetyl coenzyme A carboxylase (ACC) (26), of which the ACC2 is the predominant isomorph in human skeletal muscle. AMPK is also believed to affect transcription of several target genes such as glucose transporter 4 (Glut4) (22), hexokinase II (HK2) (14), and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (39) and interact in a complex partnership with the energy-sensing molecule sirtuin 1 (Sirt1) in regulation of PGC-1α activity (37). Interestingly, birth weight has also been inversely associated with skeletal muscle AMPK activity in sheep (45) and positively associated with skeletal muscle PGC-1α mRNA expression in human twins (19). We have previously documented a range of metabolic abnormalities in insulin-sensitive tissues of young LBW subjects that potentially could be linked to altered expression of AMPK and/or PGC-1α (13, 15, 28, 32).

In search of a simple, general public health promotion message, it is often assumed that the health benefits achieved following exercise are comparable across populations. Recently, this notion has changed somewhat, as several studies have documented the existence of various subgroups of “nonresponders” lacking adaptations to physical activity (4, 38). Especially important in the context of T2D risk is the observation that more than 30% of a very large human cohort did not improve insulin sensitivity after 20 wk of exercise training (3). To this end, De Filippis et al. (8) observed that insulin-resistant subjects have a compromised increase in skeletal muscle PGC-1α mRNA content following an acute exercise bout compared with controls, indicating that insulin-resistant muscle might also be “exercise resistant”. In other words, insulin-
resistant subjects may be unable to benefit sufficiently from the health-promoting effects of exercise training, initiating and maintaining a vicious cycle eventually leading to overt T2D. These observations highlight that investigations of skeletal muscle, not only in the basal state but also in response to acute exercise, is warranted in prediabetic human populations in search for pathogenic defects.

In this context, very little is known on exercise training adaptability of LBW subjects and whether this could be linked to the increased T2D risk in adulthood in this population. At present, only epidemiologically based data have been reported, and so far they show conflicting results both as to whether the level of physical activity during adulthood is determined by birth weight (17, 34) and whether physical activity modulates the association between LBW and development of metabolic diseases (27, 33).

Therefore, we aimed to investigate effects of both acute exercise and prolonged exercise training on skeletal muscle AMPK signaling in an LBW and normal birth weight (NBW) control setup, hypothesizing that acute AMPK activation with concomitant downstream effects as well as long-term training effects would be compromised in LBW subjects compared with NBW controls.

MATERIALS AND METHODS

Ethical approval. This study was approved by The Regional Ethics Committee of the Capital Region of Denmark (protocol no. H-A-2009-040) and conducted in accordance with the principles of the Helsinki Declaration. All subjects were informed both orally and in writing of the purpose and risks related to the study and gave written consent to participate.

Subjects. Through the Danish Birth Registry we recruited in total 30 LBW subjects (birth weight below 10th percentile) and 23 NBW control subjects (birth weight in the 50th-90th percentile range) born between 1987 and 1989 and living at the time in the Copenhagen area. All subjects were male, singletons, born at term, nondiabetic, and with no family history of diabetes.

In an attempt to match groups for baseline aerobic capacity and ensure that the exercise training intervention would represent a significant increase in physical activity level, we included only subjects being physically active less than 10 h/wk (including transportation-related biking, walking, etc.).

Experimental protocol. On the first day (a Friday), besides basic anthropometric measurements, VO2peak was determined for all subjects by using an incremental test to exhaustion on an ergometer cycle (Monark Ergomedic 839E, Monark, Sweden). Oxygen uptake, carbon dioxide release, and heart rate were measured continuously with an online respiratory gas exchange system (Oxycon pro, Jaeger, Germany).

On the second day (the following Monday), subjects fasted overnight and underwent a 3-h euglycemic-hyperinsulinemic clamp (80 mU·m−2·min−1) with basal and insulin-stimulated skeletal muscle biopsies obtained in vastus lateralis muscle of one leg (data from that leg were used throughout the study). On the third day (the following Wednesday), subjects met over-night fasted and rested in the supine position before a basal skeletal muscle biopsy was obtained. Subjects then performed 1 h of cycle exercise at 65% of VO2peak with continuous monitoring of heart rate, oxygen uptake, and carbon dioxide release before a second skeletal muscle biopsy was excised immediately after exercise. Subjects then rested in the fasted state, although water was offered ad libitum, until a final skeletal muscle biopsy was obtained 4 h into recovery (all 3 biopsies were obtained in the leg not used on day 2). Venous blood samples were collected from the antecubital vein for determination of metabolite and hormone levels 10 min prior to all three skeletal muscle biopsies on that day. Subjects then received a standardized meal and underwent a lumbar-emission X-ray absorptionmetry scan (Hologic QDR series, Discovery A; Hologic, Bedford, MA).

All three examination days were repeated in the same manner after completion of the 12-wk training regimen. The postraining acute-exercise protocol was performed at the same relative workload (65% of post-training VO2peak).

In order to standardize circumstances regarding possible confounders from acute-exercise effects and/or diet, all subjects were told to avoid alcohol and physical activity 5 days prior to all examination days. All subjects underwent dietary registration for 5 days prior to the acute-exercise day before training and were instructed to eat according to that diet registration for the 5 days prior to the acute exercise performed after the training regimen.

