NEW PERSPECTIVES ON THE ENDOCRINOLOGY OF PHYSICAL ACTIVITY AND SPORT

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NEW PERSPECTIVES ON THE ENDOCRINOLOGY OF PHYSICAL ACTIVITY AND SPORT

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EDITORIAL
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Editorial: New Perspectives on the Endocrinology of Physical Activity and Sport

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Keywords: sports endocrinology, biomarkers, overtraining syndrome, relative energy deficiency in sport, athlete

Editorial on the Research Topic

New Perspectives on the Endocrinology of Physical Activity and Sport

This edition of Frontiers in Endocrinology includes a compilation of papers seeking to determine endocrine markers that could be useful to predict performance, monitor training load, and assess low energy availability (EA) risk. We recommend beginning with Kraemer et al., who provide an in-depth review of complex interactions among androgens, growth hormones (GHs), insulin-like growth factor 1 (IGF-1) and its superfamily, glucocorticoids, and various binding proteins, regulation, and signaling pathways in the setting of exercise and circadian influence. Improved understanding of these anabolic: catabolic mechanisms will help the reader appreciate the difficulty in determining simple endocrine markers for exercise monitoring. Next, Pierce et al. address the important concept that women and men may have different hormonal responses to similar exercise training. During and after performing a loaded squat protocol, the two sexes demonstrated a similar hierarchy of serum proteins in the 3 GH molecular weight (MW) fractions (>60; 30-60; <30 kDa), but distinct response kinetics. Additionally, women and men differed in the IGF-1 MW fraction, and therefore specific IGF-1 superfamily members, that increased with the exercise intervention. In Nindl et al., some of these same authors compared concentration changes of blood and muscle interstitial IGF-1 and IGF binding proteins (IGFBPs) (collected *via* microdialysis probes in the vastus lateralis) pre- and post-unilateral jumping until exhaustion on a sledge. This study was only performed in men, but it demonstrated notable differences in local versus systemic IGF-1 and IGFBP responses to exercise. Combining these concepts of studying women and men and assessing local and systemic hormonal effects of exercise interventions is important for future study design to better inform effective, sex-specific training protocols.

In 2014, the International Olympic Committee (IOC) coined the term, "Relative Energy Deficiency in Sport" (RED-S), to highlight the myriad health and performance consequences of low EA in both female and male athletes. The IOC authors acknowledged that menstrual dysfunction and bone decrements are well-known sequelae of low EA (i.e., Female Athlete Triad), but encouraged further research into the other bodily systems and sports performance realms affected by low EA in broader athlete populations (1). In this edition, three papers address this topic. Hooper et al. assessed potential RED-S effects in 7 collegiate female cross-country athletes over 6 months. The women had no significant

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changes in body composition from pre- to post-Fall cross-country season, but had decreases in 25OH vitamin D and ferritin. Prior to outdoor Track season, a time of recovery, body mass increased, along with resting metabolic rate (RMR) and the aforementioned blood markers in the group as a whole. Interestingly, significant triiodothyronine (T3; a marker of low EA) changes were not observed. Multiple athletes reportedly had somewhat low EA, but only one experienced severely decreased RMR and performance, which improved with a nutritional and training intervention. Her ferritin was elevated, possibly indicating an inflammatory response (2). Stenqvist et al. studied similar markers during 4-weeks of intense training in 20 adult male cyclists. Short-term additions of high intensity training sessions to their baseline training over a month led to increased endurance performance and total testosterone. T3 and RMR decreased, and cortisol increased, as seen in prior work on athletes with RED-S (3). The group as a whole did not significantly change their testosterone:cortisol ratios, but individual ratio increases positively correlated with performance improvement. This is consistent with prior work on overtraining syndrome (OTS) (4). While the former case series and the latter intervention trial are both small, they illustrate the importance of considering multiple markers when assessing RED-S risk and of understanding that athletes have different EA thresholds for optimal functioning.

Hackney addressed RED-S versus OTS in his review on hypogonadism in exercising men, discussing variable causes of hypogonadism including hormonal inhibitory factors of stress, overtraining, inadequate EA, current or historic anabolic androgenic steroid use, traumatic brain injury, and chronic exercise without performance decrements [Exercise Hypogonadal Male Condition (EHMC)]. Distinguishing causes of hypogonadism is critical, as some of them are truly dysfunctional, but EHMC is debatably an appropriate adaptation, as seen among highly physically active men in non-industrial populations (5). Hackney's emphasis on different causes and consequences of low testosterone in male athletes is important in the RED-S versus OTS debate. Cadegiani and Kater (6) attempted to further clarify markers of training adaptation versus OTS in their analyses of the Endocrine and Metabolic Responses on Overtraining Syndrome (EROS) trial. In EROS, 25 healthy athletes, 14 OTS athletes, and 12 non-athletes (all male) were compared. OTS athletes were selected using interviews, performance metrics, biochemical testing, and other OTS guidelines; 117 markers were assessed in the three groups (7). In the EROS-CORRELATIONS paper (Cadegiani and Kater), the authors assessed the interplay of biomarkers to better differentiate OTS from healthy athletes. Fat mass inversely correlated with testosterone:estradiol ratio, which predicted, together with cortisol, prolactin and GH responses to stimulation, the measured RMR:predicted RMR ratio. Hypothalamic response to stress stimulation was diffuse and not hormone-specific. In the EROS-PREDICTORS paper (Cadegiani and Kater), carbohydrate intake predicted earlier hormonal responses to stimulation. Speed of muscle recovery after training was directly predicted by any source of caloric intake; protein intake predicted improved body composition.

The last two papers introduced new parameters for exploration. Eklund et al. examined the second to fourth digit ratio (2D:4D) – suggested to result from higher prenatal testosterone exposure (8) –in 104 female Olympic athletes and 117 non-athlete controls, along with serum and urine steroid profiles. The right hand 2D:4D was lower in athletes versus controls and associated with better strength and endurance performance. The ratio was negatively correlated with some urine testosterone metabolites but not with serum testosterone levels, possibly from differences in androgen metabolism only detected *via* urinary sampling. While compelling, the proposition that fetal androgen exposure predicts physical performance should be viewed with caution, as there are numerous contributors to athletic success. This may be an area for further exploration in some sports disciplines. Last, Munoz et al. considered the use of irisin, a muscle-contraction-induced myokine, as a metabolic biomarker of health-related fitness, testing normal- and overweight female students. Hand grip strength and irisin concentration correlated in the overweight group, but further studies are warranted to determine relevance in athletes.

In conclusion, as highlighted in Hackney's review, early, sensitive, and specific tools are needed to clearly distinguish dysfunction (e.g., OTS or low EA) from adaptation in athletes, accounting for gender and sport disciplines. New potential markers of interest have been presented in this edition, but they require validation with more comprehensive exploration at the local and systemic level in larger cohorts, considering the high inter-individual variation in highly functioning athletes. Studies in diverse populations of athletes and controls should attempt to employ the most standardized methodology for hormonal and metabolic assessment to further unveil adaptations that occur in athletes.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Association of Irisin Serum Concentration and Muscle Strength in Normal-Weight and Overweight Young Women

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Background: Irisin is a muscle-contraction-induced myokine. In previous studies, it has been related to exercise type, fitness and physical activity; however, evidence is not consistent. Thus, the aim of this study was to research the association between health-related fitness and irisin in young women.

Methods: The study was designed as a prospective cross-sectional one. Young, healthy, nonsmoking women were enlisted. The sample comprised 40 overweight (OW) and 40 normal-weight (NW) individuals. The average age was 18.63 ± 0.63 and 18.78 ± 0.73 years, respectively. Components of health-related fitness, metabolic parameters, serum irisin and body composition were analyzed.

Results: Statistically significant differences were found in physical tests between NW and OW groups for one-leg standing, hand grip strength, vertical jump, modified push-up, fitness index and maximal oxygen uptake (VO_{2MAX}). There were no differences in concentrations of serum irisin between the groups. We found a positive correlation between irisin and hand grip strength ($r = 0.374$, $p = 0.023$). In a multivariate analysis adjusted by body fat, a significant association between irisin and hand grip strength was observed in OW group ($\beta = 0.380$, $p = 0.026$); as well, a positive association between irisin and one-leg standing test in NW group ($\beta = 0.311$, $p = 0.044$) was found.

Conclusions: According to our findings, hand grip strength could be linked to irisin concentration in overweight young women.

