Pharmacological enhancement of leg and muscle microvascular blood flow does not augment anabolic responses in skeletal muscle of young men under fed conditions

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Skeletal muscles serve crucial locomotory and postprandial metabolic [e.g., amino acid (AA) and glucose disposal] functions (52). Given such vital functions, understanding the control of nutrient delivery in the context of muscle metabolism is important. The day-to-day stability of muscle mass in healthy, habitually active individuals (not engaged in formal exercise training) is the result of a dynamic equilibrium in muscle protein turnover, whereby rates of muscle protein synthesis (MPS) are balanced with those of breakdown (MPB) (4, 27). This equilibrium depends on the postprandial supply of essential amino acid (EAA) components of dietary protein, which provide the substrate and “signal” to recoup postabsorptive muscle protein losses. This role of EAAs has been delineated in both human (8, 32, 36) and animal work (1, 2, 15).

Before EAA can act either as substrates or signals for MPS, they must leave the bloodstream, transverse the interstitial fluid, and be transported into myocytes (12). Potentially, then, both delivery and transendothelial EAAs transport could be rate limiting for the uptake of circulating AA by muscle (13, 41), although the latter is unlikely since transsarcolemmal transport of AA is so rapid that it is unlikely to be rate limiting for MPS in healthy muscle (31). The ability of skeletal muscle to mount anabolic responses to AA may be based on not only AA availability per se but also the distribution of blood between “nutritive” and “nonnutritive” routes (12, 14, 18). Whereas the optimal nutritive network consists of tortuous capillaries contacting myocytes, the nonnutritive network supplies muscle connective tissue and adipocytes and has minimal myocyte contact (33, 45). Whereas feeding has been shown to stimulate both limb (i.e., femoral and brachial) (26, 28, 34) and muscle nutritive (30, 33) blood flow, relationships between muscle AA utilization and blood flow remain poorly defined. Nonetheless, evidence is mounting that recruitment of muscle microvasculature is important in maintaining protein (40) and carbohydrate (CHO) (42, 43) metabolic homeostasis.

A secondary effect of nutrient intake is the release of insulin. Sugars are well known for stimulating insulin secretion, and similarly, certain AAs (e.g., leucine, arginine) are also potent insulin secretagogues (21, 23), with EAA-mediated postprandial secretion of insulin amplified by sugars (19). Crucially, insulin itself modulates microvascular blood flow via nitric oxide (NO)-dependent vasodilation of precapillary arterioles (29, 43). As with EAAs, insulin has also been shown to possess anabolic effects on muscle, via actions to suppress MPB (49) or to stimulate MPS via increasing delivery of endogenous EAAs to muscle. For example, Timmerman et al. (40) reported that increases in MPS following femoral artery insulin titrations were related to EAA delivery via enhancement of microvascular flow in an insulin availability-dependent manner. Therefore, altering delivery of insulin and EAAs to muscle could have profound effects on postprandial muscle anabolism.
by means of a medical questionnaire, physical examination, and blood samples. Blood samples were taken every 20 min. A primed, the femoral artery of one leg for methacholine infusions and arterial sampling). A 20-gauge polyethylene catheter was also inserted into had polyethylene catheters inserted into the antecubital vein of both from exercise for 72 h prior to each study day and from alcohol and was approved by The University of Nottingham Ethics Committee participate after all procedures and risks were explained. This study of daily living and recreation but did not routinely participate in any