Training regimen. After completion of the three pretraining examination days, the training regimen started on the following Monday. The endurance training was performed on stationary ergometers (Bodybike Classic Supreme, Bodybike, Denmark) at the subjects’ home residence. Subjects trained for 1 h four times a week at a intensity corresponding to 65% of their individual VO2peak for 12 wk. Based on the pretraining acute exercise study, a target heart rate corresponding to 65% of VO2peak was calculated to determine training intensity. The training regimen was monitored closely using Polar heart rate monitors (Polar CS400, Polar, Finland). Heart rate data for every exercise bout was recorded and analyzed to monitor and give individual feedback on exercise intensity and compliance. A mid-way VO2peak test followed by a 30-min exercise bout was also performed in the laboratory to adjust exercise training intensity as subjects adapted to the training regimen.

Skeletal muscle biopsies were obtained from separate incisions of the vastus lateralis muscle spaced by 5 cm using local anesthesia (Lidocaine) and a 5-mm Bergstrom needle applied with suction. For the postexercise biopsy, the local anesthesia and incision of the skin were made prior to exercising, allowing the biopsy to be obtained within 20–30 s after cessation of exercise. Biopsies were immediately frozen in liquid nitrogen and stored at −80°C until further processing.

Homogenate and lysate preparation. Approximately 20–40 mg wet wt of the muscle biopsies was used for muscle homogenate and lysate preparation, using the same protocol as described previously (2).

SDS-PAGE and Western blotting. Protein expression and phosphorylations were measured using SDS-PAGE with self-casted Tris-HCl gels suitable for the molecular weight of the investigated proteins (7.5–12%). Semidry transfer of proteins to polyvinylidene difluoride membranes (Immobilon Transfer Membrane, Millipore, MA) was used. After primary (overnight) and secondary antibody treatment (45 min), membranes were incubated in a chemiluminescent substrate (Immobilon Western Chemiluminescent Substrate, Millipore, Millipore, MA) and the signal was detected on a Kodak Image Station 2000MM (Kodak, Denmark). Data were expressed relative to a pooled human or rat skeletal muscle control sample.

AMPK activity assay. In order to obtain separate activity measurements of the three AMPK complexes present in human vastus lateralis muscle (α1β2γ1 γ2, α2β2γ3, and α2β2γ1 γ2) (2, 42), γ3, α1-, and α2-containing AMPK heterotrimer complexes were, in that order, immunoprecipitated in microtiter wells from the same 300 µg of muscle lysate using protein G-agarose beads (Millipore, MA) and AMPK isoform-specific antibodies. The total AMPK activity was also calculated by adding together the individual activities of the three AMPK complexes. The activity assay was performed as described previously (23).

GS activity assay. Muscle GS activity was measured in triplicates using a 96-well plate assay (Unifilter 350 Plates; Whatman, Cambridge, UK). Homogenate samples were analyzed in the presence of 0.17 and 8 mmol/l glucose 6-phosphate (G-6-P). GS activity data are reported as the percentage of fractional velocity (%FV; 100 × activity
in the presence of 0.17 mmol/l G-6-P divided by the activity observed at 8 mmol/l G-6-P.

Muscle lactate, creatine, phosphocreatine, and adenine nucleotide measurements were performed as described previously (43).

Antibodies. AMPK\textsubscript{\alpha1}, -\alpha2, -\beta2, and -\gamma3 protein levels as well as phosphorylation of GS sites 1b, 2a, and 3a+\textsubscript{+b} were evaluated using primary antibodies raised in sheep provided by Prof. D. G. Hardie (University of Dundee). GS protein levels were measured using a primary antibody provided by Prof. Oluf Pedersen (University of Copenhagen). The following primary antibodies were also used: AMPK\textsubscript{\gamma1} (#32508, Abcam, Cambridge, UK), AMPK\textsubscript{\gamma3} (#52-5717, Zymed, after 2008 produced by Life Technologies, UK), pAMPK\textsubscript{\gamma\textsubscript{1}, -\alpha1, -\beta1, -\gamma1} (P0448 and P0163, Dako).

mRNA expression. RNA was extracted using TRI Reagent (Sigma-Aldrich, St. Louis, MO). cDNA was synthesized using a Quantitect Rev. Transcription Kit according to the manufacturer’s recommendations (Qiagen, Valencia, CA). The cDNA (mRNA) content of a given gene was determined by real-time PCR using Taqman assay as previously described (20). Primers and TaqMan Probes with 5'-FAM and 3'-TAMRA labeling were purchased from TaqCopenhagen. The number of cycles until a sample reaches a given fluorescence level is defined as the cycle threshold (C\textsubscript{T}). A serial dilution of a pooled representative sample was run on each plate and used to construct a standard curve, from which the C\textsubscript{T} values of the samples were transformed to a relative mRNA amount. For each sample, the specific target mRNA content was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA content in the given sample. GAPDH mRNA content was not affected by the intervention.

Glycogen. Muscle glycogen content was measured in muscle homogenates (150 μg of protein) as glycosyl units after acid hydrolysis determined by a fluorometric method.

Statistical methods. All statistical tests were performed in SAS (v. 9.1; SAS Institute, Cary, NC). A stepwise approach with two-tailed repeated-measures ANOVA was applied to investigate the effect of the intervention (e.g., training and acute exercise) and group (birth weight) and the interaction between these. The interaction was removed from the model when no significant interaction was observed.

The ANOVA model was applied in the following manner.

1) Basal values (Pre) before and after the exercise training intervention were investigated.

2) Basal (Pre) and exercised values (Post) before the exercise training intervention were investigated.