Keywords: irisin, health-related to fitness, body composition, muscular strength, fat mass, hand grip strength

INTRODUCTION

In recent years, the global prevalence of overweight and obesity has increased (1). The age-standardized prevalence of obesity raised from 3.2% in 1975 to 10.8% in 2014 in men and from 6.4 to 14.9% in women (2). The association between excess weight and the development of chronic degenerative diseases is well-known (3). Therefore, a large part of the research has been addressed

in order to search for preventive and therapeutic targets, focusing largely on adipose tissue and its different types (4).

In recent decades, skeletal muscle has been recognized as an endocrine organ, secretor of myokines, some induced by muscle contraction and proposed as intermediates between the absence of physical activity and the onset of chronic degenerative diseases related to obesity (5).

In 2012, irisin was first described as a hormone, product of cleave of a type 1 membrane protein encoded by the Fibronectin type 3 domain containing protein 5 (FNDC5), a gene capable of increasing energy expenditure, promoting weight loss and decreasing the resistance to insulin produced by diet (6) through mechanisms related to the browning of adipose and subcutaneous adipose tissue with a consequential increase in thermogenesis (7).

Irisin is a myokine induced by the contraction of skeletal muscle with implications in beneficial effects attributed to physical exercise (8). Cross-sectional and intervention studies have been carried out to link it to different types of physical exercise, components of fitness and physical activity, finding contradictory results (9–12).

Health-related physical fitness comprises aerobic fitness, musculoskeletal fitness, motor ability and body composition. Each component is measured using a different test. The monitoring of these components is relevant to avoid the risk of diseases associated with sedentism and also to promote the increase of physical capacity for daily activities (13). The aim of the present study was to associate irisin with health-related physical fitness components in young women.

MATERIALS AND METHODS

Study and Subjects

This is a cross-sectional study carried out on young women students of *Universidad Autónoma del Estado de México* (UAEMex), aged between 18 and 20 years. Exclusion criteria were pregnancy, smoking, diabetes mellitus, cardiorespiratory diseases, thyroid disorders, hepatic failure, renal failure and inflammatory joint diseases or myopathies, as well as those who used drugs indicated for the diseases above. A total of 80 participants were included, 40 with normal weight (NW) and 40 with overweight (OW). BMI was 21.87 $\pm$ 1.55 and 27.01 $\pm$ 1.55 kg/m^2, respectively.

This study was approved by the local Ethics and Research Committee (registration number 2016/06). All the procedures were performed according to relevant guidelines and regulations. Written informed consent was obtained from the participants.

Measurements and Biochemical Parameters

We carried out a medical history, subsequently all measurements were performed after prior standardization, we measured blood pressure considering the average of two measurements with an interval of 2 min between each. Height was measured with a stadiometer seca® (Hamburg, Germany) and weight was measured by means of bioelectrical impedance Tanita® (Arlington, Ill, USA). BMI was calculated as weight (kg) divided by height squared (m^2). Waist circumference was measured at the midpoint between the lowest rib and the iliac crest; while hip circumference, at the lateral position by measuring the circumference at the most prominent point. Body composition was evaluated by dual-energy X-ray absorptiometry (DXA) using a GE Lunar bone densitometer, GE Healthcare® (Little Chalfont, UK) wearing minimal clothing and no metallic objects.

Blood samples were taken between 08:00 and 09:00 h after fasting between 8 and 12 h. Plasma glucose was measured with the oxidized glucose method (Randox Laboratories Ltd, Antrim, UK); triglycerides with a colorimetric method following enzymatic hydrolysis performed with the lipase technique; total cholesterol was measured by cholesterol esterase; HDL cholesterol (HDLC) by the clearance method; uric acid was measured by the enzymatic colorimetric method. All biomedical assays were performed with a Selectra XL instrument (Randox Laboratories Ltd, Antrim, UK).

Serum irisin concentration was measured using the enzyme linked immunosorbent assay (ELISA) kit BioVendor (Brno. Czech Republic).

Assessment of Health-Related Fitness

Health-related fitness was measured through the performance of tests corresponding to each type of fitness, as described below (14):

Motor fitness was assessed with the one-leg standing test, for which participants chose the leg they prefer to stand on, while the heel of the other leg was placed in the knee against the anterior site of the supporting leg, the thigh rotated outward and arms hung relaxed. The result was the longest time participants maintained the correct position twice (15).

Skeletal muscle fitness was assessed using hand grip strength, vertical jump and modified push-ups. Hand grip strength was measured with a dynamometer, Takei Scientific Instruments Co., Ltd. (Niigata-City, Japan) which was handled with the dominant hand keeping the arm straight and slightly away from the body. Participants squeezed firmly and gradually, until they reached the maximum strength, the best result of two performances was considered the score (16). Vertical jump consisted in jumping as high as possible after marking the height reached by the middle finger of the right hand of the participants standing with the arm raised and straight. The score was the maximum vertical difference in centimeters between standing height and that reached in the two jumps (17). Modified push-up test was performed face down; it consisted in placing the palms of hands at the beginning of the back and rising by flexing the arms so that the elbows remained completely straight. The result was the total number of correct push-ups performed over 40 s (18).

Cardiorespiratory fitness was assessed through a 2-kilometer walk test in an electric treadmill without elevation walking as fast as possible for the participant. The score of this test was determined through cardiorespiratory fitness (CF) and VO_{2MAX} following the formulas:

CF = 304–walking time (min) $\times$ 8.5 + walking time (s) $\times$ 0.14 + heart rate (beats/min) $\times$ 0.32 + BMI (kg/m^2) $\times$ 1.1)–age (years) $\times$ 0.4.

VO_{2MAX} × (ml/min/kg) = 116.2–2.98 × walking time (min)−011 × heart rate (beats/min)−0.14 × age (years)−0.39 × BMI (kg/m^2) (19, 20).

Data Analysis

The descriptive analysis was expressed using means and standard deviations. Shapiro-Wilk test was performed to assess the distribution of variables. Differences between continuous variables were analyzed with Student's t test or Mann-Whitney U test, as appropriate. The analysis of continuous quantitative outcome variables was performed using Pearson correlation or Spearman's, as appropriate. Multivariate linear regression models were calculated adjusted for confounder variables. Variables were logarithmically transformed to fit in the model. Statistical analyses were run using Statistical software for Social Sciences (IBM SPSS Statistics for Windows, Version 22.0 Armonk, NY: IBM Corp).

RESULTS

Baseline subject characteristics are summarized in **Table 1**. Age, systolic blood pressure, diastolic blood pressure were similar between the groups. Waist circumference, hip circumference, percentage of total body fat and muscle mass were higher in OW group. Glucose, total cholesterol, LDL cholesterol, triglycerides and uric acid were also different between the groups.

Health-related fitness was assessed through physical tests in both groups. NW group showed better performances in one-leg standing, vertical jump, modified push-ups, cardiorespiratory fitness and VO_{2MAX}. On the other hand, OW group had a better performance in hand grip strength (**Table 2**). The overweight group had higher irisin concentrations compared with NW group, though there were no statistically significant differences (**Figure 1**).

The correlation between health-related fitness tests and irisin concentration was calculated for the total population and for both groups. A statistically significant positive correlation was found between hand grip strength and irisin concentration in the total population (**Figure 2**). This correlation remains in OW group ($r = 0.374$, $p = 0.023$); however, NW group did not show any statistical significance ($r = 0.129$, $p = 0.433$). Multivariate linear regression models, adjusted for total fat, were produced; we found association between hand grip strength and irisin concentration in the total population and in OW group (**Table 3**).

TABLE 1 | Baseline subject characteristics.

	Normal weight $n = 40$	Overweight $n = 40$	*p*
Age (years)	18.63 ± 0.63	18.78 ± 0.73	0.329
Body weight (kg)	55.20 ± 4.88	67.10 ± 6.92	0.001*
Height (cm)	158.93 ± 5.62	157.80 ± 5.88	0.389
BMI (kg/m^2)	21.87 ± 1.55	27.01 ± 1.55	0.001*
Waist circumference (cm)	76.21 ± 5.34	86.53 ± 7.13	0.001*
Hip circumference (cm)	92.89 ± 3.05	100.60 ± 5.78	0.001*
Body fat (%)	36.09 ± 3.75	42.33 ± 3.31	0.001*
Muscle mass (kg)	33.44 ± 2.97	36.88 ± 3.28	0.001*
Systolic blood pressure (mmHg)	95.80 ± 8.76	98.89 ± 8.72	0.119
Diastolic blood pressure (mmHg)	66.53 ± 4.91	68.04 ± 3.92	0.132
Glucose (mg/dL)	89.90 ± 11.01	96.60 ± 10.67	0.007*
Total cholesterol (mg/dL)	158.63 ± 29.66	188.48 ± 35.41	0.001*
HDLC(mg/dL)	38.08 ± 3.81	38.35 ± 3.57	0.745
LDLC (mg/dL)	99.65 ± 25.26	118.70 ± 24.87	0.002*
Triglycerides (mg/dL)	98.61 ± 52.29	137.44 ± 62.37	0.003*
Uric acid (mg/dL)	3.74 ± 0.79	4.38 ± 1.21	0.006*
Irisin (ng/ml)	108.51 ± 70.08	126.63 ± 63.24	0.250

*Data are presented as Mean ± SD. *p < 0.05 was considered statistically significant. BMI, Body mass index; HDLC, high-density lipoprotein cholesterol; LDLC, high-density lipoprotein cholesterol.*

DISCUSSION

The present study describes the relation between irisin levels and health-related fitness in young women and the possible effect of overweight. We found a statistically significant positive correlation between hand grip strength and irisin concentration.