Metabolic study procedures. Subjects were instructed to refrain from exercise for 72 h prior to each study day and from alcohol and caffeine for 24 h. Subjects fasted from 2100 the night before (water ad libitum) and reported to the laboratory at 0900. On arrival, subjects had polyethylene catheters inserted into the antecubital vein of both arms (one for tracer infusion and the other for perfusate microbubble infusion) and the femoral veins of both legs (for venous blood sampling). A 20-gauge polyethylene catheter was also inserted into the femoral artery of one leg for methacholine infusions and arterial blood samples. Blood samples were taken every 20 min. A primed, continuous infusion of (0.7 mg/kg, 1 mg·kg⁻¹·h⁻¹) of [1,2-¹³C₂]leucine and (0.3 mg/kg, 0.6 mg·kg⁻¹·h⁻¹) ring-[¹³C₆]phenylalanine tracer (99 atom%; Cambridge Isotopes, Cambridge, MA) was started at 0 h and maintained for the duration of the study with an increase (leucine: 1.5 mg·kg⁻¹·h⁻¹, phenylalanine: 0.91 mg·kg⁻¹·h⁻¹) when iv “feeding” began to prevent dilution of tracer. At 130 min, a primed, continuous infusion of Glamin (Fresenius Kabi, Germany; for composition see Table 1) was started (102 mg·kg⁻¹·h⁻¹), and 20% dextrose was infused at a variable rate to maintain blood glucose between 7 and 7.5 mM. During this period, blood glucose concentrations were measured frequently (every 5 min), and dextrose infusion was adjusted accordingly to maintain a steady-state blood glucose concentration. At 160 min, intra-arterial methacholine (Methapharm, Switzerland) administration began with methacholine (1.200 μg/mL in 0.9% NaCl) given at a rate of 3–18 ml/min to achieve an LBF twice that of the contralateral leg. This unilateral infusion model depended on the short half-life of methacholine (6). Muscle biopsies of vastus lateralis muscle were taken under sterile conditions at 0, 120, and 250 min, using the conchotome biopsy technique (17), with 1% Lidocaine (B. Braun Melsungen, Germany) as local anaesthetic. At 0 and 120 min, the biopsies were obtained from the leg randomly designated as “basal”, with biopsies from each leg (basal and methacholine) at 250 min. Muscle was rapidly dissected free of fat and connective tissue, washed in ice-cold saline, and snap-frozen in liquid N₂ and stored at −80°C until further analysis (Fig. 1).

Procedure for measurement of LBF. After 50 min of patients lying supine, basal measurements of LBF (femoral artery) were made over a 40-min period. This was repeated at 180 min (50 min after the start of Glamin and dextrose infusions) with measurements in response to feeding alone in one leg and in response to feeding plus methacholine in the leg receiving the intra-arterial methacholine infusion. Three measurements on each leg were used to obtain the final value, with no significant variation observed between them. LBF was measured using Doppler ultrasound (U222, Philips Ultrasound, CA). All measurements were taken with the subject supine, with no visual or aural stimuli. A linear array 9 to 3 MHz frequency probe (Philips Ultrasound) was used to measure mean blood velocity and arterial lumen diameter in the common femoral arteries of both legs. Measurements were made 2–3 cm proximal to the bifurcation of the femoral artery to minimize the effect of turbulence; the insonation angle was <60°. Arterial lumen diameter (mm) was measured by video callipers for each measurement and defined as the maximum distance between the media-adventitia interface of the near wall and the lumen-intima interface of the far wall of the vessel. LBF (l/min) was calculated as mean blood velocity (cm/s) × π × [femoral artery radius (mm)²]/1,000 × 60. Using the Doppler ultrasound technique to measure basal LBF, we found a coefficient of variation of 9% for three independent measurements under each condition assessed, suggesting that we could reliably detect changes of ~18% of the basal value.

Procedure for measurement of MBV. At 110 and 240 min (110 min after start of Glamin and dextrose infusions) measurements of MBV in the vastus lateralis muscle were made by contrast-enhanced ultrasound (CEUS), as previously described (33). In contrast to LBF measurements, MBV was obtained from both legs at both time points, allowing comparisons of basal to fed and basal to fed-plus-methacholine conditions. In brief, a linear array transducer (L9-3; Philips Ultrasound) was attached to each thigh for the duration of the study to allow cross-sectional imaging of the vastus lateralis muscle. A 1.5-ml suspension of perfusate microbubbles (Definity, Lantheus Medical Imaging) was diluted to 20 ml and infused at a rate of 1.2 ml/min. Real-time imaging was performed at a low mechanical index [MI; (0.08)] for 9 min to allow attainment of a steady-state microbubble signal. After this period, a high MI (1.20) “flash” was used to destroy the microbubbles, allowing for recording of the replenishment of the microbubbles in the vasculature within the ultrasound beam during a 45-s replenishment period.

