

Plasma Endogenous Endotoxin Core Antibody Response to Exercise in Endurance Athletes



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ABSTRACT

The study aimed to investigate the impact of laboratory-controlled exertional and exertional-heat stress on concentrations of plasma endogenous endotoxin core antibody (EndoCAB). Forty-four (males $n = 26$ and females $n = 18$) endurance trained ($\dot{V}O_{2max}$ 56.8min/kg/min) participants completed either: P1–2h high intensity interval running in 23 °C ambient temperature (T_{amb}), P2–2h running at 60% $\dot{V}O_{2max}$ in 35 °C T_{amb} , or P3–3h running at 60% $\dot{V}O_{2max}$ in 23 °C T_{amb} . Blood samples were collected pre- and post-exercise to determine plasma IgM, IgA, and IgG concentrations. Overall resting pre-exercise levels for plasma Ig were 173MMU/ml, 37AMU/ml, and 79GMU/ml, respectively. Plasma IgM concentration did not substantially change pre- to post-exercise in all protocols, and the magnitude of pre- to post-exercise change for IgM was not different between protocols ($p = 0.135$). Plasma IgA and IgG increased pre- to post-exercise in P2 only ($p = 0.017$ and $p = 0.016$, respectively), but remained within normative range (35–250MU/ml). P2 resulted in greater disturbances to plasma IgA ($p = 0.058$) and IgG ($p = 0.037$), compared with P1 and P3. No substantial differences in pre-exercise and exercise-associated change was observed for EndoCAB between biological sexes. Exertional and exertional-heat stress resulted in modest disturbances to systemic EndoCAB responses, suggesting EndoCAB biomarkers presents a low sensitivity response to controlled-laboratory experimental designs within exercise gastroenterology.

Introduction

It is now well established that endurance exercise promotes integrity disturbances to the gastrointestinal epithelium due to primary pathways (i. e., circulatory- and neuroendocrine-gastrointestinal) associated with exercise-induced gastrointestinal syndrome (EIGS) [1–4]. The secondary outcomes of these disturbances may include intestinal epithelium cell injury, with or without epithelial rupture, and hyperpermeability. Such perturbations to intestinal epithelial integrity may result in the translocation of pathogenic content from the intestinal lumen (i. e., bacterial endotoxins, such as lipopoly-

saccharide (LPS)) into circulation, heightening the systemic inflammatory response that is commonly observed with prolonged and strenuous exercise stress [5, 6]. These EIGS biomarkers appear to be exacerbated when exercise is performed in the heat (i. e., ≥ 35 °C ambient temperature (T_{amb})) resulting in ≥ 39.0 °C core body temperature [7–10], and of longer exercise duration (i. e., ≥ 3 h) [11]. Exacerbation of these biomarkers suggests that exertional-heat stress and exertional stress duration may play a key role in the magnitude of exercise-associated disturbance to gastrointestinal integrity, and subsequently the magnitude of translocation of intestinal

lumen originating bacterial pathogenic agents. Such translocation, in response to compromised epithelial integrity, may have clinical (e. g., sepsis) or sub-clinical (e. g., gastrointestinal symptoms (GIS)) implications [4, 12–16], which may be associated with impairment to exercise [17–19].

Systemic endotoxemia has previously been classified as a pre- to post-exercise change of ≥ 5 pg/ml in bacterial endotoxins, with a concomitant reduction in anti-endotoxin antibody immunoglobulins (Ig), namely IgM and IgG, as defined by earlier field studies; however, the clinical significance of these exercise-induced changes is yet to be clarified due to potential exposure and immune tolerance [12, 13, 20–24]. Moreover, it is common for both field and laboratory-controlled studies to measure plasma endotoxin concentration without the supportive analysis of anti-endotoxin antibodies. Nevertheless, recent laboratory-controlled studies have incorporated endogenous endotoxin core antibodies (EndoCAB) within the suite of EIGS biomarkers and suggest that measuring anti-endotoxin antibodies may provide a more comprehensive and reliable interpretation of endotoxin translocating activities in response to endurance exercise [9, 10, 25], compared with endotoxin assessment (e. g., LAL chromogenic endpoint assay) alone. This proposal is due to: 1) sample collection, processing, and analysis procedural issues [26–28]; 2) liability of circulating bacterial endotoxins to consistent immune and hepatic clearance [29–31]; and 3) lack of assessment procedures to globally detect a vast array of bacterial endotoxins with pathogenic properties [32–35]. These potential limitations may mask the full magnitude of exercise-associated endotoxemia and its contribution as a confounding factor to accurately determine exercise-associated endotoxemia.

Produced primarily by lymphocyte B cells and/or plasma cells, EndoCAB react to a number of gram-negative bacterial species antigens [22]. As such, plasma EndoCAB concentration is suggested to be a marker for systemic endotoxin exposure [36]. Within a healthy human population, resting plasma concentrations range between 35–250 MU/ml. These values are in accordance with median rangers observed in healthy blood donors with high antibody titre [37, 38]. In response to a modest acute transient endotoxin exposure (e. g., ≤ 0.3 EU/ml), there is a transient increase in circulating EndoCAB (e. g., ~ 100 –250 MU/ml), associated with immune activation of lymphocyte-B and/or plasma cells [36, 39, 40]. With a substantial systemic endotoxin load (e. g., 0.5–1.0 EU/L), as per the case of sepsis, a depression in EndoCAB is observed (e. g., ≤ 35 MU/ml), likely attributed to an increase in antibody utilization that overrides baseline and *in-situ* production levels. This functional aspect suggests EndoCAB may play an important part in the overall and correct interpretation of exercise-associated systemic endotoxin, and subsequent systemic inflammatory response profile, as part of EIGS assessment [9, 10, 25, 41].

