Changes in circulating microRNAs levels with exercise modality

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During exercise, skeletal and cardiac muscles are able to send biological signals such as myokines or natriuretic peptides to other tissues. In contrast, in some conditions, molecules released by muscles are not signals but markers of tissue damage. Exercise-induced muscle damage is very frequent following unaccustomed eccentric exercise, which involves the active lengthening of muscle during tasks such as walking downhill, and results in perturbation of myofiber membrane integrity allowing cytosolic or contractile proteins to leak out in bloodstream.

Besides peptides and proteins, microRNAs (miRNA) are of increasing interest as signaling molecules or injury biomarkers but have currently been scarcely studied in exercise physiology. MicroRNAs are small noncoding RNA involved in the posttranscriptional regulation of gene expression and play a role in a wide range of biological and pathological processes.

Numerous miRNAs are highly tissue and cell specific (15, 18). For instance, muscle-specific miRNAs such as miR-1, 133a, 133b, 206, 208a, 208b, and 499 have been described in skeletal and/or cardiac muscles (10). They are involved in cardiac and skeletal muscle development, myoblast proliferation or differentiation and tissue remodeling. Their functions and roles have been recently reviewed (11, 19, 33). Changes in muscle miRNAs expression have been described in the presence of altered contractile activity. Hence, muscle unloading induced a significant decrease in miR-206, 208b, and 499 (1, 21) within muscle tissue. In a functional overload model, primary transcripts for miR-1, 206, and 133a were increased, with no changes or a decrease in the corresponding mature miRNAs (20). Finally, endurance exercise transiently up-regulated miR-1 expression in mice, while both miR-1 and 133a in human skeletal muscle increased immediately after exercise cessation (25, 30). In addition, other miRNAs, not restricted to muscle tissue, have been shown to play important roles in muscle cells; e.g., mir-181 is up-regulated in regenerating skeletal muscle (24) and is increased in mice quadriceps after a running bout (30). Mir-214 is involved in muscle cell differentiation (13) and in cardiomyocyte survival during cardiac injury (2). Thus current literature shows that miRNAs expression is altered within muscle in response to changes in contractile activity or muscle damage, suggesting they could be involved in muscle adaptation or repair.

Recently, noncellular miRNA have been found in various body fluids including plasma or serum. There is growing interest in using circulating miRNA as biomarkers of various pathologies, especially in cancer detection (22), but also as biomarkers of tissue injuries (17). In rats, systemic administration of muscle toxic drugs induces an increase in plasma miR-133 (17) and in rodents as well as in human, circulating cardiac miRNAs are specific and early biomarkers of cardiac infarction (35). Additionally, accumulating experimental arguments suggest that secreted miRNAs are mediators of intercellular communication [review in (8)]. In a pioneer study, Baggish et al. (3) investigated circulating levels of various miRNAs in healthy subjects and described a variety of expression profiles at the end of an acute exhaustive cycling exercise bout and/or after 90 days endurance training. This work demonstrated that exercise can affect circulating miRNA expression, and the authors proposed they could be exercise biomarkers or possible physiological mediators of exercise-induced adaptations. While this work focused on angiogenesis and inflammation-related miRNA, studies investigating circulating miRNAs acute changes in response to exercise are scarce.

In the present work, our goal was to investigate if circulating muscle-specific (hsa-mir-1, 133a, 133b, 208a, 208b, and 499) or muscle related (hsa-mir-181 and 214) miRNAs are affected by exercise in human, and whether exercise modality (i.e., damaging vs. nondamaging exercise modes) can differentially influence their expression. To achieve this goal, we alterna-
tively measured plasma levels of miRNAs in response to an uphill (mainly concentric exercise) or a downhill exercise (high eccentric component), together with classical markers of exercise-induced muscle damage (muscle soreness, plasma markers, muscle strength) and exercise intensity (heart rate and ratings of perceived exertion). We postulated that a higher miRNA expression in response to concentric exercise would suggest miRNAs are signaling molecules, whereas higher miRNA in response to the eccentric exercise would suggest muscle miRNA are released in response to muscle damage.