Obesity and overweight represent the main risk factors for the development of cardio-metabolic diseases. According to epidemiological studies, obesity and overweight incidence

TABLE 2 | Results of physical tests.

	Normal weight $n = 40$	Overweight $n = 40$	*p*
One-leg standing (seconds)	49.38 ± 16.09	38.45 ± 18.95	0.007*
Hand grip strength (kg)	23.23 ± 3.69	25.81 ± 4.50	0.007*
Vertical jump (cm)	24.56 ± 4.75	21.66 ± 4.36	0.006*
Modified push-ups (number of correctly performed)	15.50 ± 5.84	12.80 ± 5.88	0.043*
Cardiorespiratory fitness	52.78 ± 17.82	39.11 ± 15.08	0.001*
VO_{2MAX}(ml/min/kg)	28.10 ± 6.91	22.73 ± 7.04	0.001*

*Data are presented as Mean ± SD. *p < 0.05 was considered statistically significant. VO_{2MAX}: maximal oxygen uptake.*

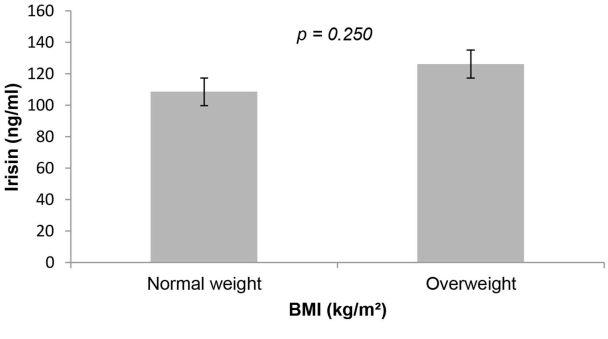

FIGURE 1 | Irisin concentration in normal and overweight participants. $p < 0.05$ was considered as statistically significant. BMI, body mass index.

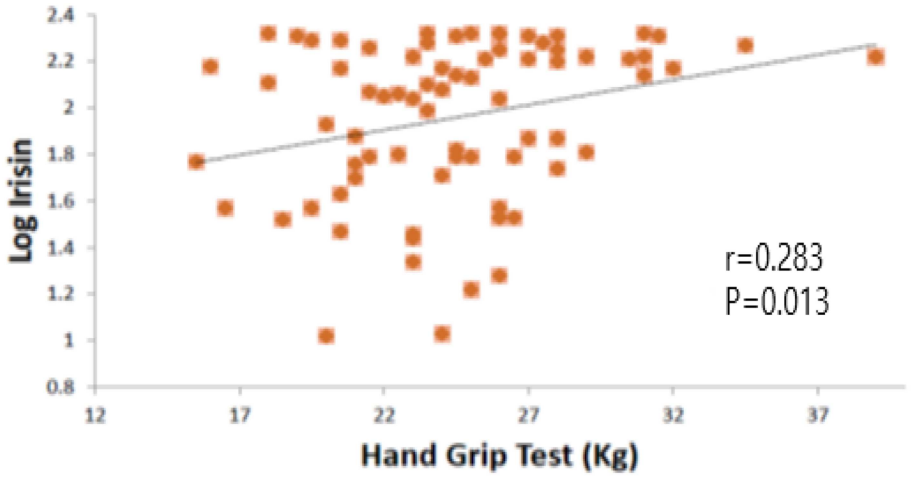

FIGURE 2 | Log irisin and hand grip test correlation. $p < 0.05$ was considered statistically significant.

TABLE 3 | Multivariate linear regression models adjusted by fat mass between irisin concentration and health-related physical tests.

	Total subjects		Normal weight		Overweight	
	β	*p*	β	*p*	β	*p*
One leg standing	0.197	0.089	0.311	0.044	0.059	0.736
Hand grip strength	0.265	0.019	0.196	0.236	0.380	0.026
Vertical jump	0.140	0.355	0.230	0.161	−0.043	0.810
Modified push-ups	0.175	0.134	0.198	0.211	0.114	0.525
Cardiorespiratory fitness	−0.066	0.578	−0.099	0.536	−0.085	0.630
VO_{2MAX}	−0.038	0.748	−0.131	0.412	0.023	0.898

$p < 0.05$ was considered statistically significant. VO_{2MAX}: maximal oxygen uptake; BMI: body mass index.

have grown as of the early eighties (21). In our study, we did not include diabetic patients, though metabolic risk factors such as lipid profile and fasting blood glucose seem to be different between NW and OW subjects. When we compared physical performance tests, cardiorespiratory, motor and some tests related to muscular fitness decreased in OW. Highlighting the effect of reduction of health-related fitness with body weight excess (22). Poor cardiorespiratory fitness has been considered an important cardiovascular risk factor and also a mortality predictor (23, 24). Shazia et al. (25) described the influence of excess body fat on aerobic fitness in young women.

In our results, irisin concentration was higher in OW group; however, it was not statistically significant. Previous studies have suggested that serum irisin concentration is higher in obese and overweight subjects compared with normal-weight subjects (26). These findings can be attributed to possible irisin resistance in presence of overweight. Park et al. (27) postulate that higher irisin concentrations in obese and overweight subjects could be related to a greater amount of fat and lean mass, and also to a possible compensatory role by irisin. Fukushima et al. (28) considered adipose tissue an influential factor in irisin secretion, especially in states of excess body fat. Previous studies have associated irisin concentration with cardiovascular fitness (29). In our study, we did not find a significant correlation between irisin and cardiorespiratory condition index or VO_{2MAX}. It is not still clear if fasting irisin may have a correlation with cardiorespiratory condition or if it changes in response to intense exercising (30). In like manner, no statistical significance was found between irisin and vertical jump or modified push-ups. Hecksteden et al. (31) reported lack of association between irisin concentration and physical fitness after muscle and aerobic endurance training in adults.

We did not find any statistically significant correlation between physical tests and irisin. Although we did not find any statistically significant correlation between other physical tests and irisin, a positive correlation has been found between hand grip strength and irisin. Hand grip strength is considered a fast and simple test, proposed to be an indirect marker of muscle strength (16); A poor hand grip performance has been reported as a predictor for further development of type 2 diabetes mellitus (32). Therefore, hand grip strength may be an indirect marker of irisin. This concurs with a previous paper by Chang, who found a positive correlation between irisin and hand grip (33).

Since adipose tissue may influence the secretion of irisin (34), we adjusted irisin levels for body fat, and its association with hand grip strength remained significant after this adjustment. It should be mentioned that OW group also showed significantly higher amounts of muscle mass. The reason why statistical correlation between hand grip strength and irisin levels was found in the OW but not in the NW group may be due to higher muscle mass typically found in OW, compared to NW. Our findings are consistent with Kim et al. (35), who reported a statistically significant positive association between hand grip strength and irisin concentration in women after performing a resistance training program.

A possible association between one-leg standing test and irisin concentration was found only in NW group. One-leg standing is a useful test to identify bone deterioration and a decreased ability to perform this test is associated with increased risk of fractures (36). In our study, we included young, healthy women without bone-fracture risk factors; however, a relation between the osseous system and skeletal muscle, where irisin exercises endocrine functions on osteoblasts (37), has been mechanically and biochemically studied. *In vitro* and *in vivo* studies suggest that irisin stimulates osteoblasts to promote the formation of new bone tissue and improves strength and bone mass; though, studies in humans under different conditions are inconclusive (38).