The acoustic index (AI) generated from the resonating microbubbles is proportional to the microbubble concentration in the region of interest (47); four destruction-replenishment cycles were recorded for each MBV measurement. Data from these recordings were exported to quantification software (Q-Lab; Philips) for analysis. Regions of interest (ROI) were drawn freehand and selected to be free of connective tissue and large vessels. The selected ROI was copied for each recording to ensure the ROI was identical for basal and fed or fed-plus-methacholine conditions. The AI obtained during the first 0.5

Table 1. Glinam composition per 1,000 ml

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Content, g</th>
<th>Constituent</th>
<th>Content, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>16.0</td>
<td>Leucine</td>
<td>7.90</td>
</tr>
<tr>
<td>Arginine</td>
<td>11.30</td>
<td>Lysine-acetate:</td>
<td>12.70</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>3.40</td>
<td>Corresp. to lysine</td>
<td>9.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>5.60</td>
<td>Methionine</td>
<td>5.60</td>
</tr>
<tr>
<td>Glycyl-glutamine:</td>
<td>30.27</td>
<td>Phenylalanine:</td>
<td>5.85</td>
</tr>
<tr>
<td>Glycyl-tyrosine:</td>
<td>10.27</td>
<td>Prolane</td>
<td>6.80</td>
</tr>
<tr>
<td>Corresp. to glycine</td>
<td>20.0</td>
<td>Serine</td>
<td>4.50</td>
</tr>
<tr>
<td>Glycyl-tyrosine:</td>
<td>3.45</td>
<td>Threonine</td>
<td>5.60</td>
</tr>
<tr>
<td>Corresp. to glycine</td>
<td>0.94</td>
<td>Tryptophan</td>
<td>1.90</td>
</tr>
<tr>
<td>Corresp. to tyrosine:</td>
<td>2.28</td>
<td>Valine</td>
<td>7.30</td>
</tr>
<tr>
<td>Histidine</td>
<td>6.80</td>
<td>Citric acid to pH 5.8</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.60</td>
<td>Water</td>
<td>1,000 ml</td>
</tr>
</tbody>
</table>
Fig. 1. Acute study protocol.
(Bio-Rad) at 100 V for 30 min and membranes blocked in 5% low-fat milk in TBS-T (Tris-buffered saline and 0.1% Tween 20; both Sigma-Aldrich, Poole, UK) for 1 h; membranes were rotated overnight with primary antibodies Ip-eNOSSer162 (Abcam, Cambridge, UK; p-AktSer473, p-S6K1Thr389 (p70), and p-eEF2Thr56; all Cell Signaling Technology, Boston, MA) at 1:2,000 dilution at 4°C. Membranes were washed (3 × 5 min) in TBS-T and incubated for 1 h at room temperature in HRP-conjugated secondary antibody (Cell Signaling Technology) before further washing (3 × 5 min) in TBS-T and incubation for 5 min with ECL reagents (enhanced chemiluminescence kit; Immunstar, Bio-Rad). Blots were imaged and quantified by peak density with bands within the linear range of detection using the Chemidoc XRS system (Bio-Rad). Coomassie staining was used to correct for loading (48). Plasma insulin was measured in undiluted samples on high-sensitivity insulin ELISAs (DRG Instruments, Marburg, Germany) using a microplate reader according to the manufacturer’s protocol.

Statistical analyses. Statistical analyses were performed using GraphPad Prism version 5.00 (La Jolla, CA). All data are reported as means ± SE, with significance set at \( P < 0.05 \). Two-way ANOVA with repeated measures and Bonferroni post hoc analysis were adopted to compare LBF, muscle protein synthesis, muscle protein breakdown, net muscle protein balance, arterial plasma phenylalanine concentrations, and protein phosphorylation between the conditions. Paired Student’s t-tests were used to compare muscle MBV between basal and fed or basal and fed-plus-methacholine conditions.