**METHODS**

**Ethical approval.** Experimental protocol was performed according to the Helsinki Declaration of 1975 as revised in 2008 on ethical principles for medical research involving human subjects. The project was approved by the scientific committee of Aspetar and by the ethics committee of Shaifallah Medical Genetics Center (Institutional Review Board - Project number 2011-003). Participants gave their informed, written consent prior to the commencement of the experiment, once the experimental procedures, associated risks, and potential benefits of participation had been explained.

**Participants.** Nine healthy male subjects (27 to 36 yr old) were included in the study. Mean body mass index was 24.59 ± 0.76 kg/m². They were recreationally active, involved in individual (running, swimming, cycling) or collective sports (football). Participants declared 1- to 4-h physical activity in the week, with no important variation in the 3 mo preceding the experiment. They had no significant occupational physical activity. All subjects were unacustomed to eccentric exercise and were not involved in any regular resistance training program for at least 3 mo before the experiment. Exclusion criteria were chronic or recent (>3 mo history of traumatic muscle injury, history of heat-related illness, 3 /H11349 criteria were chronic or recent (>3 mo history of traumatic muscle injury, history of heat-related illness, contact sports practice (>2 h/wk).

**Experimental design.** Participant performed two randomly assigned, separate experimental sessions (downhill exercise or uphill exercise) with at least a 6-wk washout period. During this period subjects were asked to maintain their normal lifestyle (they were not involved in resistance, aerobic, and/or flexibility training). They were asked not to perform physical activity 48 h before and during the experimental sessions. Blood samples, muscle strength, and delayed onset muscle soreness (DOMS) were assessed preexercise and immediately postexercise as well as 24, 48, and 72 h after the recovery period. Blood samples were additionally drawn 2 and 6 h after the end of exercise.

**Exercise protocol.** Downhill exercise consisted of one bout of 30 min of backward downhill walking exercise on a motorized treadmill (Cosmos, Nussdorf-Traunstein, Germany), at a constant velocity of 1 m/s, with a grade of 25%, with an additional load carriage of 12% of body wt (loaded backpack), as described by Racinais (27). This exercise protocol was previously found to induce significant damages of the plantar flexor muscles.

The uphill exercise was performed under the same conditions as the downhill session (i.e., 30 min, 1 m/s, grade of 25%; additional load carriage of 12% of body wt), but the rotational direction of the treadmill belt was inverted to make the participant walk uphill. This exercise was therefore matched to downhill exercise in terms of distance covered.

**Exercise bouts were performed in a controlled thermoneutral environment (20°C/30% relative humidity). To prevent any potential unknown daily variations in miRNAs that could interfere with our results, exercise trials were performed at the same time of day (between 8:30 and 10:00 am). Participants were allowed to drink ad libidum during exercise and recovery. Heart rate (HR) was recorded (5-s intervals) via a wireless Polar monitoring system (Polar Electro Oy, Kempele, Finland). Ratings of perceived exertion (RPE) were obtained every 5 min using the 6–20 Borg scale.

**Delayed onset muscle soreness assessment.** A subjective evaluation of the extent of DOMS in the plantar flexor muscles was performed prior to assess muscle strength by using a 10-cm visual scale ranging from 0 (“normal absence of soreness”) to 10 (“extremely painful”). They were instructed to indicate the general soreness of the entire muscle area when moving or using it.

**Strength assessment.** Isometric ankle plantar-flexion torque of the right foot was measured using a dynamometric pedal (Captels, St Mathieu de Treviers, France). The subject’s seating position was standardized with pelvis, knee, and ankle angulations of 90°, the foot securely strapped on the pedal by three straps. Subjects were instructed to perform three maximal isometric contractions for a 4- to 5-s duration with a rest interval of 30 s. They were vigorously encouraged to perform maximally during voluntary efforts, and the maximal voluntary contraction (MVC) torque was computed as mean value recorded over all trials for a given experimental session. Prior to strength assessment (except for postexercise session), subjects carried out a standardized warm-up consisting of 10 isometric plantar flexions (4-s duration interspaced with 10-s rest periods). Contraction intensities were self-adjusted by the subjects to be progressively increased up to MVC level during the last three trials.