With this in mind, the current study therefore aimed to investigate the impact of exertional and exertional-heat stress on systemic EndoCAB (i. e., IgG, IgM, and IgA, collectively) concentration. Based on previous exercise gastroenterology research outcomes and clinical manifestation of changes to EndoCAB in response to systemic endotoxin exposure, it was hypothesised that the proposed exertional stress would result in increased concentrations of EndoCAB, but exertional-heat stress would result in a depressed response.

Materials and Methods

Participants

Forty-four ($n = 26$ males and $n = 18$ females) individuals trained in endurance running volunteered to participate in the study and experimental procedures (► **Table 1**). All participants provided written informed consent, which received approval from the local ethics committee, and conformed to the 2008 Helsinki Declaration for Human Research Ethics and meet the ethical standards of the International Journal of Sports Medicine [42]. The laboratory's standard exclusion criteria has been previously reported [17]. For female participants, the experimental trial was scheduled during the early-mid follicular phase of their menstrual cycle ($n = 14$), when taking oral contraceptive pill ($n = 1$), or postmenopause ($n = 3$). Resting estrogen levels (DKO003/RUO; DiaMetra, Italy) were measured for verification (< 50 pg/ml) and did not differ between trials [43].

Preliminary measures

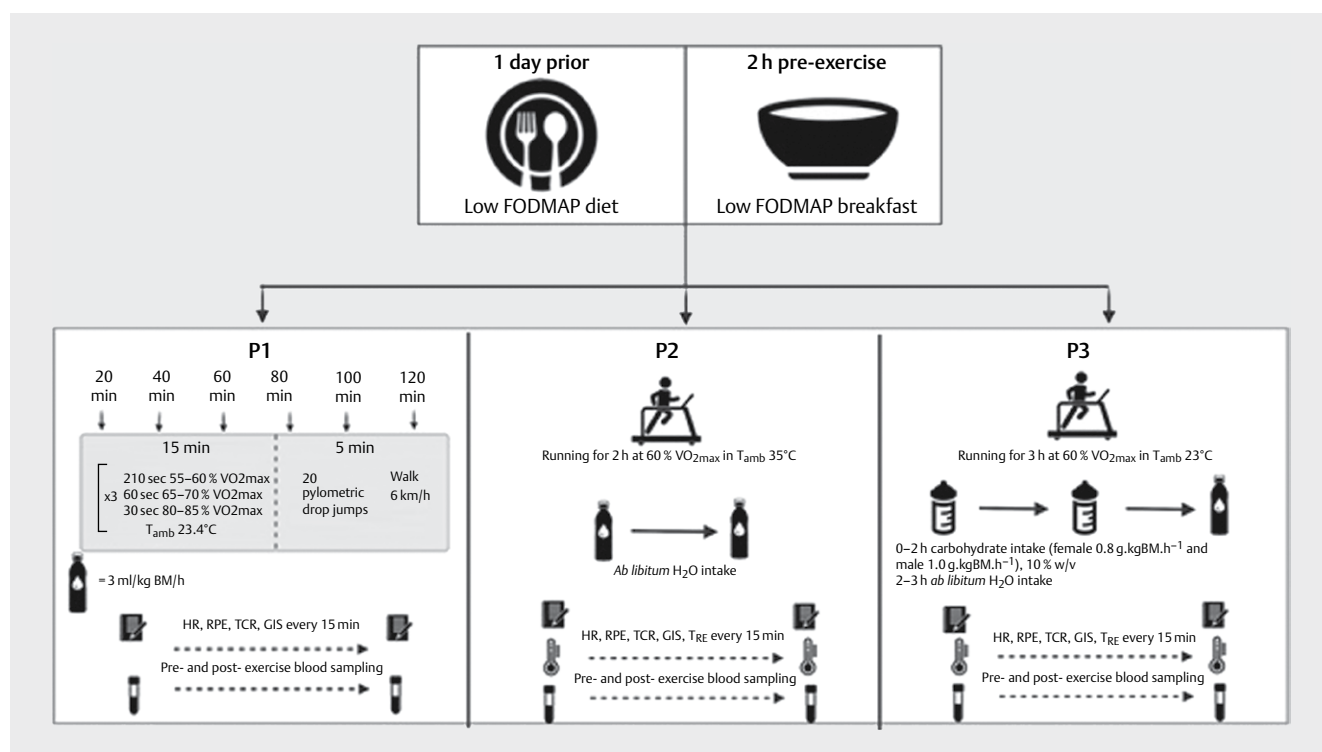
Prior to the first experimental trial, baseline measurements for height (Stadiometer, Holtain Limited, Crosswell, Crymch, United Kingdom), body mass and body composition by multi-frequency bioelectrical impedance analysis (MBIA, Seca 515 MBICA, Seca Group, Hamburg, Germany), and $\dot{V}O_{2\max}$ (Vmax Encore Metabolic Cart, Carefusion, San Diego, CA, United States) were recorded; and familiarisation of the running exercise procedures was undertaken. $\dot{V}O_{2\max}$ was estimated by means of a continuous incremental exercise test to volitional exhaustion on a motorized treadmill (Technogym, Cesena, Italy), as previously reported [44]. To determine running speeds for the respective exercise trials, the speed at approximately 60% of $\dot{V}O_{2\max}$ at 1% gradient (10.1 ± 1.6 km/h) was determined and verified from the $\dot{V}O_2$ -work rate relationship. In addition, for the high intensity interval training simulation (HIIT), the speed at approximately 50 (7.3 ± 1.0 km/h), 55–60 (8.6 ± 1.4 km/h), 70–75 (10.5 ± 1.6 km/h), and 80–85 (12.3 ± 2.0 km/h) % $\dot{V}O_{2\max}$ and 1% gradient was extrapolated and verified.