The present study was limited by lack of comparability with a group of men; thereby, it was not possible to discuss the influence of gender on irisin and on the components of health-related fitness. Our sample included normal-weight and overweight women, it is suggested performing studies with higher BMI that allow observing the effect of fat percentage on irisin correlation and on components of health-related fitness. On the other hand, it is recommended carrying out intervention studies aimed at improving each of the components of health-related fitness that allow better analyzing the impact of each component on irisin concentration.

To sum up, irisin was not correlated with cardiorespiratory fitness test and its indexes such as VO_{2MAX} and cardiorespiratory fitness index. In the same way, there was no correlation between irisin and vertical jump and modified push-ups. However, we found an association between hand grip strength and irisin in overweight young women. In the normal weight group, one-leg standing test was associated with irisin concentration.

DATA AVAILABILITY

Primary data is available from the authors.

ETHICS STATEMENT

The authors certify that they complied with the ethical guidelines for authorship and publishing. The protocol was accepted by the local IRB and all the participants signed an informed consent letter.

AUTHOR CONTRIBUTIONS

IM and EC executed the research procedures, sample collection, laboratory analyses and data interpretation, designed the study, clinical management and laboratory analyses, interpreted data, contributed to the discussion, and reviewed and edited the manuscript. TC executed research procedures, sample collection, laboratory analyses, contributed to the discussion, and reviewed and edited the manuscript. JS executed laboratory analyses, contributed to the discussion, and reviewed and edited the manuscript. MC executed sample collection, laboratory analyses, contributed to the discussion, and reviewed and edited the manuscript. LM sample collection, laboratory analyses, contributed to the discussion, and reviewed and edited the manuscript. GH interpreted data, contributed to the discussion, and reviewed and edited the manuscript. JG executed the research procedures, sample collection and data interpretation, designed the study, clinical management, and laboratory analyses, interpreted data, contributed to the discussion, and reviewed and edited the manuscript. All the authors read and approved the final manuscript.

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Inter-correlations Among Clinical, Metabolic, and Biochemical Parameters and Their Predictive Value in Healthy and Overtrained Male Athletes: The EROS-CORRELATIONS Study

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Objectives: The Endocrine and Metabolic Responses on Overtraining Syndrome (EROS) study identified multiple hormonal and metabolic conditioning processes in athletes, and underlying mechanisms and biomarkers of overtraining syndrome (OTS). The present study's objective was to reveal independent predictors and linear correlations among the parameters evaluated in the EROS study to predict clinical, metabolic, and biochemical behaviors in healthy and OTS-affected male athletes.

Methods: We used multivariate linear regression and linear correlation to analyze possible combinations of the 38 parameters evaluated in the EROS study that revealed significant differences between healthy and OTS-affected athletes.

Results: The testosterone-to-estradiol (T:E) ratio predicted the measured-to-predicted basal metabolic rate (BMR) ratio; the T:E ratio and total testosterone level were inversely predicted by fat mass and estradiol was not predicted by any of the non-modifiable parameters. Early and late growth hormone, cortisol, and prolactin responses to an insulin tolerance test (ITT) were strongly correlated. Hormonal responses to the ITT were positively correlated with fat oxidation, predicted-to-measured BMR ratio, muscle mass, and vigor, and inversely correlated with fat mass and fatigue. Salivary cortisol 30 min after awakening and the T:E ratio were inversely correlated with fatigue. Tension was inversely correlated with libido and directly correlated with body fat. The predicted-to-measured BMR ratio was correlated with muscle mass and body water, while fat oxidation was directly correlated with muscle mass and inversely correlated with fat mass. Muscle mass was directly correlated with body water, and extracellular water was directly correlated with body fat and inversely correlated with body water and muscle mass.

Conclusions: Hypothalamic-pituitary responses to stimulation were diffuse and indistinguishable between the different axes. A late hormonal response to stimulation, increased cortisol after awakening, and the T:E ratio were correlated with vigor and

fatigue. The T:E ratio was also correlated with body metabolism and composition, testosterone was predicted by fat mass, and estradiol predicted anger. Hydration status was inversely correlated with edema, and inter-correlations were found among fat oxidation, hydration, and body fat.

Keywords: hormonal conditioning, endocrinology of physical activity, sports endocrinology, hormones and sports, endocrine and metabolic responses on overtraining syndrome (EROS) study, overtraining syndrome

INTRODUCTION

The benefits of extensive exercise have exceeded previous expectations, including primary prevention, active part of the treatment, improvement of life quality, and prognosis under chronic or incurable diseases, and prevention of complications and recurrence, of a wide range of diseases, including cardiovascular (1, 2), hypertension, type 2 diabetes mellitus (T2DM) (1), dyslipidemia, cancers (3–6), cognitive function (7), and all quality of life domains (8–10).

Many of the benefits from physical activity are linked to multiple adaptive changes that leads to improvements in neuromuscular (11), cardiovascular (12), musculoskeletal, autonomic, and other systems that active individuals undergo. However, these benefits may only occur when physically active individuals sleep and eat appropriately, alongside with training (13, 14).

Indeed, overtraining syndrome (OTS), which affects between 40 and 60% of the elite athletes during their careers, is likely a major manifestation of the harms of an imbalance between excessive training, insufficient recovery, non-refreshing sleep, insufficient caloric, protein, and/or carbohydrate intake, and concurrent psychological stress, including excessive cognitive effort, social, familiar, or financial issues (15–17). This imbalance is the likely underlying reason of the paradoxical loss of physical performance in OTS, which is not able to be justified by any apparent dysfunction (15). However, OTS is still a controversial issue since several characteristics of OTS, including underlying mechanisms, pathophysiology, and biological markers are universally accepted or clarified, as the prevailing findings on previous studies were inconsistent (15–17).

To address all the unanswered questions on OTS, and also to better understand the multiple conditioning processes that athletes seem to undergo, we conducted the Endocrine and Metabolic Responses to Overtraining Syndrome (EROS) study (18–24). In that study, we evaluated basal and exercise-independent hormonal responses to stimulation tests, multiple biochemical markers, including muscular, inflammatory, immunologic, and nutritional parameters, specific eating, psychological, and social patterns, and the body metabolism and composition of healthy athletes, athletes affected by OTS, and sedentary individuals with similar baseline characteristics (age, sex, and body mass index—BMI). The list of parameters evaluated by the EROS study are detailed in **Table 1**, together with the selection process for the present analysis, to be described further.

Among the 117 markers evaluated in the EROS study, we identified 50 novel parameters for OTS-affected and healthy athletes, including amplified and prolonged GH, cortisol, and prolactin responses to a stimulation test, increased testosterone, lactate clearance, catecholamines, basal metabolic rate (BMR), fat oxidation, and hydration in healthy athletes, and blunted hormonal responses (compared to healthy athletes), increased creatine kinase (CK), aromatase activity, estradiol, anger, depression, fatigue, mental confusion and fat mass, and reduced testosterone, hydration, muscle mass, BMR, fat oxidation, and moods in athletes affected by OTS (23). The major findings of the EROS study are described in **Figure 1**.

The findings unveiled by the EROS study supported the hypothesis of the existence of multiple adaptations of clinical, metabolic, biochemical, and body parameters that athletes, while the majority of the physiological adaptive changes are compromised in OTS, which may explain the hallmark of OTS, the loss of performance (23, 24).

Despite the multiple and broad adaptive changes previously demonstrated to occur in athletes, and the more than 50 novel markers and processes identified in both healthy and OTS-affected athletes in the present study, the relationships between parameters that are affected by training and/or OTS are unclear (18–21). Associations, interactions, synergisms, stimulations, and inhibitions between hormones, inflammatory, immunologic, muscular, metabolic, and clinical markers, and psychological, eating, sleep, and training patterns, have been poorly assessed previously, and have not been identified in the EROS study, once our primary objective was to detect differences between OTS and healthy athletes, and sedentary control among the 117 parameters evaluated, using three-group and pairwise comparisons, which were published in different arms (18–24).

The unexpected large number of markers identified in both populations of athletes allowed us hypothesize the existence of a web of multiple sorts of interactions between parameters of different natures, which could result in the wide range of benefits and improved performance observed in healthy athletes, and the paradoxical decrease of sports performance, fatigue, reduced libido, and body changes in OTS (24). The correlations to be identified between the newly uncovered parameters could provide a new understanding of the complex processes of conditioning processes that athletes typically undergo, and the convoluted mechanisms that lead to OTS (23, 24).

In summary, despite the large number of discoveries (18–22, 24), our primary findings do not demonstrate the multiple sorts of relationship between those markers that participate in the adaptative processes of the athletes and those that have

TABLE 1 | Eligible markers for the present analysis.