3) Basal (Pre) and exercised values (Post) after the exercise training intervention were investigated.

4) In situations where acute effects of exercise were observed in analysis 2 or 3, the pre-post (Δ) values before the exercise training intervention and the Δ values after the exercise training intervention were investigated to determine the impact of training on the response to acute exercise.

5) Basal (Pre) and 4 h into recovery (4 h) values before the exercise training intervention were investigated.

6) Basal (Pre) and 4 h into recovery (4 h) values after the exercise training intervention were investigated.

7) In situations where recovery effects were observed in 5 or 6, the Pre-4 h Δ before the exercise training intervention and the Pre-4 h Δ after the exercise training intervention were investigated (only performed for mRNA data).

Pearson correlation coefficients were used. P < 0.05 was considered statistically significant. Data are presented as means ± SE throughout.

RESULTS

Clinical characteristics. In total, 11 subjects chose to end their participation before completing the training regime (9 LBW and 2 NBW subjects). This was mainly due to training regimen compliance issues (7 LBW and 1 NBW), leaving the total number of subjects to complete the study at 42, 21, in each group.

LBW subjects had significantly lower birth weight and lower present height, weight, BMI, and lean body mass than the NBW group. Aerobic capacity, blood pressure, fat mass, and fat distribution were similar in the two groups. The following main effects of training were identified: decreased body weight, BMI, total fat mass, whole body fat percentage, trunk fat, and higher aerobic capacity (Table 1).

Both groups displayed similar characteristics regarding resting heart rate, maximal heart rate, heart rate corresponding to 65% of VO\textsubscript{2peak}, and respiratory exchange ratio (RER) during acute exercise. Relative workload during acute exercise was similar, but absolute workload was significantly lower in the LBW group. A main effect of training was found on maximal heart rate (lower after training) and absolute workload eliciting

Table 1. Clinical subject characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Before training (n = 30)</th>
<th>Midway (n = 24)</th>
<th>After training (n = 21)</th>
<th>Before training (n = 23)</th>
<th>Midway (n = 22)</th>
<th>After training (n = 21)</th>
<th>P Value (Main Effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight, g</td>
<td>2773 ± 28</td>
<td>2773 ± 28*</td>
<td>2773 ± 28*</td>
<td>3773 ± 33*</td>
<td>3773 ± 33*</td>
<td>3773 ± 33*</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Age, yr</td>
<td>22 ± 0</td>
<td>22 ± 0</td>
<td>22 ± 0</td>
<td>22 ± 0</td>
<td>22 ± 0</td>
<td>22 ± 0</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Height, cm</td>
<td>178 ± 1</td>
<td>184 ± 2*</td>
<td>184 ± 2*</td>
<td>184 ± 2*</td>
<td>184 ± 2*</td>
<td>184 ± 2*</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>71.9 ± 1.6</td>
<td>71.0 ± 1.7</td>
<td>70.2 ± 1.7</td>
<td>83.2 ± 3.0</td>
<td>82.9 ± 3.0</td>
<td>81.3 ± 2.8</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>BMI, kg/m\textsuperscript{2}</td>
<td>22.7 ± 0.5</td>
<td>22.6 ± 0.5</td>
<td>22.4 ± 0.5</td>
<td>24.5 ± 0.8</td>
<td>24.5 ± 0.7</td>
<td>24.2 ± 0.7</td>
<td>P &lt; 0.03</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.88 ± 0.01</td>
<td>0.89 ± 0.01</td>
<td>0.89 ± 0.01</td>
<td>0.85 ± 0.01</td>
<td>0.87 ± 0.01</td>
<td>0.86 ± 0.01</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Aerobic capacity, ml•min\textsuperscript{-1}•kg\textsuperscript{-1}</td>
<td>48.3 ± 1.0</td>
<td>52.4 ± 1.1</td>
<td>55.1 ± 1.3</td>
<td>48.0 ± 1.5</td>
<td>52.4 ± 1.6</td>
<td>54.5 ± 1.6</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Lean body mass, kg</td>
<td>56.9 ± 1.0</td>
<td>56.7 ± 1.0</td>
<td>56.5 ± 1.4</td>
<td>56.0 ± 1.4</td>
<td>56.0 ± 1.5</td>
<td>56.0 ± 1.5</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Total fat, kg</td>
<td>12.6 ± 0.8</td>
<td>11.8 ± 0.9</td>
<td>11.9 ± 0.9</td>
<td>14.9 ± 1.8</td>
<td>14.9 ± 1.8</td>
<td>13.6 ± 1.7</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Whole body fat, %</td>
<td>17.1 ± 0.8</td>
<td>16.3 ± 1.0</td>
<td>17.2 ± 1.3</td>
<td>16.0 ± 1.2</td>
<td>16.0 ± 1.2</td>
<td>16.0 ± 1.2</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Trunk fat, %</td>
<td>16.2 ± 1.0</td>
<td>15.4 ± 1.3</td>
<td>16.4 ± 1.5</td>
<td>14.7 ± 1.3</td>
<td>14.7 ± 1.3</td>
<td>14.7 ± 1.3</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Systolic resting BP</td>
<td>123 ± 2</td>
<td>121 ± 2</td>
<td>124 ± 2</td>
<td>124 ± 2</td>
<td>124 ± 2</td>
<td>124 ± 2</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Diastolic resting BP</td>
<td>67 ± 1</td>
<td>65 ± 2</td>
<td>66 ± 1</td>
<td>68 ± 2</td>
<td>68 ± 2</td>
<td>68 ± 2</td>
<td>P &lt; 0.0001</td>
</tr>
</tbody>
</table>

Values are means ± SE. LBW, lean birth weight; NBW, normal birth weight. *Significant group difference on data before training.