Experimental procedure

On a separate occasion, ≥ 1 week after the incremental exercise test, participants were provided with a low FODMAP diet (9.8 ± 2.1 MJ/day (143 ± 31 kJ/kg/day) energy, 351 ± 64 g/day (5.1 ± 0.9 g/kg/day; 62% of total energy intake contribution) carbohydrate, 92 ± 33 g/day protein (1.3 ± 0.5 g/kg/day; 16% of total energy intake contribution), 56 ± 21 g/day fat (0.8 ± 0.3 g/kg/day; 22% of total energy intake contribution), 44 ± 8 g/day fibre, and 2 ± 0 g/day total FODMAP) the day before the experimental trial to reduce GIS confounded from the lead-in diet [7, 45]. Food and fluid provisions were designed in accordance with current nutrition guidelines for endurance athletes, with estimated total daily energy expenditure determined via the Cunningham equation, and adjusted for a resting non-training day [46]. In addition, dietary provisions were aimed to provide < 2 g FODMAP per meal using a FODMAP specific database (Monash University, FoodWorks Professional 7, Xyris, Brisbane, Australia) [7, 45]. Participants reported to the laboratory at 0800h after consuming the standardised pre-trial low FODMAP meal (2.9 ± 0.7 MJ), 99 ± 28 g carbohydrate, 25 ± 6 g protein, 20 ± 5 g fat, 11 ± 4 g fibre, 1 ± 1 g total FODMAP), consumed at 0700h. The

► **Table 1** Descriptive participant characteristics of volunteers undertaking one of three different experimental running exercise protocols (P1-HIIT: 2 h high intensity interval training running with plyometric drop jumps in temperate ambient conditions, P2-EHS: 2 h steady state running in hot ambient conditions, and P3-SS: 3 h steady state running in temperate ambient conditions).

	All n=44	P1-HIIT n=17	P2-EHS n=14	P3-SS n=13
	Male = 26 Female = 18	Male = 10 Female = 7	Male = 9 Female = 5	Male = 7 Female = 6
Age (yr)	36 (34 to 39)	32 (28 to 36)	35 (31 to 39)	45 (29 to 50)
Height (m)	1.73 (1.71 to 1.76)	1.74 (1.70 to 1.78)	1.75 (1.70 to 1.79)	1.72 (1.67 to 1.77)
Body mass (kg)	68.5 (65.6 to 71.5)	68.7 (63.4 to 73.9)	67.3 (61.4 to 73.1)	69.7 (64.2 to 75.2)
Body fat mass (%)	18.8 (17.1 to 20.6)	18.1 (14.9 to 21.3)	18.0 (14.8 to 21.2)	20.7 (17.3 to 24.1)
$\dot{V}O_{2max}$ (min/kg/min)	56.8 (54.1 to 59.4)	54.5 (51.5 to 57.5)	63.2 (57.6 to 68.8)	52.8 (48.7 to 56.9)
Training load (min/week)	504 (440 to 568)	410 (310 to 509)	623 (483 to 762)	499 (421 to 577)
Modality		Endurance running n=5 Ultra-endurance running n=7 Triathlon n=5	Endurance running n=8 Ultra-endurance running n=6	Endurance running n=13
Mean (95% CI)				



► **Fig 1** Schematic illustration of the three experimental exercise protocols. P1-HIIT: 2 h high intensity interval training running with plyometric drop jumps in temperate ambient conditions, P2-EHS: 2 h steady state running in hot ambient conditions, and P3-SS: 3 h steady state running in temperate ambient conditions. Abbreviations: FODMAP, fermentable oligo-, di-, monosaccharides and polyols; HR, heart rate; RPE, rating of perceived exertion; TCR, thermal comfort rating; GIS, gastrointestinal symptoms; T_{amb}, ambient temperature; $\dot{V}O_{2max}$, maximal oxygen uptake; BM, body mass; w/v, water volume equivalent.

meal was consumed 2 h prior to the start of exercise, simulating real-life translational practice in the target population [47, 48]. A dietary log containing the prescriptive diet determined ingestion compliance and food waste. Participants refrained from strenuous exercise 48 h before the respective trial.

Prior to the commencement of exercise at ~0900h, participants were asked to void before nude body mass measurement, and MBIA to determine total body water (TBW). Blood samples were then collected by venepuncture from an antecubital vein into lithium heparin (6 ml, 1.5 IU/ml heparin) and K₃EDTA (4 ml, 1.6 mg/ml EDTA)

vacutainers. Pre-exercise resting rectal temperature (T_{re}) was then recorded, with participants inserting a thermocouple 12 cm beyond the external anal sphincter (Alpha Technics Precision Temperature 4600 Thermometer, Oceanside, CA). As part of exercise gastroenterology intervention studies reported elsewhere [7, 11, 49–51], participants undertook one of three endurance exercise protocols (► **Fig 1**).

Protocol 1 (P1-HIIT): Starting in a euhydrated state (plasma osmolality: 291 ± 8 mOsmol/kg and TBW: 60.7 ± 3.4%) participants (n = 17) undertook 2 h high intensity interval running exercise (HIIT)

session in temperate ambient conditions (ambient temperature (T_{amb}) $23.4 \pm 0.9^\circ\text{C}$ and $43 \pm 7\%$ relative humidity (RH)), with dual fan wind speed set at 10.6 km/h. The protocol involved 3 rounds of running for 3.5 min at $55\text{--}60\% \dot{V}O_{2max}$, 1 min of running at $65\text{--}70\% \dot{V}O_{2max}$, and 30-s running at $75\text{--}80\% \dot{V}O_{2max}$, followed by 20 plyometric drop (50 cm) jumps of alternating legs. Participants then returned to the treadmill to walk until the 20-min cycle was completed. This was repeated 6 times. During exercise, participants were provided with room temperature water equivalent to 3 ml/kgBM/h (208 ± 32 ml/h). Total distance over the 2-h protocol was 16.2 ± 2.5 km with 120 plyometric drop jumps. Exercise-associated body mass loss and post-exercise plasma osmolality were $1.8 \pm 1.0\%$ and 293 ± 10 mOsmol/kg, respectively.