Tests	Markers	Initially elected? (Provide independent data, provide additional data (in relation to other parameters evaluated), substantiated/validated)	Significantly different between OTS-affected and healthy athletes? (If "Yes," included in the present analysis)
BASAL BIOCHEMICAL TESTS			
Basal hormones	(1) Total testosterone (ng/dL)	Yes	**Yes**
	(2) Estradiol (pg/mL)	Yes	**Yes**
	(3) IGF-1 (pg/mL)	Yes	No
	(4) TSH (μUI/mL)	Yes	No
	(5) Free T3 (pg/mL)	Yes	No
	(6) Total catecholamines (μg/12 h)	Yes	**Yes**
	(7) Total metanephrines (μg/12 h)	Yes	No
	(8) Noradrenaline (μg/12 h)	Yes	No
	(9) Epinephrine (μg/12 h)	Yes	No
	(10) Dopamine (μg/12 h)	Yes	**Yes**
	(11) Metanephrines (μg/12 h)	Yes	No
	(12) Normetanephrines (μg/12 h)	Yes	No
Muscular, inflammatory, immunologic, and other basal biochemical markers	(13) Erythrocyte sedimentation rate (ESR, mm/h)	Yes	No
	(14) Hematocrit (%)	Yes	No
	(15) C-reactive protein (CRP, mg/dL)	Yes	No
	(16) Lactate (nMol/L)	Yes	**Yes**
	(17) Vitamin B12 (pg/mL)	Yes	No
	(18) Ferritin (ng/mL)	Yes	No
	(19) Neutrophils (mm^3)	Yes	**Yes**
	(20) Lymphocyte (mm^3)	Yes	**Yes***
	(21) Eosinophils (mm^3)	Yes	No
	(22) Creatine kinase (CK, U/L)	Yes	**Yes**
	(23) Low-density lipoprotein cholesterol (LDLc, mg/dL)	Yes	No
	(24) High-density lipoprotein cholesterol (HDLc, mg/dL)	Yes	No
	(25) Tryglycerides (mg/dL)	Yes	No
	(26) Medium corpuscular volume (MCV)	No (interpretation may vary)	-
	(27) Platelets (10^3/mm)	No (platelet-to-lymphocyte was used instead)	-
Ratios	(28) Testosterone-to-oestradiol ratio	Yes	**Yes**
	(29) Testosterone-to-cortisol ratio	Yes	No
	(30) Neutrophil-to-lymphocyte ratio	Yes	**Yes**
	(31) Platelet-to-lymphocyte ratio	Yes	**Yes***
	(32) Catecholamine-to-metanephrine ratio	No (Non-validated marker)	-
HORMONAL FUNCTIONAL TESTS			
Insulin tolerance test (ITT)	(33) Basal ACTH (pg/mL)	Yes	No
	(34) ACTH during hypoglycaemia (pg/mL)	Yes	No
	(35) ACTH 30 min after hypoglycemia (pg/mL)	Yes	**Yes**
	(36) ACTH increase during ITT (pg/mL)	Yes	**Yes**
	(37) Basal cortisol (μg/dL)	Yes	No
	(38) Cortisol during hypoglycaemia (μg/dL)	Yes	**Yes**
	(39) Cortisol 30 min after hypoglycemia (μg/dL)	Yes	**Yes**
	(40) Cortisol increase during ITT (μg/dL)	Yes	No
	(41) Basal GH (μg/L)	Yes	**Yes**
	(42) GH during hypoglycaemia (μg/L)	Yes	**Yes**
	(43) GH 30 min after hypoglycemia (μg/L)	Yes	**Yes**
	(44) Basal prolactin (ng/mL)	Yes	**Yes**
	(45) Prolactin during hypoglycaemia (ng/mL)	Yes	**Yes**
	(46) Prolactin 30 min after hypoglycemia (ng/mL)	Yes	**Yes**

(Continued)

TABLE 1 | Continued

Tests	Markers	Initially elected? (Provide independent data, provide additional data (in relation to other parameters evaluated), substantiated/validated)	Significantly different between OTS-affected and healthy athletes? (If "Yes," included in the present analysis)
	(47) Prolactin change during ITT (ng/mL)	Yes	No
	(48) Basal ACTH/cortisol ratio	No (Non-validated marker)	-
	(49) ACTH/cortisol ratio during hypoglycemia	No (Non-validated marker)	-
	(50) ACTH/cortisol ratio 30 min after hypoglycaemia	No (Non-validated marker)	-
	(51) Basal serum glucose (mg/dL)	No (does not provide useful data)	-
	(52) Serum glucose during hypoglycemia (mg/dL)	No (does not provide useful data)	-
	(53) Capillary glucose during hypoglycemia (mg/dL)	No (does not provide useful data)	-
	(54) Adrenergic symptoms during hypoglicemia (0–10)	No (Non-validated marker)	-
	(55) Neuroglycopenic symptoms during hypoglicemia (0–10)	No (Non-validated marker)	-
Cosyntropin stimulation test (CST)	(56) Basal cortisol (μg/dL)	No (does not provide additional data)	-
	(57) Cortisol at 30 min after infusion (μg/dL)	Yes	No
	(58) Cortisol at 60 min after infusion (μg/dL)	Yes	No
	(59) Difference between basal cortisol on day 1 (CST) and day 3 (ITT) (%)	No (non-validated marker)	-
Salivary cortisol rhythm (SCR)	(60) Salivary cortisol (ng/dL) at awakening	Yes	No
	(61) Salivary cortisol (ng/dL) 30 min after awakening	Yes	**Yes**
	(62) Salivary cortisol (ng/dL) at 4 p.m.	Yes	No
	(63) Salivary cortisol (ng/dL) at 11 p.m.	Yes	No
	(64) Cortisol awakening response (CAR)	Yes	No
	(65) Difference between 8 a.m. and 4 p.m. salivary cortisol (%)	No (non-validated marker)	-
CLINICAL PARAMETERS			
Sleeping and social characteristics	(66) Duration of night sleep (h/night)	Yes	No
	(67) Self-reported sleep quality (0–10)	No (out of the scope of the present study)	No
	(68) Self-reported libido (0–10)	Yes	**Yes**
	(69) Number of hours of activities besides professional training (h/day)	Yes	No
	(70) Initial imnsonia (Y/N)	No (qualitative marker)	-
	(71) Terminal imnsonia (Y/N)	No (qualitative marker)	-
	(72) More than two wake-ups during sleep (Y/N)	No (qualitative marker)	-
	(73) Work and/or study (Y/N)	No (qualitative marker)	-
	(74) Libido during resting periods / vacations (0–10)	No (qualitative marker)	-
	(75) Calorie intake (kcal/kg/day)	No (out of the scope of the present study)	-
	(76) Carbohydrate intake (g/kg/day)	No (out of the scope of the present study)	-
	(77) % calories from carbohydrate (%)	No (out of the scope of the present study, and intrinsically linked to other markers)	-
	(78) Protein intake (g/kg/day)	No (out of the scope of the present study)	-
	(79) % calories from protein (%)	No (out of the scope of the present study, and intrinsically linked to other markers)	-
	(80) Fat intake (g/kg/day)	No (out of the scope of the present study)	-
	(81) % calories from fat (%)	No (out of the scope of the present study, and intrinsically linked to other markers)	-
	(82) Carbohydrate intake > 3 g/kg/day (Y/N)	No (out of the scope of the present study and a qualitative marker)	-
	(83) Daily whey protein consumption (Y/N)	No (out of the scope of the present study and a qualitative marker)	-
	(84) Followed a diet plan (Y/N)	No (out of the scope of the present study and a qualitative marker)	-

(Continued)