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65% of $\dot{V}O_{2\text{peak}}$ (increased after training). Both groups showed similar training intervention compliance rates among subjects who completed the study (Table 2).

**Protein expression.** No effects of acute exercise were found on expression of any of the investigated proteins. Therefore, the following statistical analyses were based on a mean value calculated from the three biopsies taken before and after the training intervention, respectively. We observed the following intrindidividual variations in protein expression data [calculated as (highest data point − lowest data point)/mean of all 3 data points × 100 and given as means ± SE]: AMPKα1, 51% ± 4 before training and 55% ± 5 after training; AMPKα2, 30% ± 2 before training and 29% ± 2 after training; AMPKβ2, 35% ± 3 before training and 32% ± 2 after training; AMPKγ1, 59% ± 5 before training and 55% ± 5 after training; AMPKγ3, 46% ± 4 before training and 49% ± 5 after training; ACC2, 42% ± 4 both before and after training and GS: 64% ± 6 before training and 61% ± 4 after training.

Protein levels were increased for AMPKα1 ($P < 0.0001$), AMPKγ1 ($P < 0.01$), and GS ($P < 0.001$) after 3 mo of training. AMPKα2, AMPKβ2, and ACC2 protein levels remained unchanged, whereas AMPKγ3 protein levels were decreased ($P < 0.01$) after training. These protein levels and training effects did not differ between groups, and no group × training interactions were observed (Fig. 1, A–E, and G, Fig. 2, and Fig. 4A).

**AMPK and ACC phosphorylation.** Basal levels of AMPKα Thr172 phosphorylation were increased after training ($P < 0.02$). Before training, AMPKα Thr172 phosphorylation increased with acute exercise ($P < 0.0001$). This increase tended to be higher in the LBW group ($P < 0.06$ for interaction between acute exercise and group). After exercise training, no acute effects were observed. The analyses on pre-post Δ values before and after training confirmed that the acute response was reduced after training ($P < 0.0001$). Both before and after training, basal values of AMPKα Thr172 phosphorylation were similar to 4-h recovery values (Fig. 1F). We observed higher basal Ser211 phosphorylation of ACC after training ($P < 0.0001$). Before training, Ser211 phosphorylation increased with acute exercise ($P < 0.0001$). This increase was higher in the LBW group than in the NBW group ($P < 0.03$ for interaction between acute exercise and group). The acute exercise response (pre-post Δ) was reduced after training compared with before training ($P < 0.001$). Before training, 4-h recovery levels of Ser211 phosphorylation were still elevated compared with basal values ($P < 0.01$), whereas pre- and recovery values were similar after training (Fig. 1H).

**AMPK activity.** Basal AMPKα1β2γ1-associated activity was increased with training ($P < 0.0001$). The α1β2γ1-associated activity was decreased with acute exercise before ($P < 0.006$) but not after training. At 4 h into recovery, α1β2γ1-associated activity was not different from basal values either before or after training (Fig. 3A).

Basal AMPKα2β2γ1-associated activity was increased with training ($P < 0.001$). Before training, α2β2γ1-associated activity was increased with acute exercise in the LBW group only ($P = 0.04$), whereas after training α2β2γ1-associated activity remained unchanged with acute exercise in both groups. The acute response was also significantly different before compared with after training ($P < 0.02$). At 4 h into recovery, α2β2γ1-associated activity was not different compared with basal values either before or after training (Fig. 3B).

The α2β2γ3-associated activity was increased with acute exercise both before and after training ($P < 0.0001$ for both), and the acute response was significantly reduced after training compared with before training ($P = 0.0002$). Four hours into recovery, α2β2γ3-associated activity was comparable to basal values both before and after training (Fig. 3C).

Basal total AMPK activity was increased after training ($P < 0.0001$). Total AMPK activity increased with acute exercise before training ($P = 0.001$) but not after training. The acute effect was also significantly reduced after training compared with before training ($P < 0.03$). Four hours into recovery, the total AMPK activity was comparable to basal values both before and after training (Fig. 3D).

**GS phosphorylation, GS activity, and glycogen content.** Basal GS site 1b phosphorylation was lower after training than before training ($P < 0.001$). Site 1b phosphorylation was decreased with acute exercise before training ($P < 0.01$) but not after training. The acute-exercise response was significantly reduced after training compared with before training ($P = 0.04$). Four hours into recovery, site 1b phosphorylation was no longer different from basal levels before training but was elevated compared with basal values after training ($P < 0.002$; Fig. 4B).

Basal levels of GS site 2+2a phosphorylation were not different before and after training. Site 2+2a phosphorylation was elevated with acute exercise both before ($P < 0.002$) and after ($P = 0.001$) training. At 4 h into recovery, levels of site 2+2a phosphorylation were lower than basal levels both before ($P < 0.0001$) and after ($P < 0.01$) training (Fig. 4C).