Protocol 2 (P2-EHS): Starting in a euhydrated state (plasma osmolality: 296 ± 4 mOsmol/kg and TBW: $59.4 \pm 3.5\%$) participants ($n = 14$) undertook 2 h of exertional-heat stress (EHS), comprising running exercise on a motorised treadmill at the previously determined speed representing $60\% \dot{V}O_{2max}$ in hot ambient conditions (T_{amb} $35.7 \pm 0.9^\circ\text{C}$ and $23.3\% \pm 3.2\%$ RH), with dual fan wind speed 10.6 km/h. Room temperature water was provided ad libitum (662 ± 279 ml) for autonomy over drinking patterns to minimise programmed drinking induced occurrence of GIS. Total distance over the 2-h protocol was 21.5 ± 3.3 km. Exercise-associated body mass loss and post-exercise plasma osmolality were $2.1 \pm 0.9\%$ and 297 ± 6 mOsmol/kg, respectively.

Protocol 3 (P3-SS): Starting in a euhydrated state (plasma osmolality: 293 ± 9 mOsmol/kg and TBW: $57.5 \pm 3.5\%$) participants ($n = 13$) undertook 3-h steady state (SS) running exercise on a motorised treadmill at the previously determined speed representing $60\% \dot{V}O_{2max}$ in temperate ambient conditions (T_{amb} $23.1 \pm 1.2^\circ\text{C}$ and $43.6 \pm 5.5\%$ RH), with dual fan wind speed set at 10.6 km/h. During exercise, participants were provided with a room temperature carbohydrate (dextrose-fructose solution) beverage containing 64 ± 15 g/h, 10% w/v, 509 mOsmol/kg) at 0 min and every 20 min thereafter for the first 2 h, and allowed to consume water ad libitum. Total water intake during the first 2h equated to 650 ± 147 ml/h. In the 3rd hour, room temperature water was provided ad libitum, equating to 276 ± 15 ml. Total distance over the 3 h protocol was 27.3 ± 2.1 km. Exercise-associated BM loss and post-exercise plasma osmolality were $1.1 \pm 0.4\%$ and 292 ± 9 mOsmol/kg, respectively.

The exercise protocols were employed in accordance with previous exercise experimental models known to disturb gastrointestinal epithelial integrity to levels of clinical and performance significance. Standard physiological strain variables (i. e., T_{re} , heart rate (HR), rating of perceived exertion (RPE), McGinnis 13-point thermal comfort rating (TCR), and body mass) were measured at regular intervals during running, as previously reported [7, 11, 49–51]. Immediately after exercise, a blood sample was collected and nude body mass measured, as previously described.

Sample analysis

Whole blood hemoglobin was determined by a HemoCue system (Hb201; HemoCue, Ängelholm, Sweden), and hematocrit was determined by the capillary method with a microhematocrit reader (ThermoFisher Scientific), both from heparin whole blood samples. Haemoglobin and haematocrit values were used to estimate changes

in plasma volume (P_V) relative to baseline and used to correct plasma variables. The remaining heparin and K_3 EDTA whole blood samples were centrifuged at 4000 rpm (1500 g) for 10 min within 15 min of sample collection. Plasma was aliquoted into 1.5 ml micro-storage tubes and frozen at -80°C until analysis, except for 2 x 50 μ l heparin plasma that was used to determine plasma osmolality (P_{Osmol}) in duplicate (CV: 1.7%) by freeze-point osmometry (Osmomat 030, Gonotec, Berlin, Germany). Plasma concentration of endogenous endotoxin core antibodies (EndoCAB) IgM, IgA, and IgG were determined by ELISA (EndoCAB, HK504, Hycult Biotech, Uden, Netherlands). All variables were analysed in duplicate as per manufacturer's instructions, with standards and controls on each plate, and each participant assayed on the same plate. The CV for plasma IgM, IgA, and IgG were 7.9%, 7.7%, and 13.2%, respectively.

Data analysis

Confirmation of adequate statistical power for the primary research are previously described [7, 11, 49–51]. Participants and researchers at the time of data collection were unaware that the data would be used for analysis of pre- and post-exercise plasma anti-endotoxin antibody concentration in response to various exertional and exertional-heat stress protocols. Based on the statistical test, mean, standard deviation, and effect size; and applying a standard alpha (0.05) and beta value (0.80) the current participant sample size is estimated to provide adequate statistical power (power* 0.80–0.99) for detecting significant exercise magnitude and sub-group differences (G^* Power 3.1, Kiel, Germany), and is in accordance with participant sample sizes previously used to explore gastrointestinal epithelial integrity biomarkers for EIGS [9, 10, 25, 52, 53]. Descriptive data in text are presented as mean \pm standard deviation (SD). Primary and secondary variable data in text and tables are presented as mean and 95% confidence interval, unless otherwise indicated. For clarity, data in figures are presented as individual responses and mean. All data were checked for normal distribution by Shapiro-Wilks test of normality, prior to applying appropriate parametric or non-parametric statistical tests. Paired sample t-tests or non-parametric equivalents (Wilcoxon signed-rank) were used to assess pre- to post-exercise anti-endotoxin antibody concentration within exercise bouts. One-way ANOVA or non-parametric equivalents (Wilcoxon signed-rank) with post hoc analysis, were used to assess pre-exercise anti-endotoxin antibody concentration and Δ pre- to post-exercise response magnitudes between exercise bouts. Statistics were analysed using SPSS statistical software (v.27.0, IBM SPSS Statistics, IBM Corp., Armonk, NY, USA) with significance accepted at $p < 0.05$.