TABLE 1 | Continued

Tests	Markers	Initially elected? (Provide independent data, provide additional data (in relation to other parameters evaluated), substantiated/validated)	Significantly different between OTS-affected and healthy athletes? (If "Yes," included in the present analysis)
	(85) Post-workout carbohydrate intake > 0.5 g/kg (Y/N; only applicable for athletes)	No (out of the scope of the present study and a qualitative marker)	-
Psychological patterns	(86) Profile of Mood State (POMS) scale (total score:−32 to +120)	Yes	**Yes**
	(87) Anger subscale (0–48)	Yes	**Yes**
	(88) Confusion subscale (0–28)	Yes	**Yes**
	(89) Depression subscale (0–60)	Yes	**Yes**
	(90) Vigor subscale (0–32)	Yes	**Yes**
	(91) Fatigue subscale (0–28)	Yes	**Yes**
	(92) Tension subscale (0–36)	**Yes**	**Yes**
	(93) Have you been sick in the last 2 weeks? (Y/N) ?	No (alone does not determine status, and a qualitative marker)	-
	(94) How was your last training session compared to the projected goals? (Extremely easy to extremely hard)	No (alone does not determine status)	-
	(95) How do your muscles feel? (Nothing at all to extremely painful)	No (alone does not determine status)	-
	(96) How friendly do you feel today? (0–6)	No (alone does not determine status)	-
	(97) How worthless do you feel today? (0–6)	No (alone does not determine status)	-
	(98) How miserable do you feel today? (0–6)	No (alone does not determine status)	-
	(99) How helpful do you feel today? (0–6)	No (alone does not determine status)	-
	(100) How bad-tempered do you feel today? (0–6)	No (alone does not determine status)	-
	(101) How unworthy do you feel today? (0–6)	No (alone does not determine status)	-
	(102) How peeved do you feel today? (0–6)	No (alone does not determine status)	-
	(103) How cheerful do you feel today? (0–6)	No (alone does not determine status)	-
	(104) How sad do you feel today? (0–6)	No (alone does not determine status)	-
	(105) How do you fell today? (0–10)	No (alone does not determine status)	-
Body metabolism analysis	(106) Measured-to-predicted basal metabolic rate (BMR, %)	Yes	Yes
	(107) Percentage of fat burning compared to total BMR (%)	**Yes**	**Yes**
Body composition	(108) Body fat percentage (%)	Yes	**Yes**
	(109) Muscle mass percentage (%)	Yes	**Yes**
	(110) Body water percentage (BW, %)	Yes	**Yes**
	(111) Extracellular water compared to total BW (%)	Yes	**Yes***
	(112) Visceral fat (cm^2)	Yes	**Yes**
	(113) Chest-to-waist circumference ratio	Yes	**Yes***
	(114) Waist circumference (cm)	No (interpretation may vary, and alone does not determine status or diagnosis)	-
	(115) Chest circumference (cm)	No (interpretation may vary, and alone does not determine status or diagnosis)	-
	(116) Biceps circumference (cm)	No (interpretation may vary, and alone does not determine status or diagnosis)	-
	(117) Hip circumference (cm)	No (interpretation may vary, and alone does not determine status or diagnosis)	-

$p > 0.05$ but < 0.1 between OTS-affected and healthy athletes, but different between athletes (both groups) and sedentary, with possible clinical significance. Bold values: parameters that were selected for the present analysis.

roles in the pathogenesis of OTS. Therefore, in the present study we aimed to uncover the web of multiple interactions that participate in the conditioning processes that occur in athletes, and the underlying mechanisms of the pathophysiology of OTS, derived from an exhaustive yet reasonable joint *post-hoc* analysis of the primary findings of the EROS study, using

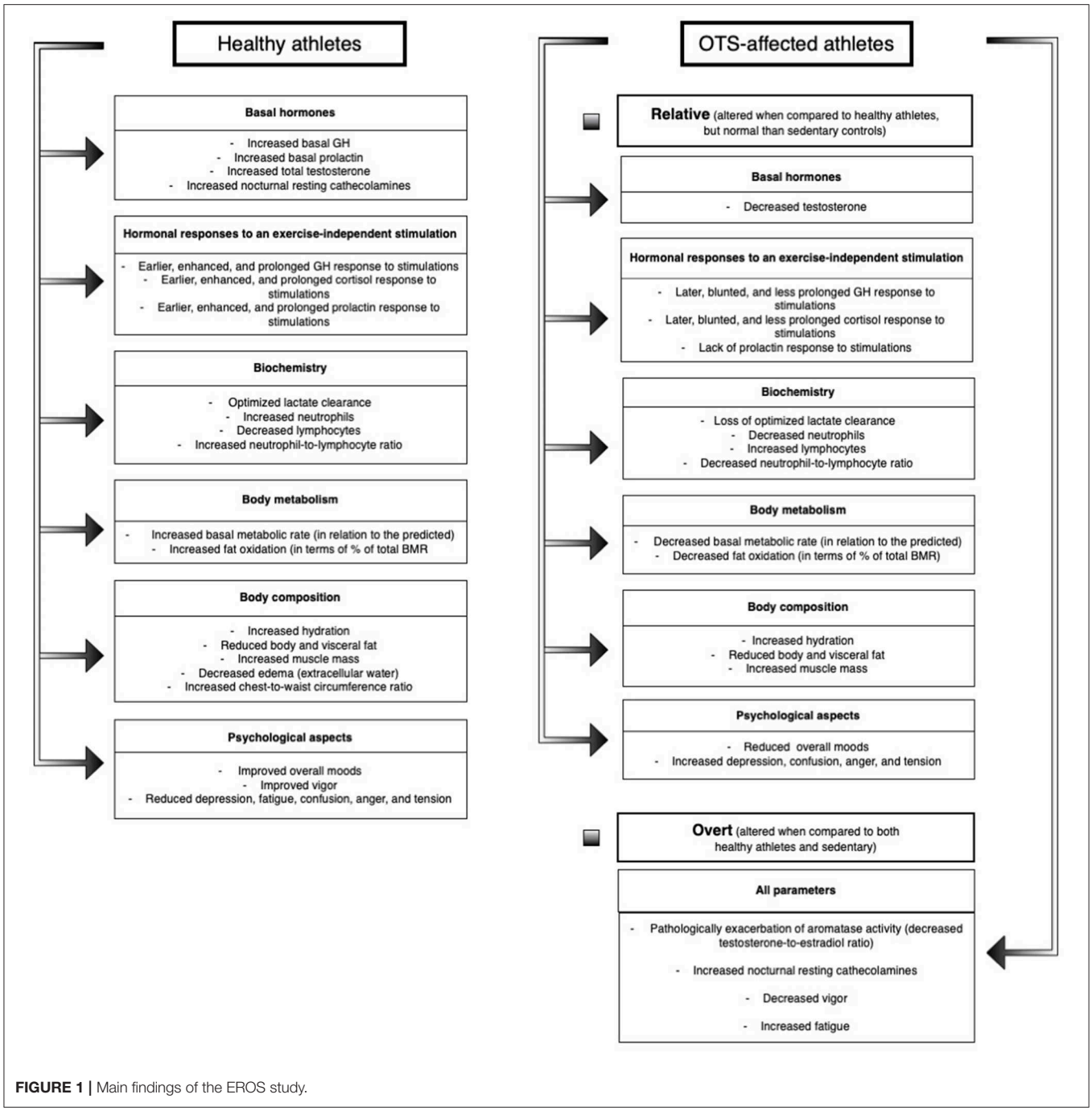

FIGURE 1 | Main findings of the EROS study.

different and more complex statistical analyses (e.g., multivariate linear regression, logistic regression, and linear correlation analyses) that those employed in the primary arms of the study, which is adequate owing to the large number of data generated by the EROS study (more than 11,000 results among 117 parameters).

Our ultimate objective was to identify independent predictors and linear correlations, and determine causal relationships and inter-influences, among hormonal, immunologic, inflammatory, muscular, psychological, metabolic, and body composition parameters, aiming to uncover behavior patterns and dysfunctional pathways in OTS.

MATERIALS AND METHODS

Subject and Parameter Selection

The detailed methodology was of the EROS study was previously published (18–22), also available at a depository (https://osf.io/bhpq9/). The present study was approved by the ethical

committee of the Federal University of São Paulo (Approval Number: 1093965).

We recruited participants through social media (Facebook, Instagram, Whatsapp), and were initially evaluated for age, sex, weight and height, clinical characteristics, and if they were suspected for Overtraining Syndrome (OTS), healthy athletes (ATL), or non-physically active (NPAC), and training (if athletes). Inclusion criteria for all participants, criteria for all athletes, and specific criteria for OTS are shown in **Figure 2**. Employing a two-step selection process, we avoided athletes with an insufficient amount of training for the adaptive changes to exercises, non-full sedentary, extremes of age, misdiagnosis of OTS, and presence of confounding hormones, medications, and diseases.

For OTS candidates, we employed the diagnostic flowchart proposed by the latest guideline on OTS (15), the joint guideline of the European College of Sport Science and American College of Sports Medicine, from 2013, which requires the presence of decrease of at least 10% in training intensity, volume, pace, power, strength, or overall performance, decrease of the time-to-fatigue of at least 20%, both verified by a professional coach, increased sense of effort, changes in behavior and decreased energy levels, with or without sleep disturbances, infections or injuries, which persisted for at least 1–2 months, despite the attempts to recover, and which is not caused by conditions that could inherently lead to decrease of performance, including inflammations, infections, and frank hormonal dysfunctions.