### Table 2. Heart rate and acute-exercise-related subject characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>LBW</th>
<th>Before training</th>
<th>After training</th>
<th>NBW</th>
<th>Before training</th>
<th>After training</th>
<th>$P$ Value Main Effects</th>
<th>Training</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting heart rate, beats/min</td>
<td></td>
<td>62 ± 2</td>
<td>59 ± 2</td>
<td></td>
<td>60 ± 2</td>
<td>59 ± 2</td>
<td>$P &lt; 0.0001$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal heart rate, beats/min</td>
<td></td>
<td>195 ± 1</td>
<td>193 ± 1</td>
<td></td>
<td>196 ± 2</td>
<td>191 ± 2</td>
<td>$P &lt; 0.0001$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean heart rate during acute exercise, beats/min</td>
<td></td>
<td>156 ± 2</td>
<td>154 ± 2</td>
<td></td>
<td>160 ± 2</td>
<td>159 ± 2</td>
<td>$P &lt; 0.0001$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intensity during acute exercise, %$\dot{V}O_{2\text{peak}}$</td>
<td></td>
<td>64.4 ± 0.5</td>
<td>64.0 ± 0.2</td>
<td></td>
<td>64.7 ± 0.3</td>
<td>63.6 ± 0.2</td>
<td>$P &lt; 0.0001$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean RER during acute exercise</td>
<td>0.89 ± 0.01</td>
<td>0.90 ± 0.01</td>
<td>0.90 ± 0.01</td>
<td></td>
<td>0.90 ± 0.00</td>
<td>0.91 ± 0.01</td>
<td>$P &lt; 0.0001$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean workload during acute exercise, W</td>
<td>137 ± 3</td>
<td>161 ± 6</td>
<td>161 ± 6</td>
<td></td>
<td>148 ± 3</td>
<td>171 ± 4</td>
<td>$P &lt; 0.0001$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Training intervention compliance rate, %exercise bouts performed</td>
<td></td>
<td>98.4 ± 0.9</td>
<td>98.9 ± 0.6</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Resting heart rate was measured after 30 min of resting in bed. RER, respiratory exchange ratio.
Basal levels of GS site 3a+b phosphorylation were lower after training than before (P < 0.02). Site 3a+b phosphorylation was reduced with acute exercise both before and after training (P < 0.0001 for both), but the acute-exercise response was significantly reduced after compared with before training (P < 0.004). Before training, site 3a+b phosphorylation was still reduced 4 h into recovery compared with basal values (P < 0.0001), whereas after training basal and 4-h recovery values were not different (Fig. 4D).

Basal GS activity was lower after training than before (P < 0.0001). GS activity increased with acute exercise both before and after training (P < 0.0001 for both), and after training, the increase with acute exercise was higher in the LBW group than in the NBW group (P < 0.04). The acute increase in GS activity was lower after training compared with before training (P < 0.0001). GS activity was still increased 4 h into recovery compared with basal levels both before and after training (P < 0.0001 for both). After training, the increase from basal to 4 h into recovery was higher in the LBW group than in the NBW group (P = 0.03; Fig. 4E).

Basal levels of glycogen were increased after training (P < 0.0001) and the increase with training tended to be less pronounced in the LBW than in the NBW group (P < 0.07). Glycogen stores were reduced with acute exercise both before
and after training ($P < 0.0001$ for both). This reduction in muscle glycogen with acute exercise was significantly decreased after training compared with before training ($P < 0.03$). Glycogen stores remained reduced 4 h into recovery compared with basal levels both before and after training ($P < 0.0001$ for both; Fig. 4).

**Muscle lactate, creatine, phosphocreatine, and adenine nucleotide measurements.** Muscle ATP and phosphocreatine (PCr) levels were unaffected by acute exercise both before and after training. Before training, levels of creatine, lactate, free ADP, free AMP as well as the free ADP/ATP and free AMP/ATP ratios were all increased with acute exercise ($0.004 < P < 0.0001$ for all tests), whereas the PCr/(PCr + creatine) ratio decreased with acute exercise ($P < 0.0001$). After training, muscle lactate levels were still increased with acute exercise ($P < 0.003$), whereas all other measurements (Table 3) were unaffected by acute exercise. In line with these findings, a main effect of training was observed on the acute-exercise response on creatine levels, and tendencies toward a training effect were also observed on ADP and AMP measurements ($0.05 < P \leq 0.1$ for all tests). No group differences in acute exercise or training response were observed (Table 3).

**Gene mRNA expression.** No acute exercise, group, or training effects on GAPDH mRNA levels were present (Fig. 5A).

PGC-1α mRNA levels increased with acute exercise before training ($P < 0.03$) but not after. Four hours into recovery, PGC-1α mRNA levels were higher than basal levels both before and after training ($P < 0.0001$ for both), and this response tended to be decreased after training compared with before training ($P = 0.07$; Fig. 5B).

Glut4 mRNA levels increased with acute exercise both before ($P < 0.0001$) and after training ($P < 0.04$). Four hours into recovery, Glut4 mRNA levels were higher than basal levels both before ($P < 0.0001$) and after training ($P < 0.004$). This response to acute exercise tended to be decreased after training compared with before training ($P < 0.1$; Fig. 5C).

HK2 mRNA levels increased with acute exercise before training ($P < 0.05$) but not after training. Four hours into recovery, HK2 mRNA levels were higher than basal levels both before ($P < 0.001$) and after training ($P < 0.0004$), and
this response was decreased after training compared with before training ($P < 0.04$; Fig. 5D).