Results

Dietary and exercise control

No significant difference was observed between foods and fluids provided to participants and actual consumption of these food and fluids in P1-HIIT, P2-EHS, and P3-SS. Overall, >95% of the foods and fluids provided to participant were consumed within the three experimental designs. All participants confirmed they refrained from strenuous exercise for 48 h before each trial.

► **Table 2** Pre- and post-exercise anti-endotoxin antibody plasma concentrations across the three different experimental running exercise protocols (P1-HIIT: 2 h high intensity interval training running with plyometric drop jumps in temperate ambient conditions, P2-EHS: 2 h steady state running in hot ambient conditions, and P3-SS: 3 h steady state running in temperate ambient conditions).

	P1-HIIT			P2-EHS			P3-SS		
	n = 17			n = 14			n = 13		
	IgM (MMU/ml)	IgA (AMU/ml)	IgG (GMU/ml)	IgM (MMU/ml)	IgA (AMU/ml)	IgG (GMU/ml)	IgM (MMU/ml)	IgA (AMU/ml)	IgG (GMU/ml)
Pre-exercise	229 (150 to 308)	32 (22 to 42)	90 (21 to 160)	106 ^{aa} (87 to 125)	40 (22 to 57)	54 (27 to 80)	173 (82 to 263)	39 (26 to 52)	90 (31 to 149)
Post-exercise	215 (135 to 294)	32 (21 to 43)	169 (-70 to 408)	127 (92 to 162)	49 * (28 to 69)	80 * (40 to 121)	133 (87 to 178)	39 (28 to 50)	91 (9 to 172)
Mean (95 % CI): * $p < 0.05$ vs. pre-exercise; ^{aa} $p < 0.01$ vs. P1-EHS.									

Physiological and thermoregulatory strain

A significant change was observed during P1-HIIT for RPE ($p < 0.001$), but not HR (160 (157 to 163) bpm) or TCR (8 (8 to 9)), with RPE increasing from the first 20-min HIIT cycle (11 (10 to 11)) to cycle 4 (80-min; 13 (12 to 14)) and onwards (120-min; 14 (12 to 15)). T_{re} was measured pre- and immediately post-exercise, with a significant increase ($p < 0.001$) observed from pre- (36.5 (36.1 to 36.8)°C) to post-exercise (37.9 (37.6 to 38.1)°C).

A significant change was observed for RPE ($p = 0.02$) during P2-EHS with a significant increase at the end of exercise (120 min) (13 (11 to 14)) compared to 15 min into exercise (10 (9 to 11)). From pre-exercise resting values (37.0 (36.8 to 37.3)°C), T_{re} significantly increased from 30-min exercise until the end of exercise (peak T_{re} : 38.9 (38.5 to 39.4)°C) ($p < 0.001$). HR increased from 144 (134 to 153) bpm (15-min) to 158 (145 to 172) bpm (120-min), but the increase was not significant. TCR remained constant throughout P2-EHS (9 (9 to 9)).

A significant change was observed for RPE ($p = 0.002$) and T_{re} ($p = 0.036$) on P3-SS. RPE increased from 90 min into exercise until completion (13 (12 to 15)) compared with 30 min (11 (10 to 11)). T_{re} increased in the last 30 min of exercise until completion (peak T_{re} : 38.6 (38.3 to 38.8)°C) compared with pre-exercise (36.9 (36.7 to 37.1)°C). HR increased from 133 (128 to 138) bpm (15 min) to 141 (134 to 148) bpm (180 min), but the increase was not significant. TCR remained constant throughout P3-SS (8 (7 to 8)).

Effect of exercise protocols on plasma EndoCAB concentration

Pre- and post-exercise plasma concentrations for each respective protocol are described in ► **Table 2**. Overall resting pre-exercise levels for plasma IgM, IgA, and IgG were (mean and 95 % CI), 173 (132 to 214) MMU/ml, 37 (29 to 44) AMU/ml, and 79 (48 to 109) GMU/ml, respectively. Resting pre-exercise plasma IgM concentration significantly differed between protocols ($p = 0.035$); whereby, P2-EHS presented the lowest mean baseline values (► **Table 2**). No significant differences were observed between protocols for pre-exercise plasma IgA ($p = 0.742$) and IgG ($p = 0.308$) concentrations. In P1-HIIT and P3-SS, plasma concentrations of all EndoCAB did not significantly differ from pre- to post-exercise. In P2-EHS, plasma concentrations of anti-endotoxin antibodies of IgA ($p = 0.017$) and IgG ($p = 0.016$), but not IgM ($p = 0.158$), significantly increased from pre- to post-exercise.