**Blood sampling.** Blood was drawn from the antecubital vein with a new puncture at each time point. No catheter was used in order to avoid local inflammation and alteration of miRNA expression. Blood was collected into two separated tubes (Becton Dickinson, Franklin Lakes, USA), one EDTA tube for plasma sample (5 ml), and one serum separating tube for serum (SST) (7 ml). Plasma was separated immediately by centrifugation (2,000 g, 4°C, 10 min) and aliquots (250 μl) were frozen (~80°C). SST tubes were allowed to clot at ambient temperature, 15 min, and centrifuged. Serum was analyzed daily for creatine kinase (CK), aspartate aminotransferase (AST) activities, myoglobin, and cardiac troponin I concentrations.

**Serum markers measurement.** CK, myoglobin AST, and cardiac troponin I were measured in serum using Dimension RxL/XP clinical chemistry analyzer (Siemens Healthcare, Erlangen, Germany) with corresponding Flex reagent cartridges. Specific performance characteristics of the measures were as follow: coefficient of variation 0.5% to 1.9%, 1.9% to 2.3%, 0.02% to 0.08%, 0.8% to 2.7% (within run) and 2.7% to 2.9%, 3.2% to 3.6%, 0.05% to 0.35% and 2.1% to 5.2% (total) for CK, myoglobin, troponin, and AST respectively.

**miRNA extraction.** Plasma aliquots were thawed at 4°C and centrifuged (10,000 g, 4°C, 10 min), 200 μl were used for total RNA extraction. MirVana Paris isolation kit (Ambion, Austin, USA) was used according to manufacturer protocol for liquid samples with an additional precipitation step: column elution was performed with 180-μl sterile water, 18-μl sodium acetate 3M (Sigma), 400-μl ethanol (70%), and 1-μl linear acrylamide (Ambion, Austin, USA) were added. Tubes were vortexed and RNA were allowed to precipitate overnight at ~20°C. After centrifugation (12,000 g, 4°C, 10 min), supernatant was carefully removed and pellets were allowed to dry 30 min. RNA were finally suspended in 12-μl sterile water, incubated at 50°C, 10 min, and frozen at ~80°C.

**cDNA synthesis.** MicroRNA complementary DNA was synthesized in a 10-μl final volume with Universal cDNA Synthesis Kit (Exiqon, Vedbaek, Denmark). Reaction mix contained 2-μl 5X buffer, 2-μl nuclease free water and 1-μl Enzyme mix. For each sample, 5-μl reaction mix and 5-μl total RNA (diluted 1/6) were gently mixed and incubated at 42°C, 60 min. Reaction was stopped with a final step at 95°C, 5 min, and cDNA were frozen (80°C).

**qPCR.** PCR were performed on a 96 wells LightCycler 480 instrument (Roche Applied Science, Mannheim, Germany). Each sample was first controlled with an individual measurement of hsa- miR-103 as a quality control. Pick & Mix microRNA PCR panels (Exiqon, Vedbaek, Denmark) were designed with 22 miRNAs per sample. Reaction was performed in a 10-μl final volume per well, licensed under the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/)
with 5-μl SYBR Green master mix, Universal RT (Exiqon, Vedbaek, Denmark), and 5-μl cDNA (diluted 1/40). PCR protocol consisted in 1 activation cycle (95°C, 10 min), 45 amplification cycles (denaturation 95°C, 10 s, and annealing/amplification 60°C, 1 min), and a melting curve analysis step. Single measurements were performed except for hsa-mir-21 that was measured in duplicate on each plate to assess PCR repeatability: mean ΔCq was 0.13 (0.001 to 0.828) corresponding to a mean coefficient of variation of 0.4% (0.001% to 2.2%).

Normalization. Quantification cycles (Cq) were determined using the second derivative maximum method. An interplate calibration was performed according to manufacturer’s protocol. Quantification was performed according to the 2^(-ΔΔCq) method. Potent reference miRNAs were chosen based on literature. The number of reference miRNAs and their stability were examined using GeNorm software (34). Finally, five references (hsa-miR-20a, hsa-miR-103, hsa-miR-21, hsa-miR-192, and hsa-miR-185) were necessary to validate the normalization. Final quantification was the geometric mean of the quantifications performed with each reference.