Basal levels of Sirt1 mRNA were higher after training ($P < 0.05$). Sirt1 mRNA levels increased with acute exercise before training ($P < 0.03$) but not after training. Four hours into recovery, Sirt1 mRNA levels were higher than basal levels before ($P < 0.001$) but not after training (Fig. 5E).

**Blood parameters.** Basal plasma glucose concentration was reduced after training ($P = 0.03$). Before training, plasma glucose was decreased by acute exercise, mostly in the LBW group ($P < 0.006$ for training $\times$ group interaction). After training, acute exercise led to an increase in plasma glucose ($P = 0.02$), and in line with this an effect of training was observed on acute-exercise response ($P < 0.0001$). Plasma glucose was lower 4 h into recovery compared with basal values both before and after training ($P < 0.0001$ for both).

Both insulin and C-peptide levels were decreased with acute exercise before training ($P < 0.0001$ for both) but not after training. Insulin and C-peptide levels were lower 4 h into recovery compared with basal values both before and after training ($P < 0.0001$ for all 4 tests).

Plasma lactate levels were increased with acute exercise both before and after training ($P < 0.0001$ for both). Four hours into recovery, plasma lactate was similar to basal levels both before and after training (Table 4).

**Correlations.** Exercise-induced total AMPK activity correlated significantly with AMPK Thr172 phosphorylation both before ($r = 0.69$, $P < 0.0001$) and after training ($r = 0.37$, $P < 0.02$). Glycogen content was negatively associated with both exercise-induced $\alpha_2B2\gamma1$ and $\alpha_2B2\gamma1$ activity before training ($r = -0.53$, $P < 0.0001$ for both correlations). After training, glycogen content correlated negatively with exercise-induced $\alpha_2B2\gamma3$ activity only ($r = -0.63$, $P < 0.0001$). Glycogen content did not correlate significantly with exercise-induced $\alpha_1B2\gamma1$ activity either before or after training.

**DISCUSSION**

In this study, we hypothesized that the skeletal muscle AMPK signaling response to acute exercise and prolonged endurance training was compromised in LBW subjects compared with NBW controls and that this might in part explain the higher risk of developing T2D in this population. In contrast, we found evidence of increased exercise-induced AMPK activation and ACC2 Ser273 phosphorylation in skeletal muscle of LBW subjects.

As AMPK is generally considered an energy sensor in the cell, it is, in the context of the thrifty phenotype hypothesis (12), tempting to speculate that undernutrition in utero leading to LBW could also promote programming of a more sensitive AMPK system in LBW subjects. However, our data showed no effect of birth weight on AMPK subunit expression, confirming our previous findings (23). We also observed similar changes in AMPK expression profile and basal activity levels in both groups with exercise training. These observations indicate that LBW subjects have a normal regulation of AMPK expression and basal activity in response to exercise training.

To our knowledge, the current human cohort is the largest to date investigating in depth skeletal muscle AMPK signaling in relation to exercise training. In general, our data confirm the changes in AMPK expression reported previously in human skeletal muscle following an exercise training intervention (11, 42). Nevertheless, despite similar AMPK expression, exercise-induced AMPK Thr172 and ACC2 Ser273 phosphorylation was increased in LBW subjects, indicating that in utero programming of the AMPK system might involve birth weight-dependent differences in upstream AMPK regulators rather than differences in AMPK protein expression.

On the other hand, the latter observation may also reflect a diminished ability to maintain energy balance during exercise in LBW subjects. However, our whole body measurements as
well as muscle nucleotide data do not support this line of thought, suggesting that other mechanisms are responsible for the increased AMPK activation observed in these subjects. We have previously found normal in vivo mitochondrial function but reduced insulin-stimulated glycolytic flux in LBW subjects with very similar clinical characteristics (6), pointing at the capacity of glucose metabolism as a possible point of defect in LBW subjects. We (35, 43) and others (44) have previously observed higher exercise-induced AMPK activation in glycogen-depleted compared with glycogen-loaded skeletal muscle, indicating that group differences in glycogen stores might explain differences in AMPK activation. However, the main group effect found on glycogen stores in this study was mostly driven by a group difference after training. Hence, the minor differences in glycogen stores before training are unlikely to have caused the significant group differences observed in AMPK Thr172 and ACC2 Ser221 phosphorylation before training. After training, glycogen stores were increased in the NBW control group only, which might suggest a reduced activity of glycogen synthase (GS) in LBW subjects. However, our data do not support any defect in GS activation during acute exercise or 4 h into recovery in muscle of LBW subjects. On

Table 3. Muscle lactate, creatine, PCR, and adenine nucleotide measurements before and after acute exercise