Plasma EndoCAB concentration between protocols

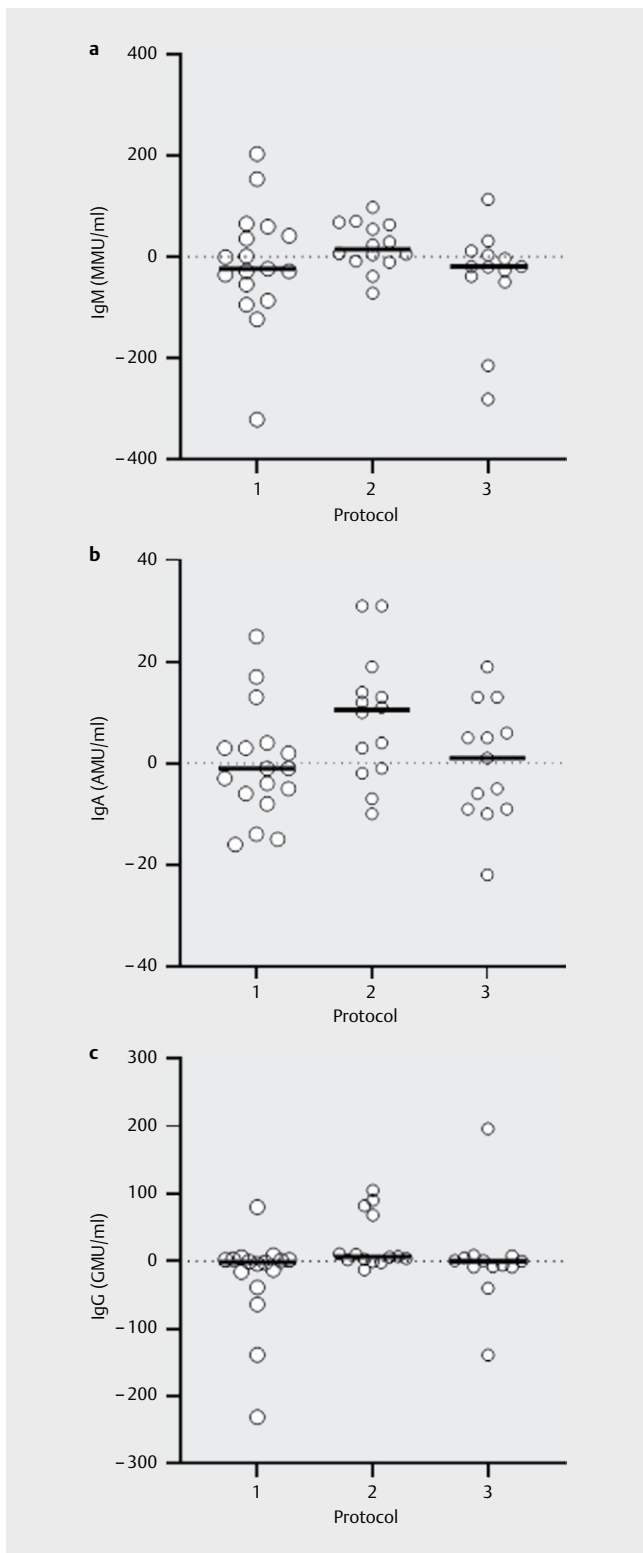
► **Fig 2** illustrates the mean and individual participant magnitude of pre- to post-exercise change for plasma concentrations of anti-endotoxin antibody between protocols. There was no significant difference in the magnitude of pre- to post-exercise change for plasma IgM concentration between protocols ($p = 0.135$). There was no significant difference, but a trend in the magnitude of pre- to post-exercise change for plasma IgA concentration between protocols ($p = 0.058$); whereby a greater change was seen in P2-EHS, compared with P1-HIIT and P3-SS. A significant difference between protocols for the magnitude of pre- to post-exercise change for plasma IgG concentration was observed ($p = 0.037$). However, when significance was adjusted by the Bonferroni correction for multiple tests, no significant difference existed between the magnitude of pre- to post-exercise change for anti-endotoxin antibody IgG across the protocols ($p > 0.05$).

Plasma EndoCAB by biological sex

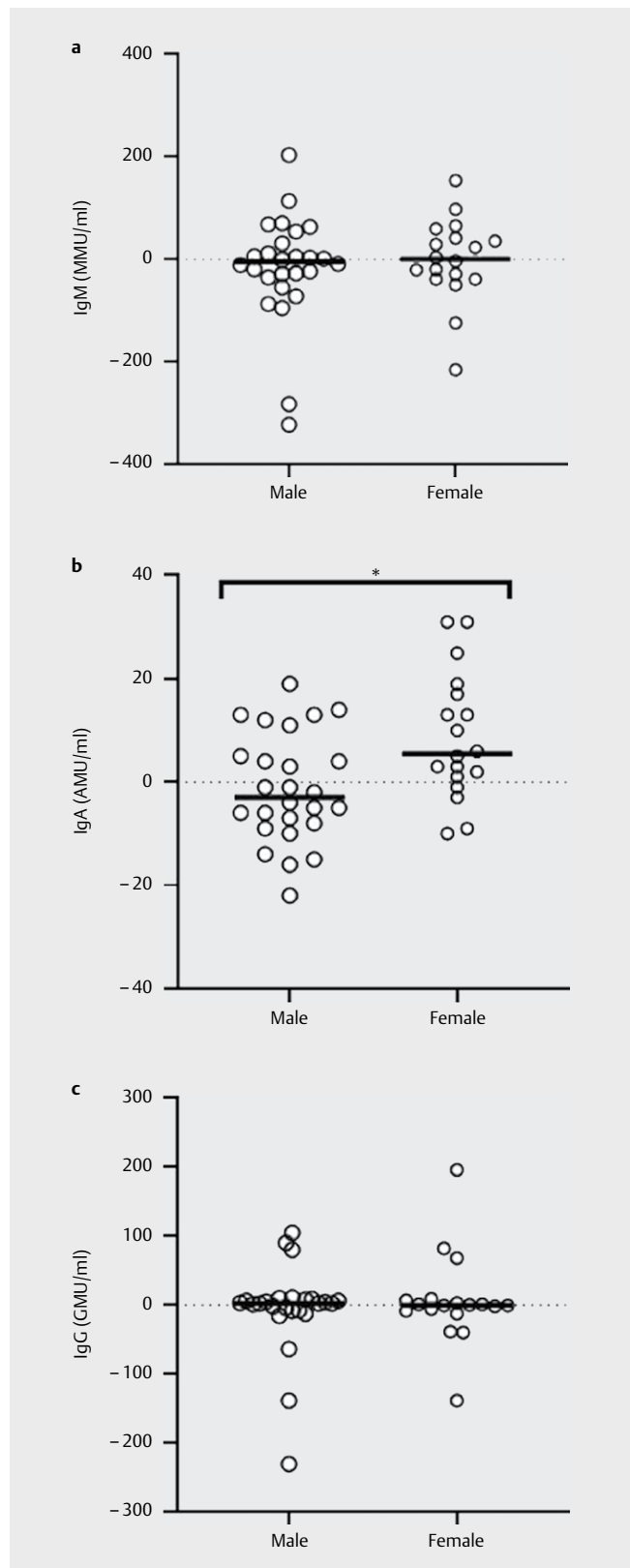
Pre-exercise anti-endotoxin antibody concentrations of IgM, IgA and IgG were the same in both male and female participants across the three exercise protocols. There was a significant difference between biological sex in plasma concentration of anti-endotoxin antibody IgA ($p < 0.05$), but not IgM or IgG, in response to exercise, with a mean increase in female participants (8.61 (2.48 to 14.70) AMU/ml) and a decrease in male participants (-1.34 (-5.57 to 2.90) AMU/ml) from pre- to post-exercise (► **Fig 3**).