RESULTS

Plasma glucose, insulin, and arterial plasma phenylalanine concentrations. The dextrose requirement to obtain and maintain a blood glucose value of 7–7.5 mM was 69.4 ± 8.2 ml/min over the period of AA infusion, with a trend for a continuous increase in dextrose infusion during this period. Plasma insulin values were higher under fed (9.7 ± 1.4 vs. 31.3 ± 5.7 \( \mu \)U/ml, \( P < 0.001 \)) compared with fasting conditions and remained elevated throughout the AA infusion period (Fig. 2B). In terms of arterial plasma phenylalanine concentrations, there was an increase over basal in arterial plasma phenylalanine concentrations during the AA infusion (from 60.7 ± 2.8 to 121.4 ± 3.3 \( \mu \)M, \( P < 0.05 \)), which was identical to when methacholine was infused (119.2 ± 6.3 \( \mu \)M; Fig. 2C).

LBF. LBF was significantly greater in response to the AA and dextrose infusion (from 0.51 ± 0.02 to 0.57 ± 0.04 l/min, \( P < 0.05 \)), with a further significant increase after methacholine infusion (from 0.57 ± 0.04 to 1.15 ± 0.08 l/min, \( P < 0.001 \); Fig. 2A).

MBV. Infusion of 1.2 ml/min Definity microbubbles achieved a plateau in acoustic index signal at 5 min 40 s (range: 4 min 50 s to 6 min 20 s) after a lag phase of ~60 s (33). Based on this, an infusion time of 9 min was used before the measurements of MBV were obtained. The AA and dextrose infusion resulted in an increased MBV of 25% (\( P < 0.05 \)), with the addition of methacholine resulting in an increase of 79% over basal (\( P < 0.01 \)). There was no increase in flow rate constant (B-value) in response to feeding (0.15 ± 0.01 vs. 0.19 ± 0.03) or feeding-plus-methacholine (0.15 ± 0.03 vs. 0.22 ± 0.04; Fig. 3).

FSR. In contrast to the results of LBF and MBV, the addition of methacholine did not further enhance the FSR responses over AA and dextrose infusion. FSR was increased in response to the AA and dextrose infusion (0.04 ± 0.004 vs. 0.08 ± 0.01%/h, \( P < 0.05 \)) but was not further enhanced by the addition of methacholine (0.07 ± 0.01%/h; Fig. 4A). Increases in FSR did not correlate with increases in MBV in either condition (fed, \( P = 0.44 \); fed-plus-methacholine, \( P = 0.78 \); Fig. 5).

\( \text{R}_{\text{a}}, \text{R}_{\text{b}}, \text{and net protein balance}. \) MPB (\( \text{R}_{\text{a}} \)) was not altered in response to either experimental manoeuver (Fig. 4C). Net protein balance (NPB) was increased by AA infusion (−4.4 ± 2.4 vs. 16.4 ± 5.7 nmol Phe·100 ml leg\(^{-1}\)·min\(^{-1}\), \( P < 0.05 \)), but this increase was not further augmented by methacholine (24.0 ± 7.7 nmol Phe·100 ml leg\(^{-1}\)·min\(^{-1}\), \( P < 0.01 \) vs. basal; Fig. 4D). \( \text{R}_{\text{a}} \) was significantly increased only in the feeding-
higher than in the feeding alone leg (78.4 ± 4.6 vs. 78.4 ± 9.5 nmol Phe·100 ml⁻¹·min⁻¹, P < 0.05) and was also significantly higher than in the feeding alone leg (78.4 ± 9.5 vs. 55.0 ± 7.8 nmol Phe·100 ml⁻¹·min⁻¹, P < 0.05).

**Immunoblotting.** Phosphorylation of Akt was increased by infusion of AA (1.33 ± 0.08 vs. 1.95 ± 0.21, P < 0.01) but was not further augmented by methacholine (1.89 ± 0.20, P < 0.05). The same was true for p70 phosphorylation (1.17 ± 0.08 vs. 1.72 ± 0.22, P < 0.05, and 1.55 ± 0.10, P < 0.05). Phosphorylations of eEF2 and eNOS were unchanged in either condition (Fig. 6).

**DISCUSSION**

We studied the effects of increasing plasma availability of AA, glucose, and insulin on LBF, MBV, MPS, MPB, and NPB in the absence or presence of a pharmacological vasodilator. We found that pharmacological vasodilation, despite dramatically enhancing LBF and MBV, elicited no additional muscle anabolic effect above that seen in response to our feeding regimen. These data reveal that surpassing the subtle vasoactive effects of nutrition via pharmacological enhancement of muscle MBV does not further increase fed-state muscle anabolism in skeletal muscle of younger men. This is perhaps reflective of the weak quantitative relationship we found between MBV and MPS in response to feeding.