<table>
<thead>
<tr>
<th>Unit</th>
<th>LBW Before</th>
<th>LBW After</th>
<th>NBW Before</th>
<th>NBW After</th>
<th>ANOVA, Acute Exercise Response (Pre-Post Δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
<td></td>
</tr>
<tr>
<td>ATP mmol/kg dry wt</td>
<td>23.2 ± 0.6</td>
<td>24.0 ± 0.7</td>
<td>22.1 ± 0.9</td>
<td>23.7 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>PCr mmol/kg dry wt</td>
<td>87.0 ± 2.5</td>
<td>81.0 ± 3.2</td>
<td>73.4 ± 3.5</td>
<td>74.9 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>Creatine mmol/kg dry wt</td>
<td>35.7 ± 1.5</td>
<td>46.9 ± 2.5*</td>
<td>37.3 ± 2.5</td>
<td>37.7 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Lactate mmol/kg dry wt</td>
<td>9.9 ± 0.8</td>
<td>16.2 ± 1.6*</td>
<td>12.3 ± 1.7</td>
<td>18.8 ± 2.9*</td>
<td></td>
</tr>
<tr>
<td>ADP free mmol/kg dry wt</td>
<td>0.09 ± 0.0</td>
<td>0.13 ± 0.0*</td>
<td>0.10 ± 0.0</td>
<td>0.10 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>AMP free μmol/kg dry wt</td>
<td>0.38 ± 0.1</td>
<td>0.60 ± 0.1*</td>
<td>0.57 ± 0.1</td>
<td>0.46 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>P(i)/PCR+ creatine</td>
<td>0.71 ± 0.0</td>
<td>0.63 ± 0.0*</td>
<td>0.66 ± 0.0</td>
<td>0.67 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>ADP free ·100/ATP</td>
<td>0.39 ± 0.0</td>
<td>0.55 ± 0.1*</td>
<td>0.48 ± 0.0</td>
<td>0.42 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>AMP free ·100/ATP</td>
<td>1.6 ± 0.3</td>
<td>2.5 ± 0.3*</td>
<td>2.6 ± 0.6</td>
<td>1.9 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. PCR, phosphocreatine. *Significantly different from Pre value.

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the contrary, after training our data suggest a somewhat paradoxically increased GS activity in the LBW group. Thus, it is tempting to speculate that the tendency toward a reduced ability to increase glycogen stores by exercise training in the LBW group in the present study could be related to a reduced GS activation by insulin rather than a compromised GS activation by exercise. However, our previous observations suggest impaired insulin-stimulated GS activation (30) and peripheral insulin action (31) in elderly LBW subjects only, whereas third-trimester growth was associated with insulin action in a paradoxical inverse manner in younger twins (29), which in total speaks against this line of thought for the young LBW cohort in the present study.

In contrast to the present PGC-1α/H9251 mRNA data, we have previously found decreased PGC-1α/H9251 mRNA levels in LBW subjects after 5 days of overfeeding (5) and a positive association between birth weight and basal PGC-1α/H9251 mRNA levels in human twins (19). However, this finding was observed primarily in elderly twins, suggesting that this defect might not yet be present in younger LBW subjects on an isocaloric diet and/or

Table 4. Plasma metabolite and hormone parameters before and after acute exercise

<table>
<thead>
<tr>
<th></th>
<th>Before Training (n = 30)</th>
<th>After Training (n = 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post 4 h Recovery</td>
</tr>
<tr>
<td><strong>LBW</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>5.2 ± 0.1</td>
<td>4.9 ± 0.1*</td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td>32.4 ± 4.0</td>
<td>18.0 ± 1.8*</td>
</tr>
<tr>
<td>C-peptide, pmol/l</td>
<td>501 ± 37</td>
<td>350 ± 22*</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>1.1 ± 0.1</td>
<td>3.1 ± 0.2*</td>
</tr>
<tr>
<td><strong>NBW</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>5.1 ± 0.0</td>
<td>5.2 ± 0.1</td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td>31.5 ± 4.0</td>
<td>21.6 ± 3.1*</td>
</tr>
<tr>
<td>C-peptide, pmol/l</td>
<td>485 ± 43</td>
<td>429 ± 48*</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>1.1 ± 0.0</td>
<td>3.0 ± 0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE. All measurements from blood samples collected from the antecubital vein. Measurements were made before acute exercise (Pre), immediately after (Post) acute exercise, and 4 h into recovery. *Significantly different from Pre value.
that the defect might be caused by twin-specific in utero programming. It has previously been shown that increases in PGC-1α mRNA levels during recovery after exercise correlate positively with exercise intensity (25). In the LBW group, a higher AMPK response was associated with a “normal” mRNA response for all four genes measured, indicating either that AMPK might not be the only upstream factor responsible for exercise-induced increases in these mRNA levels, which is in line with studies performed on AMPK knockout mice (16), or that unknown mechanisms might inhibit exercise-induced increases in mRNA levels, and as such, a higher AMPK activation might be needed to stimulate a given mRNA increase in LBW subjects than in NBW subjects.

Our data show that acute exercise performed at the same relative workload before and after 12 wk of exercise training induced similar responses on whole body measurements such as increases in plasma lactate levels, working heart rate, and RER value. However, one of the novel findings from this study is the observation that, during investigation of local mechanisms in exercise-stimulated human muscle, acute exercise-induced responses of AMPK activity and GS activity as well as the putative downstream targets ACC2 Ser221 phosphorylation and HK2 mRNA levels, although still present after training, were all significantly decreased after the training intervention even when acute exercise was performed at the same relative workload. As far as we are aware, previous human training studies have only compared exercise-induced AMPK activation performed at the same absolute workload before and after a relative short-term (10 days) exercise training intervention (18, 21). In our laboratory, we have previously investigated acute AMPK activation at the same absolute workload in a cross-sectional setup with trained and untrained subjects (24). Previous studies in rats also support that AMPK activation by exercise might be reduced as the muscle adapts to training (9).