Statistics. Data are presented as means ± SE. Normal distribution of the data was tested using the Kolmogorov-Smirnov test. Results were analyzed using a two-way ANOVA for paired values. We first assessed the effect of “exercise” (seven time points) to test the hypothesis that circulating miRNAs could be affected by one bout of exercise. We also tested the effect of “condition” (uphill or downhill trials) and any potential interaction between “exercise” and “condition,” to test the hypothesis that exercise modality could differentially affect circulating miRNAs. When appropriate, a Newman-Keuls post hoc test was applied to compare groups. Effect sizes (ES) have been calculated as Cohen’s d (with d ≤ 0.2 representing a trivial difference; 0.2–0.5, a small difference; 0.5–0.8, a moderate difference; and >0.8, a large difference). Analyses were performed with Statistica 7.0 software (Statssoft, Maisons-Alfort, France). Results were considered significant for P < 0.05.

RESULTS

Physiological responses. Heart rate and RPE increased in response to both forms of exercise and were significantly higher during the uphill compared with the downhill session (P < 0.001) (Fig. 1, A and B).

There was a significant effect of exercise (P < 0.001) and condition (P < 0.001) on DOMS, with a significant interaction between these factors (P < 0.001) (Fig. 1C). Uphill exercise induced a modest and nonsignificant (P = 0.07) increase in DOMS immediately postexercise, whereas downhill exercise induced an increase in DOMS with peak values observed 48 h postexercise. MVC torque was affected by exercise (P < 0.001) and condition (P < 0.01), with a significant interaction (P < 0.05) (Fig. 1D). There was a 7 ± 6% decrease in MVC torque at the end of the uphill exercise that was fully recovered with 24 h. Immediately after the downhill exercise, MVC torque dropped by 20 ± 9% and remained significantly lower than baseline until 72 h of recovery.

Plasma markers of muscle injuries. Plasma CK activity significantly increased in response to exercise (P < 0.001), peaking
after 6-h recovery (Fig. 2A). No significant difference between uphill and downhill sessions ($P = 0.12$) and no interaction between exercise and condition were found ($P = 0.22$). Plasma concentrations of myoglobin increased in response to exercise ($P < 0.001$), peaking after 2-h recovery (Fig. 2B), but no significant difference between conditions ($P = 0.15$) and no interaction were found ($P = 0.42$). Exercise induced an increase in plasma AST activity ($P < 0.001$) with peak values observed after 24 h of recovery (Fig. 2C). AST values were significantly higher in downhill condition ($P < 0.05$) without interaction ($P = 0.80$). Finally, cardiac troponin I remained undetectable throughout the experiment.

**Plasma miRNAs.** We first focused on muscle specific miRNAs. Exercise significantly affected plasma levels of hsa-mir-1 ($P < 0.001$), hsa-mir-133a ($P < 0.001$), and hsa-mir-133b ($P < 0.001$) with peak values observed 6 h after exercise (Fig. 3). Effect sizes comparing preexercise values to peak values were hsa-mir-1 (1.11; CI95% -0.21 to 2.43), 133a (1.23; CI95% -0.11 to 2.58), and 133b (1.31; CI95% 0.08 to 2.54). There was a significant effect of condition for both hsa-mir-1 ($P < 0.05$) and hsa-mir-133a ($P < 0.05$) with higher values observed in downhill condition, whereas statistical significance was not reached for hsa-mir-133b ($P = 0.053$). Finally, a significant interaction was found ($P < 0.05$ for hsa-mir-133a; $P < 0.01$ for both hsa-mir-1 and 133b). Post hoc analysis demonstrated significantly higher values in downhill condition 2 h and 6 h after exercise for hsa-mir-1 ($P < 0.05$ and $P < 0.001$) and 6 h after exercise for hsa-mir-133a and 133b ($P < 0.001$). Individual profiles for hsa-mir-1, 133a, and 133b are shown in Fig. 4. Plasma levels of hsa-mir-206 could not be consistently measured because of high Cq (>$40$).

Plasma levels of hsa-mir-499–5p displayed a high variability between subjects. They were significantly affected by exercise ($P < 0.01$) with a peak value 6 h after exercise, but no effect of condition nor any interaction were found (Fig. 5A). Hsa-mir-208b was significantly affected by exercise ($P < 0.001$), and the effect of condition failed to reach significance ($P = 0.068$) (Fig. 5B). However, a robust interaction was found ($P < 0.001$), and post hoc test revealed a significant difference 6 h after exercise ($P < 0.001$). Finally, the cardiac specific hsa-mir-208a remained undetectable throughout the experiment.