Design of the Study

Summary of the procedures according to each primary arm of the EROS study

All selected participants signed a written informed consent for participation in the study, approved by the ethics committee of the Federal University of São Paulo, in accordance with the Declaration of Helsinki. Then, participants underwent hormonal responses to stimulation tests, basal biochemical, inflammatory, muscular, immunologic, and hormonal parameters, nocturnal urinary catecholamines (NUC) and its metabolites, analysis of body metabolism and composition, and evaluation of psychological, social, sleep, and eating patterns.

In the EROS-HPA axis arm of the study, we evaluated peripheral and central components of the hypothalamic-pituitary-adrenal (HPA) axis (whether primary or peripheral: adrenal, or central: pituitary and hypothalamus), by employing a 250 ug cosyntropin stimulation test (CST), for direct evaluation of cortico-adrenal responses to a synthetic ACTH, an insulin tolerance test (ITT), an exercise-independent stimulation test that provokes a hypoglycemia as the stimulation for the evaluation of the integrity of the HPA axis, and salivary cortisol rhythm (SCR), for the identification of the patterns of the circadian rhythm of the cortisol.

In the EROS-STRESS test we employed the same ITT for the evaluation of the of the growth hormone (GH) and prolactin responses to hypoglycemia, and which we detailed different aspects of the test, including time-to-hypoglycemia, glycemic nadir, severity of adrenergic, and neuroglycopenic symptoms during hypoglycemia, and compared between groups.

In the EROS-BASAL arm, we evaluated basal hormones, immunologic, muscular, classical inflammatory, lipids, and vitamin. And for the EROS-PROFILE, participants underwent several questions regarding specific sleeping, eating, social, and psychological patterns, and underwent analysis of body composition and metabolism.

The full process was performed during a short period, of <10 days, between the recruitment, clinical, and biochemical inclusion and exclusion criteria, the collect of the basal biochemical parameters, all questionnaires, body composition, and metabolism, and functional tests. For all parameters we performed three-group and pairwise comparisons.

Procedures and Tests

Questionnaires

After the selection criteria, athletes (sedentary subjects were not assessed at this moment) underwent an initial specific interview about training patterns, including the type(s) the sport(s) practiced, time since starting the current sport(s), training volume and intensity (evaluated by a professional coach, on a scale from 0 to 10 compared to athletes of the same level of training), duration of training per week (min), number of rest days per week was recorded based on standardized tests, and whether they were supervised by a coach. This first questionnaire aimed to determine the baseline characteristics of the OTS and ATL Groups.

For all athletes affected by OTS, we evaluated whether and the number of days to overcome the underperformance state, changes in sensitivity to heat or to coldness, occurrence of infections, particularly upper respiratory tract infections (URTIs), and injuries, and feelings of monotony and boredom.

All participants (now including the non-physically active group—NPAC) then underwent specific questionnaires regarding sleeping, eating, social, and psychological characteristics.

In terms to eating habits, we employed a 7-day food and nutrition specific record and manually calculated mean daily carbohydrate, fat, protein, and overall caloric intake, aiming to preclude heterogeneity regarding the food analysis. The following specific aspects were evaluated: mean daily (1) carbohydrate, (2) protein, and (3) fat intake (in g/kg), (4) mean daily calorie, total (kcal/day) and per weight (kcal/kg/day), (5) the proportion of carbohydrate, protein and fat intake, (6) whether a diet plan was followed (yes or no), (7) whether there was daily whey protein ingestion, (8) whether post-training carbohydrate intake was > 0.5 g/kg, and (9) whether carbohydrate intake throughout the day was > 4.0 g/kg.

With regards to sleeping patterns, evaluation included the following aspects: (1) self-reported sleep quality (zero to ten; where zero = awful and ten = great), (2) mean duration of sleeping time, (3) whether there was difficulty falling sleep, (4) whether waking up too early and unable to sleep again, and (5) whether waking up more than two times during the night.

For the evaluation of the social aspects, we asked (1) whether participants attended work or study besides the professional training sessions, if so, (2) how many hours per day they attended to the professional activities besides the training periods, and (3)

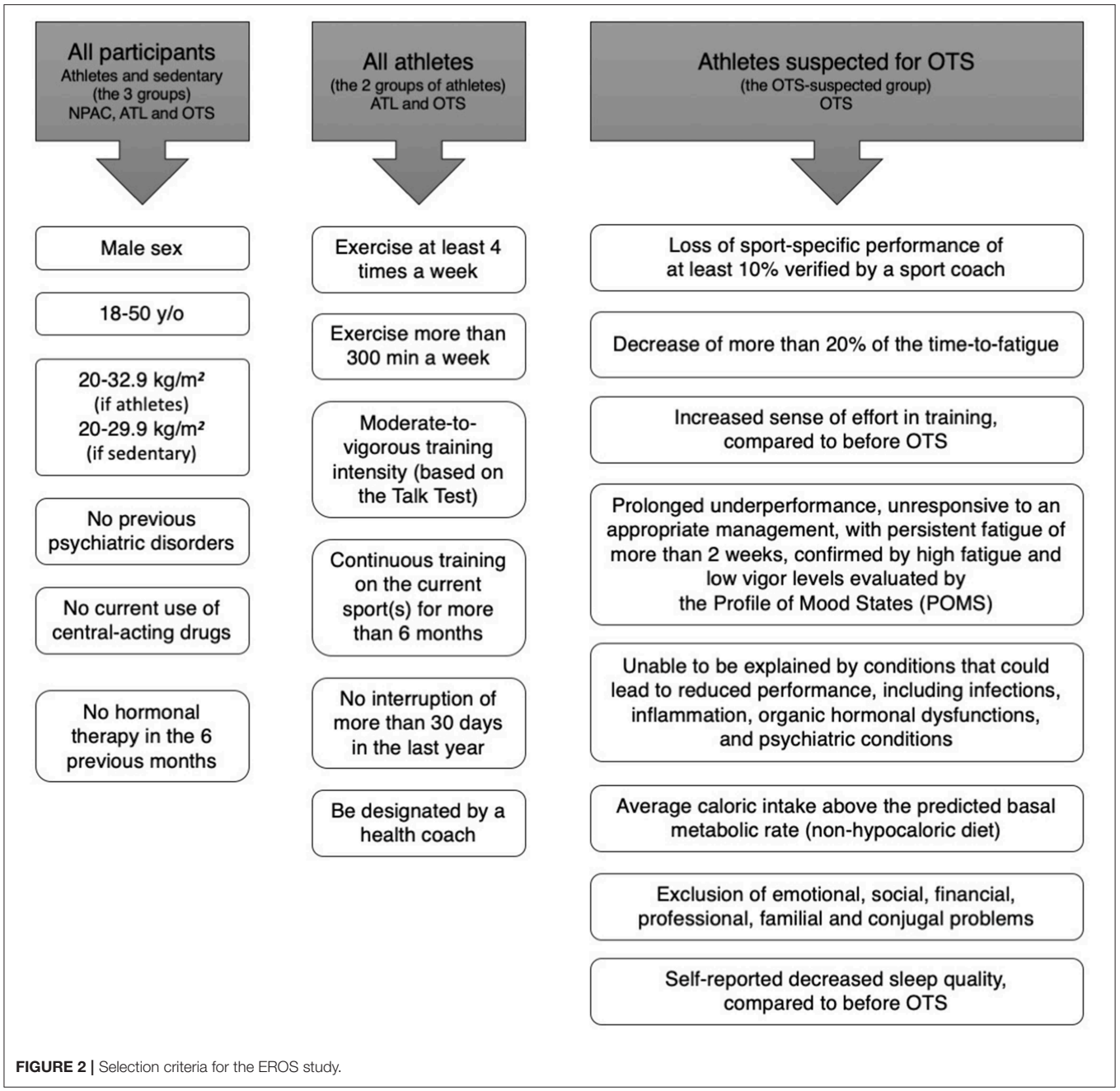

FIGURE 2 | Selection criteria for the EROS study.

their self-reported libido, from zero to ten (zero = no sex drive; ten = astonishing sex drive), compared to 1 year before, during the training periods.