In this study, we provided an AA infusion to double plasma concentrations, using plasma phenylalanine concentration as a proxy. Under these conditions, and in agreement with the work of Vincent et al., who provided mixed macronutrition (44), we were able to detect increases in both muscle MBV and LBF (26), which, although these increases did not correlate, suggests that expansion of the endothelial surface is an important physiological response to feeding. Of the signals regulating this response, both increased availability of insulin and L-arginine (the precursor for NO synthesis) have been shown to regulate muscle perfusion via NO-dependent vasodilation of precapillary arterioles (42). However, as 10 g of arginine did not enhance blood flow responses to EAA in young men (38), a combined effect with insulin is likely to be driving the increases in MBV that we observed in the fed state. As expected, methacholine enhanced fed-state LBF through its potent cholinomimetic effects; this also translated into increased MBV such that we were able to test our hypotheses.

Previous authors have attempted to define mechanistic/correlative roles of MBV in the regulation of muscle protein anabolism. For example, Timmerman et al. reported that the muscle protein anabolic effects of local insulin infusions were inhibited by the nitric oxide synthase inhibitor N<sup>3</sup>-monomethyl-L-arginine (L-NMMA) in young men (40), and enhanced by the NO donor sodium nitroprusside (SNP) (41) in older men. These data appear to point to an intimate relationship between muscle nutritive blood flow and protein anabolism. However, our finding of a lack of superposition of the effect of increased MBV on MPS (in response to both feeding and feeding plus methacholine) does not support the concept of tight blood flow-anabolic coupling. Moreover, complex relationships appear between blood flow and muscle protein turnover when using two-pool a-v balance models [as in studies (40, 41) cited above], suggesting imperfect modeling by this approach. For instance, during infusion of SNP and insulin, which doubles flow, proteolysis is increased, as is synthesis approximately threefold, when FSR increased by only 10% (41). In addition, Timmerman et al. reported that L-NMMA infusions during local insulinemia altered the route of protein accretion from stimulation of MPS to attenuation of MPB despite resultant improvements in NPB being identical (40). Since in the present study we did not measure the expected depression in MPB in response to either feeding alone or feeding plus methacholine, we contend that artifacts may exist in the a-v balance two-pool model (NB: limb blood flow is used to calculate MPB), which can skew interpretation of changes in MPB under conditions of radically altered blood flow; i.e., small shifts in tracer concen-
trations are amplified by extremes of blood flow (40, 41). We also saw no significant increase in $R_A$ in the fed-only leg, despite MPS being robustly stimulated when measured using leucine tracer incorporation. This discrepancy probably reflects both the inherent variability in the a-v balance approach and that the study is slightly underpowered when using this technique. Indeed, power analysis suggests that using the a-v balance approach to measure $R_A$, we would have needed three more subjects to obtain 95% power, thereby increasing confidence in the data and removing the possibility of Type 1 or 2 errors.

Rather than determining the effects of “local” insulinemia, we studied the effects of exogenous AA and dextrose, which then stimulated physiological systemic insulin responses. Our principal question was whether enhancing muscle MBV could influence muscle utilization of AA for MPS. To probe this, we provided intra-arterial infusions of methacholine, a cholinomimetic and activator of NOS, to robustly increase muscle MBV beyond that of the effects of feeding alone, specifically in the limb into which it was infused. Yet, despite LBF and MBV dramatically exceeding the values in the contralateral limb (showing the efficacy of our study design), increases in MPS (and improvements in NPB) were identical between the limbs. These data reveal that increasing MBV over and above the changes typically occurring as a result of feeding does not further enhance postprandial protein accretion; it must be acknowledged, however, that relationships between blood flow and anabolism are complex and will be governed not only by the specific feeding regimen but also by other physiological factors such as prior physical activity and feeding patterns. In addition, the methacholine-induced MBV response, although unphysiological compared with those observed in response to feeding, are within the range of changes seen in response to an acute exercise bout (33), suggesting that the marked anabolic effects postexercise cannot be due solely to elevated postexercise MBV. Nonetheless, it is recognized that the muscle metabolic background under exercising conditions is distinct from that under postprandial conditions. Finally, recent work has demonstrated that there exist maximum amounts of substrate [$\sim 20$ g protein (24), $\sim 10$ g EAA (11, 16), doses $\sim 3$ g of leucine (37, 50)] and limited durations of their exposure to muscle [maximum $\sim 2$ h (3, 9, 25)], which will stimulate muscle’s use of EAA for MPS. However, we conclude that these limits of AA utilization for MPS are unlikely to be due to declining MBV, especially as MBV still remained elevated at the end of the “active” anabolic period.