Plasma levels of hsa-mir-181b (Fig. 6A) and hsa-mir-214 (Fig. 6B) were not globally affected by exercise and condition, but significant interactions were found ($P < 0.05$). Post hoc analysis demonstrated a significantly increased value at the end of uphill exercise compared with baseline (effect size 1.12; after 6-h recovery (Fig. 2A). No significant difference between uphill and downhill sessions ($P = 0.12$) and no interaction between exercise and condition were found ($P = 0.22$). Plasma concentrations of myoglobin increased in response to exercise ($P < 0.001$), peaking after 2-h recovery (Fig. 2B), but no significant difference between conditions ($P = 0.15$) and no interaction were found ($P = 0.42$). Exercise induced an increase in plasma AST activity ($P < 0.001$) with peak values observed after 24 h of recovery (Fig. 2C). AST values were significantly higher in downhill condition ($P < 0.05$) without interaction ($P = 0.80$). Finally, cardiac troponin I remained undetectable throughout the experiment.

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CI95% 0.66 to 1.58) and other time points of the same condition and after downhill exercise for hsa-mir-181b (P < 0.05). Plasma hsa-mir-214 values were significantly higher at the end of uphill exercise compared with baseline (effect size 0.74; CI95% 0.13 to 1.60) and time points 2, 6, and 72 h after exercise (P < 0.05). Individual profiles are shown in Fig. 7. Finally, hsa-mir-181a plasma levels did not change throughout the protocol (Fig. 6C).

DISCUSSION

Our main findings are 1) muscle-specific miRNAs (hsa-mir-1, 133a, 133b, and 208b) are up-regulated in the early recovery period (i.e., 6 h) of the downhill exercise, and 2) muscle-related miRNAs (hsa-mir-181b and 214) are transiently up-regulated at the end of the uphill exercise. To our knowledge, we report for the first time differential regulations of circulating miRNAs in response to eccentric vs. concentric exercise in humans.

As expected, uphill condition resulted in higher exercise intensity than downhill exercise, which induced plantar flexors muscles damage as shown by persistent decreases in MVC torque. Serum CK and myoglobin increased following the same pattern in both conditions. Uphill exercise was mainly concentric, yet, because of the treadmill slope and load carrying, an eccentric component may have induced slight muscle damages. Alternatively, alterations in CK, due to transient alterations of myofibers membrane permeability, have been reported in response to nondamaging exercise and may explain the increases we found (5). Plasma CK is the most commonly used blood marker of exercise-induced muscle damage; however, many limitations have been reported such as individual, gender, or ethnicity-related variations (5, 16). Only serum AST displayed a significant effect of condition with higher values for eccentric exercise. Unfortunately, AST is not specific of muscle tissue. The present results further confirm that in some situations, classical markers can hardly discriminate between situations with different levels of muscle damage.