Common precipitation analyses in our laboratory have shown that only three AMPK complexes exist in detectable amounts in human vastus lateralis muscle (α1β2γ1, α2β2γ3, and α2β2γ1) (2, 42). Therefore, immunoprecipitating γ3, α1, and α2 in this order from the same lysate sample should reflect separate measurements of AMPK activity for these three AMPK complexes. The ~80% decrease in activity response of the α2β2γ3 complex observed after training in this study may indicate an increased ability for the muscle fibers to maintain energy status after a period of regular exercise training, a view that is in line with the present changes in creatine and adenine nucleotides with acute exercise. On the other hand, AMPK might also be a poor reflector of the cellular energy stress imposed by exercise. At least two factors might contribute to such apparent mismatch, the first being the elevated glycogen content observed after training in this study. The highly significant negative correlations between glycogen levels and exercise-induced AMPKα2β2γ3 activity observed both before and after training in this study support previous observations that glycogen content might be able to inhibit activation of this particular AMPK complex. This line of thought is supported by the finding that γ3 knockout in mice abolishes this association (1). Our observation that glycogen content was significantly associated with α2β2γ1 activity before training but not after training might indicate that exercise training is able to modulate the inhibition from glycogen via for example training-induced changes in intracellular localization of this AMPK complex. The second possible explanation for lower AMPK activation after exercise training is the decreased γ3 protein expression observed after training. As our data clearly demonstrate and previous work in our laboratory confirms (2), the α2β2γ3 complex is the primarily trimetric complex activated during exercise in human skeletal muscle, and a reduction in γ3 expression could therefore potentially influence the degree of activity of this AMPK complex during exercise. However, in the present study we observed only an ~10% decrease in γ3 expression, whereas exercise-induced α2β2γ3 activity was reduced ~80% after training, so it is unlikely that decreased γ3 expression can account for the entire decrease in exercise-induced α2β2γ3 activity observed after training.

The consequence of the extensive suppressed AMPK activation observed after training is unknown, but one interpretation is that after a prolonged period of exercise training AMPK activation seems not to be needed in response to acute exercise, when only a low stress is imposed, e.g., when the muscle contracts in a pattern that it is already adapted to (regarding mode, intensity, etc., as discussed in Ref. 10). In accordance, it has previously been demonstrated that, although endurance training was associated with decreased AMPK phosphorylation during a “familiar” exercise regimen, a bout of “unfamiliar” exercise such as strength training was still able to induce a strong AMPK response (7), which may relate to the recruitment of different skeletal muscle fibers, and/or AMPK activation might depend on different upstream signaling pathways with different types of exercise.

From a purely methodological perspective, our AMPK activity data clearly illustrate the interpretative limitations of measuring only AMPKα Thr172 and/or ACC2 Ser221 phosphorylation status in skeletal muscle as surrogate markers of AMPK activity. For example, although ~80% reduced, our AMPK activity measurements still showed highly significant acute exercise activation of the α2β2γ3 complex after training contrasting the unchanged AMPKα Thr172 phosphorylation (and total AMPK activity) data. This is probably because the α2β2γ3 complex represents only ~20% of the total AMPK complex pool (42), and although not statistically significant, it is evident that when activity of the three AMPK complexes is added to a total AMPK activity the small decrease in activity observed with acute exercise of the α1β2γ1 and α2β2γ1 complexes after training is sufficient to induce a zero “net effect” for all three complexes combined as detected when measuring AMPKα Thr172 phosphorylation and/or total AMPK activity. Our data also illustrate the need to investigate exercise activation patterns of the three different AMPK complexes separately, as they clearly behave very differently in response to acute exercise in human skeletal muscle (2). Furthermore, the degree of activation of all three AMPK complexes, and especially the α2β2γ3 complex, seems to be highly dependent on the training status of the muscle investigated.

Although not based on statistics, it is striking that seven LBW subjects but only one NBW subject chose to stop during this study due to training-related issues. In line with this, previous studies have shown that preterm LBW is associated with less physical activity during adulthood (17). However, we have studied data on the eight dropouts thoroughly and found no indication of differences in phenotype compared with subjects completing the study in both groups (data not shown), and
as such, on the basis of our data we have no reason to suspect that the subjects completing the study are “selected” as opposed to drop-out subjects.

In summary, our data indicate increased exercise-induced AMPK activation as well as increased effects on the downstream target ACC2 in LBW subjects, which might indicate a more sensitive AMPK system in this population. Even when performed at the same relative workload, acute exercise-induced AMPK activation and GS activation as well as downstream effects on ACC2 and the putative AMPK gene target HK2 are significantly reduced after 12 wk of endurance exercise training, which might be related to increased glycogen stores after training. These observations might suggest a reduced need for AMPK to control energy turnover during acute exercise. Therefore, the reduced γ3-associated AMPK activation by acute exercise observed after 12 wk of exercise training might be sufficient to maintain cellular energy balance.

ACKNOWLEDGMENTS

We thank laboratory technicians Lars Sander Koch, Marianne Modest, Karina Olsen, Betina Bolmgn, and Irene Bech Nielsen for dedicated assistance during the experiments. We also thank the subjects for participating in this study.

GRANTS

The study was funded by the 6th European Union Framework EXGENESIS Grant, The Danish Research Council of Health and Disease, The Danish Strategic Research Council, The European Foundation for the Study of Diabetes (EFSDF), The Novo Nordisk Foundation, and The Lundbeck Foundation. This project was also funded by The UNIK Project: Food, Fitness Pharma for Health and Disease, supported by the Danish Ministry of Science, Technology and Innovation. First author B. Mortensen was granted an Industrial PhD scholarship from The Danish Agency for Science Technology and Innovation, Copenhagen, Denmark.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