Although in the present study we found no effect of further increasing blood flow at rest, above that seen in response to feeding, on muscle anabolism, enhancement of nutritive flow by prior exercise has been reported to enhance blood flow and, consequently, MPS responses to nutrition [in older individuals (39)]. On this basis, it would seem unlikely that increasing blood flow per se is responsible for this effect (20); rather it is likely to be a feature of sustained (10) increases in muscle
protein turnover (for remodeling/adaptive purposes) and muscle metabolic demand. These data underscore the concept that exercise is required to enhance anabolic responses to nutrition and that this cannot be reproduced by artificially increasing muscle MBV or AA availability, probably due to the “muscle-full” effect of AA utilization by muscle for anabolic purposes, at rest (3, 9, 25), whereby MPS becomes refractory to the stimulatory anabolic effects of nutrition despite continued availability.

The last of our objectives was to investigate links between muscle MBV and anabolic signaling associated with stimulation of MPS in response to nutrition. To achieve this, we measured Akt as an index of proximal insulin signaling (49) and both p70 and eEF2 as indexes of mTORC1 signaling, probably reflective of EAA signaling (50). However, despite dramatic differences in muscle MBV responses to feeding and feeding plus methacholine, increases in Akt and mTORC1 substrate phosphorylation were identical and occurred in concert with those of the MPS and NPB responses. In addition, the phosphorylation of eNOS (53) in muscle appeared to represent a poor proxy for MBV, since, whereas we detected robust increases in MBV, we found no measurable change in eNOS phosphorylation.

Finally, we acknowledge a number of potential study limitations. The “importance” of blood flow in modulating muscle protein turnover may depend on the composition or volume of the feed; i.e., the results of this study may not be representative of all feeding strategies, and these results can draw conclusions only about young men, as neither women nor older individuals were studied. We also acknowledge the possibility of additional “off-target” effects of methacholine, which although not currently recognized, may have affected the product-precursor relationship in muscle. However, since both the KIC enrichment in the femoral venous plasma (fed, 5.52 ± 0.19 vs. fed-plus-methacholine, 5.52 ± 0.22 APE) and the intracellular phenylalanine (fed, 2.98 ± 0.17 vs. fed-plus-methacholine, 2.93 ± 0.23 APE) and leucine (fed, 2.90 ± 0.21 vs. fed-plus-methacholine, 2.88 ± 0.26 APE) enrichments from both legs were identical throughout the feeding period, we contend this was not the case. It is true that the homogenate composition from our muscle biopsy samples will contain many cell types and this may have limited our ability to detect changes in phosphorylation of eNOS in endothelial cells. Possibly, studies of the relationship between blood flow and muscle protein turnover in a condition the reverse of that explored in this study, i.e., blood flow restriction, would yield important new information about any functional links between eNOS modification and microcirculatory regulation.

To summarize, we have produced new information on relationships between whole LBF, muscle MBV, MPS, and anabolic signaling. We conclude that supply of AAs and dextrose to young men leads to raised plasma insulin availability, increased LBF and muscle MBV, and increases in MPS, NPB, and anabolic signaling. However, MBV was not closely coupled to MPS (although there may have been an early or later vascular responses that were missed), and pharmacological enhancement of LBF and MBV did not enhance muscle anabolic responses. These data further highlight the concept that muscle exerts a high degree of intrinsic control over protein metabolism (3, 9, 25). Nonetheless, whether enhancing postprandial muscle MBV is beneficial for muscle protein anabolism in the context of microvascular impairment (e.g., aging, type 2 diabetes) warrants further investigation.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