Discussion

The current study aimed to investigate the impact of exertional and exertional-heat stress on systemic EndoCAB concentration. To our knowledge this is the first to comprehensively assess EndoCAB responses to a variety of exercise stress models and using rigorous control to avoid artificial impact of confounders known to perturb the epithelial integrity of the gastrointestinal tract in response to exercise. Contrary to our hypothesis, all systemic anti-endotoxin classed antibodies measured in this current study were not substantially impacted with exertional stress, characterised by 3-h steady state and 2-h high intensity interval running exercise. Instead of the expected reduction in all EndoCAB Ig in response to exertional-heat stress, a significant increase was observed for plasma IgA (Δ pre- to post-exercise (9 (2 to 16) AMU/ml)) and IgG (27 (3 to 50) GMU/ml) concentrations, but not for plasma IgM concen-



► **Fig 2** Magnitude of pre- to post-exercise change in plasma concentrations of endogenous endotoxin core antibody (EndoCAB) IgM (A), IgA (B), and IgG (C) across the three different experimental running exercise protocols (P1-HIIT: 2 h high intensity interval training running with plyometric drop jumps in temperate ambient conditions, P2-EHS: 2 h steady state running in hot ambient conditions, and P3-SS: 3 h steady state running in temperate ambient conditions). Mean and individual responses ($n=44$). Outlier removed from ► **Fig 2C** ($n=1$): P1-HIIT IgG = 1741 GMU/ml.



► **Fig 3** Magnitude of pre- to post-exercise change in plasma concentrations of endogenous endotoxin core antibody (EndoCAB) IgM (A), IgA (B), and IgG (C) by biological sex. Mean and individual responses ($n=44$); $p < 0.05$ vs biological sex. Outlier removed from ► **Fig 3C** ($n=1$): male IgG = 1741 GMU/ml.

tration (21 (-6 to 47) MMU/ml). However, the magnitude of change for IgA and IgG was modest and within normative values for a resting healthy human population [37, 38]. The current findings suggest such indirect biomarkers to describe the magnitude of translocation of intestinal lumen pathogenic endotoxin into systemic circulation are not particularly sensitive to exercise stress. This is in the context of: 1) the rigorous confounder control within the current study (i. e., pre and during exercise food and fluid intake, hydration status, circadian variation, ambient conditions, and female menstruation cycle, measurement of physiological and thermal strain known as EIGS exacerbation factors) [3], 2) the diversity in exercise experimental models used, and 3) the overall physical strain of the experimental models being synonymous with promoting EIGS [1, 2]. Moreover, although significant differences in EndoCAB responses were observed between males and females (i. e., biological sex differences), the magnitude of difference appears modest, within normative reference values for resting healthy human populations, and of no clinical relevance.

Within a healthy individual, plasma concentrations of EndoCAB at rest are sufficient for immunocompetence and range from an arbitrary value of 35 to 250 median-units (MU)/ml, based on healthy blood donors with high Ig titres [37, 38]. EndoCAB responses in systemic circulation primarily act to tag microbial endotoxins (e. g., rough and smooth LPS and/or lipid-A from various pathogenic bacteria such as *E. coli*) for immune cell detection, neutralisation and/or clearance by innate and/or adaptive immune responses [22, 54, 55]. For example, the mechanistic explanation for EndoCAB activity may include Fc receptor antibody-dependant immune activation of phagocytes (i. e., IgA), complement-dependant cytotoxicity (i. e., IgM and IgG), and/or antibody-dependant cell-based cytotoxicity (i. e., IgA and IgG) [56–58]. It has previously been documented that in response to a modest acute transient endotoxin exposure (e. g., ≤ 0.3 EU/ml, equivalent to 30 pg/ml), there is a transient increase in circulating EndoCAB (e. g., IgM ≥ 250 MU/ml and IgG ~ 100 –250 MU/ml), due to the circulating endotoxin exposure activation, generally irrespective of the bacterial species origins of the endotoxin [36, 39, 40]. Whilst, a substantial systemic endotoxin load (e. g., 0.5–1.0 EU/L, equivalent to 50–100 pg/ml), as per the case of sepsis, a depression in EndoCAB is observed (e. g., ≤ 35 MU/ml), likely attributed to the increased antibody utilization that overrides baseline starting and *in-situ* production levels [36, 39, 40, 59]. Therefore, clinical relevance is indicated at < 35 MU/ml, suggesting a state of immunosuppression as a result of Ig consumption $>$ production rate in response to illness and/or pathogenic infection [36]. In the current study, overall resting pre-exercise levels for plasma EndoCAB IgM, IgA, and IgG were 173 (132 to 214) MMU/ml, 37 (29 to 44) AMU/ml, and 79 (48 to 109) GMU/ml, respectively. Therefore, values appear to be within normative health ranges, although it is recognised and accepted that plasma IgA concentrations are generally lower than those presented for IgM and IgG, due to IgA's predominant role within the gastrointestinal tract lumen (e. g., secretion of IgA into lumen through epithelial cells), and not necessarily within internal circulation [60]. The inclusion of IgA and IgG in the current study are a novel contribution to scientific literature, while pre-exercise resting values for IgM are similar to previous studies; whereby, mean values of 90 to 127 MMU/ml have been reported [9, 10, 25]. Although the pre-exercise resting levels of En-