We found that plasma hsa-mir-1, -133a, and -133b were significantly increased 2 to 6 h after exercise, with a larger response following the downhill exercise. Another muscle-specific miRNA, hsa-mir-208b, had similar expression pattern, but because it was not detectable in all subjects (i.e., 6 out of 9), we did not find a significant difference between conditions, although a robust interaction was noted. Finally, hsa-mir-499–5p increased in both conditions, but displayed large interindividual differences that make any interpretation difficult. Since all these miRNAs have a tissue-restricted expression, muscle tissue is necessarily the main source for plasma variations. Nevertheless, the mechanism(s) by which muscle releases miRNAs in plasma has yet to be determined. An increase in mir-1 expression has been found in muscle of running
mice (30), and has-mir-1 and 133a (but not 133b nor 206) increased within muscle tissue at the end of 1h cycling in humans (25). However, because these miRNAs levels, displaying modest increases (20–35%), returned to control values within 3h of exercise, it is therefore unlikely that such variations in miRNAs may account for the alterations we report in plasma. Furthermore, a much larger muscle mass was probably recruited in both legs in our uphill vs. downhill condition, which would have resulted in higher miRNA levels. Otherwise, we propose that plasma levels of muscle-specific miRNAs rise in response to myofibers damage. Skeletal muscle toxic injuries induced significant rises in plasma mir-133a in rats (17). Recently, high plasma levels of mir-1, 206, and 133a have been shown in a mouse and a dog model of muscle dystrophy, as well as in Duchenne muscular dystrophy (DMD) patients. Rescuing dystrophin expression in mice substantially decreased plasma miRNA levels, demonstrating they are reliable biomarkers of muscle dystrophy (6, 23). Here we report an increase in muscle-specific miRNAs in response to the completion of a damaging walking exercise. Interestingly, the peak values were found 6 h after the end of exercise, a time course similar to what was observed for circulating mir-1, 133a, 499, and 208a in experimental myocardial infarction in mice, another form of acute muscle injury (9, 35). In our study, damages mainly occurred in a restricted muscle mass (plantar flexors muscles), and despite the fact that loading paradigm was high relative to everyday experience, exercise-induced muscle damages are expected to be very modest compared with muscle dystrophy. Consistently, we report lower fold increases in miRNA levels than in DMD patients (6); however, when comparing peak with control values, we found large effect sizes. Whether similar response would be found in trained athletes is unknown. Taken together, our data suggest that circulating muscle-spe-
cific miRNA deserve more attention as a potentially interesting and clinically relevant new class of plasma biomarkers to assess skeletal muscle injuries in neuromuscular disorders, as proposed by Cacchiarelli, but also in sports medicine and research. Nevertheless, further experiments are required to evaluate this alternative. Plasma hsa-mir-1, 133a, and 208b have been proposed to be biomarkers of myocardial infarction (32). Our results indicate that they should be carefully interpreted since concomitant skeletal muscle injuries, due to an exercise bout or a fall, could significantly interfere with the results. The cardiac specific hsa-mir-208a could help discriminating between muscle and cardiac injury.

miRNAs can leak out an injured cell but can also be actively secreted (8) thereby targeting neighboring cells to exert a paracrine function. In addition to muscle-restricted miRNAs, we selected miRNAs, which are reported to have an important role in muscle biology and/or be regulated by exercise within skeletal muscle. We measured both hsa-mir-181a and 181b, encoded by two separate genes. Whereas hsa-mir-181a was not affected by exercise, we found a transient increase in plasma hsa-mir-181b with a large effect size immediately at the end of the uphill exercise. A similar profile was found for plasma hsa-mir-214 with a medium effect size. Whether mir-214 is altered in response to muscle contraction is not known, but interestingly, both mir-181b and 214 are up-regulated in response to hypoxia (14, 31), a condition that may occur within myofibers during sustained exercise (4). It could be tempting to suggest that contracting muscle could account for variations observed in plasma. Nevertheless, it is currently not possible to know the source of mir-181b and 214, since they are present in many tissues (7, 12). Blood cells account for a substantial part of circulating miRNAs (26). Since mir-181b expression is up-regulated in neutrophils and peripheral blood mononuclear cells after one bout of cycling exercise (28, 29), the variations reported here may reflect changes either in blood cell count or miRNA expression. Whether circulating miRNAs could participate in the complex interactions reported between exercise and immune system is a new question raised here.

**Conclusions.** Here we found that circulating muscle-specific and muscle-related miRNAs are affected by exercise modality according to two different patterns. Muscle specific miRNAs respond to damaging exercise and increase during the recovery period, raising the possibility that they could be alternative biomarkers of muscle injury. Hsa-mir-181b and 214 respond acutely to high-intensity exercise. In line with previous data, our data show that acute exercise can alter circulating miRNAs profiles. Further experiments are necessary to determine the significance of these changes, unspecific alterations of plasma miRNA levels in response to various stimuli, or specific secretion/signaling intended to actively participate in exercise responses or training adaptations. Now, a full profiling of circulating miRNAs would provide an extensive overview of the variations observed in response to various exercise modalities, thereby providing new orientations for future research.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

N.K. interpreted results of experiments; S.B. prepared figures; S.B. drafted manuscript; S.B., O.G., C.D., H.C., and N.K. edited and revised manuscript; S.B., O.G., S.R., C.D., H.C., and N.K. approved final version of manuscript.

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