doCAB differed substantially between the protocols within the current study and previous studies, they were within resting normative ranges.

Circulating levels of EndoCAB are reported to increase in response to mild endotoxin presence in systemic circulation, but depress in response to severe and exaggerated endotoxin load [36, 59]. These clinical characteristics between endotoxemia and EndoCAB responses have also been observed in exercise research. Whereby, prolonged duration endurance events (e. g., triathlon and marathon) and ultramarathon events have resulted in a detectable systemic endotoxin load with a concomitant reduction in EndoCAB IgM and/or IgG [20–23], in adjunct with altered systemic inflammatory cytokines, mimicking SIRS [13]. It is therefore expected that exertional and/or exertional-heat stress of sufficient magnitude would show similar outcomes. Laboratory control studies that used 2-h exertional-heat stress ($60\% \dot{V}O_{2max}$, $35^{\circ}C T_{amb}$), and reported a modest plasma endotoxin load post-exercise ($\Delta 9.6$ pg/ml), also observed a modest reduction in anti-endotoxin antibody IgM (12%), concomitant with a greater systemic inflammatory response, compared with exercise at $20^{\circ}C$ and $30^{\circ}C T_{amb}$ [9, 10, 25]. Apart from the exertional-heat stress model with water consumption to maintain euhydration [61], all other exercise models, with and without nutrient feeding during, showed pre- to post-exercise increases in plasma IgM concentration. Other studies have used more subtle stress models, and subsequently have reported none to very modest disturbances to pre- to post-exercise anti-endotoxin antibody values (e. g., 30-min 60 – 65% heart rate reserve, up to 90 min walking in temperate and hot ambient conditions [62–64]). These observations suggest exertional-heat stress and duration of exertional stress may play a key role in the magnitude of exercise-associated disturbance to gastrointestinal integrity, and subsequently the translocation magnitude of intestinal lumen originating microbial pathogenic agents.

In the current study, 2 h of high intensity interval running with plyometric jumps did not promote substantial changes to circulating EndoCAB values. This is not surprising considering this exercise stress load has been reported to result in no substantial change to sCD14, LBP, and SIR-profile [49–51]. Similarly, 3 h steady state running did not result in any substantial changes to circulating EndoCAB values, even though IgM reduced by 23% pre- to post-exercise, but was not to a significant extent and showed large individual variation. These outcomes were surprising considering this exercise stress load has previously been reported to result in substantial increases in pre- to post-exercise sCD14, LBP, and SIR-profile [11], suggesting some evidence of exercise-associated endotoxemia, but a lack of EndoCAB responding accordingly. Finally, 2 h of exertional-heat stress increased all EndoCAB to a modest degree, with IgM failing to reach significance. These outcomes were also surprising considering previous research using that same exercise and heat load has previously reported depressed IgM responses [9, 25]. It is however important to note that the peak T_{re} and TCR in previous research was greater ($39.6^{\circ}C$ and 11-very hot, respectively), compared with the current study (i. e., $38.9^{\circ}C$ and 10-hot) that did not reach the target threshold established to promote substantial gastrointestinal integrity perturbations (circulatory-gastrointestinal pathway) synonymous with EIGS (i. e., $\geq 39.0^{\circ}C$); thus, potentially providing some insight into the differences in EndoCAB

outcomes, namely IgM. Nevertheless, exertional-heat stress of the current study was accompanied by modest increases in pre- to post-exercise sCD14, LBP, and SIR-profile [7], suggesting a mild endotoxin exposure and not synonymous with sepsis associated EndoCAB systemic release and consumption [36]. Collectively and from a research and practice perspective, considering pre- and post-exercise plasma EndoCAB Ig concentrations were within the normative values, it appears even strenuous prolonged exercise experimental protocol, with or without additional heat strain, do not substantially push EndoCAB to clinical relevance (e. g., activation at > 250 MU/ml or suppression at < 35 MU/ml), which have only been observed in ultra-endurance field events.

In conclusion, exertional and exertional-heat stress, synonymous with EIGS and perturbations to intestinal epithelial integrity leading to pathogenic bacterial endotoxin translocation into systemic circulation and subsequent systemic inflammatory responses, resulted in modest disturbances to circulating EndoCAB concentration. Both pre- and post-exercise values were within normative ranges for a healthy population, suggesting the exercise-associated magnitude of change of EndoCAB biomarkers (i. e., IgM, IgA, and IgG) presents a low response sensitivity to a variety of exertional and exertional-heat stress loads. Within the EIGS, the previous suggestion to use EndoCAB Ig as a marker to detect endotoxin exposure in systemic circulation should be used with caution within an exercise model and as a supportive biomarker instead of a primary biomarker.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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