With regards to the psychological characteristics, we employed the Profile of Mood States (POMS) questionnaire with the overall and specific mood scales: (1) total score (from −32 to +200; where −32 is the best score and +200 the worst score), (2) anger (from 0 to 48), (3) confusion (from 0 to 28), (4) depression (from 0 to 60), (45 fatigue (from 0 to 28), (6) tension (from 0 to 36), and (7) vigor (from 0 to 32; vigor score is counted as negative for the total POMS score) subscales. In a different moment of the interview, we also evaluated specific self-reported feelings of (8) general well-being, (9) friendly, (10) worthless, (11) miserable, (12) helpful, (13) bad-tempered, (14) guilty, (15) unworthy, (16) peeved, (17) cheerful, (18) sad, and (19) fatigue [from zero (not fatigued at all) to ten (extremely fatigued)].

The POMS questionnaire and the specific feeling questions were performed by only one author (FAC), in an impartial way, with a constant voice and standardized words of each question in order to prevent "faking good" in ATL and "faking bad" in healthy and OTS-affected athletes, respectively. The RESTQ questionnaire, also used to evaluate athletes,

although not validated for non-physically active individual, was not employed, once NPAC were also evaluated as a second group control.

Basal Tests

Between 36 and 48 h after the last training session (in the case of the groups of athletes), we collected basal fasting levels of the following parameters: CRP; ESR; creatinine; hematocrit, medium corpuscular volume, and numbers of neutrophils, lymphocytes, eosinophils, and platelets (automated assays); CK; ferritin; high-density lipoprotein-cholesterol and triglycerides (calorimetric enzymatic assays) and low-density lipoprotein-cholesterol (Friedewald equation); **serum** lactate (enzymatic assays); serum total testosterone; estradiol (chemiluminescence assay); serum IGF-1 (chemiluminescence assay); nocturnal 12-h urinary catecholamines and metanephrines (calorimetric enzymatic assays); serum free thyronine (fT3; electrochemiluminescence assay); and serum TSH (electrochemiluminescence assay).

We then calculated the testosterone-to-estradiol, testosterone-to-cortisol, catecholamines-to-metanephrines, neutrophil-to-lymphocyte, and platelet-to-lymphocyte ratios, and compared them between the groups.

Hormonal Functional Tests

The CST, ITT, and SCR were performed in all participants, in a specific sequence.

Cosyntropin stimulation test (CST)

In the first day, we performed a stimulation test with a high doses (250 μg) of cosyntropin, a synthetic adrenocorticotropic hormone (ACTH), in order to hormonal responsiveness of the adrenal glands.

For the CST, at 8.00 a.m. (after 30-min resting and 8-h fasting) blood was collected (time 0) from the antecubital vein of the participants for serum cortisol. Immediately, 250 μg of cosyntropin was infused intravenously, slowly (during 30 s), and blood was collected at 30 min (time 1) and 60 min (time 2) for the analysis of the cortisol increase, in absolute levels [μg/dL], in response to a synthetic ACTH.

Insulin tolerance test (ITT)

Forty-eight hours after the CST, we then performed an ITT, to evaluate the integrity of the HPA, GH, and prolactin axes, once a normal response required absolute unaltered functions in all levels (hypothalamus, pituitary, and adrenals or other glands) of the axes. This is an intrinsic and independent test of the hormonal responsiveness, without interferences from external signaling or systems.

Participants followed the same protocol of at least 8-h fasting, arrival time before 7.30 a.m. and a 30- resting period prior to the beginning of the ITT. Although participants had signed the written consent and were fully aware of the risks of an ITT, before the beginning of the ITT we reminded them of the potential side effects derived from a state of hypoglycemia purposely induced by the test. After agreeing, blood was collected (time 0), and a dose of 0.1 IU/kg of regular insulin was infused in bolus. When hypoglycemia was detected, blood was collected (time 1—during hypoglycemia), 10 mL of 50% glucose solution was given intravenously, and high-glycemic index food was offered *ad libitum* (fat free ice-creams, Diletto, São Paulo, Brazil), blood was finally sampled again, 30 min (time 2) and 60 min (time 3) after the hypoglycemic episode.

The criteria for the detection of the hypoglycemia for the collect of the blood at time 1 was: (1) Asymptomatic hypoglycemia, when capillary glucose was below 30 mg/dL; (2) Moderate-to-intense adrenergic (cold sweating, shakiness, pallor, heart palpitations) and/or neuroglycopenic (mood changes, unrest, sleepiness) symptoms (a score of 5–10, from a zero to ten scale), regardless of the glucose levels; and (3) Capillary glucose below 45 mg/dL associated with any adrenergic or neuroglycopenic symptom.

Serum glucose (mg/dL), cortisol (μg/dL), ACTH (pg/mL), GH (μg/L), and prolactin (ng/mL) were collected at all times. During the ITT, time-to-hypoglycemia (minutes since the insulin infusion), and level of intensity of adrenergic and neuroglycopenic symptoms (zero to ten, self-reported) were also evaluated during the ITT. Absolute increase of cortisol, prolactin, GH, and ACTH, as well as the ACTH/cortisol ratio at all times were calculated. Among these hormones, we adjusted GH for body composition, since GH release is negatively influenced by body fat.

Given the actual risk of ITT-induced severe hypoglycemia (loss of consciousness), three doses of subcutaneous glucagon (GlucaGen HypoKit, 1 μg, NovoNordisk), syringes containing 20 mL of 50% glucose solution and an automated external defibrillator (AED) were available.

Salivary cortisol rhythm (SCR)

Between 2 and 7 days after the ITT, we collected the SCR, including the collect of the saliva at the awakening moment, at 30 min after awakening, at 4 and at 11 p.m. which were collected by the participants themselves, using laboratory kits provided by the researcher (FAC). Specific recommendations for the self-collect of the samples were provided.

All hormones from the functional tests were analyzed by specific electrochemiluminescence assays using specific commercial kits (Roche), while serum glucose was analyzed by an enzymatic assay of hexokinase.

All biochemical data were determined using standardized commercial assay kits ((18–21), https://osf.io/bhpq9). The inter- and intra-assay coefficients of variability were lower than 3.5 and 3%, respectively.

Body Composition and Metabolism

On a different day from the CST, ITT, or CST, previously scheduled, and after at least 24 h of the last training session (for OTS and ATL), we performed the evaluation of body composition using a gold-standard air-displacement pletimosgraph (Bod Pod, CosMed, USA) for analysis of body fat in terms of weight (kg) and percentage (%), and a validated and standardized electrical bioimpedance scale (InBody770, Biospace, South Korea) for analysis of visceral fat (%), muscle mass (kg), percentage of lean mass (%), body water (liters), percentage of body water (%), and percentage of extracellular water (%).

We then measured chest, biceps, and waist circumferences using a standardized and highly accurate pro-body- scanner and (Styku, USA). An indirect calorimetry (Spirostik, Geratherm Respiratory, Germany) was performed to evaluate basal metabolic rate (BMR) (kcal/day), the measured-to-predicted BMR ratio (%), after adjustments for age, weight, height and sex, and fat oxidation in relation to total metabolic rate (%).

Selection of the Markers for the Analysis of Associations, Predictions, and Correlations Between Markers

We initially excluded 48 of the 117 markers present in the EROS study, by excluding those that were intrinsically linked to other parameters (seven markers), did not determine diagnoses or status (16 markers), were qualitative indices (nine markers), markers that did not provide additional independent data (two markers), markers that were not the behavioral consequences of exercise (four markers), and invalid and/or unsubstantiated data (nine markers) (**Table 1**). We then selected the 38 from the 69 remaining markers that yielded significant differences between the two groups of athletes: OTS-affected athletes and healthy athletes (34 markers), or that were significantly different between these groups and sedentary controls ($p < 0.05$) with trends to be significantly different between healthy and OTS-affected athletes ($p < 0.1$) (four markers).

Statistical Analysis

We used multivariate linear regression to analyze all possible combinations of the 38 parameters that were evaluated in the EROS study (18–21). The intent was to identify: (1) clinical or biochemical markers (including hormonal, metabolic, and body metabolism and composition markers) as independent predictors of other markers and (2) strong linear correlations among the parameters evaluated in the EROS study (18–21). As the identification of triggers and the influence of OTS were not evaluated in the present study, they were excluded from the analyses.

We used multivariate linear regression with a removal criterion of $p > 0.01$. The standardized residual variables of the last model were examined for normality and homoscedasticity. The cut-off for the presence of multicollinearity was a tolerance index of 0.40^3 for the variables in the last model. A p-value < 0.05 was considered statistically significant for independent predictors, and $p < 0.01$ for linear correlations (with >0.40 correlation coefficient), which we considered to unveil moderate-to-strong correlations.

Although $r > 0.4$ is generally considered to be of moderate association, there is no rule or universally accepted sizes of correlation to be considered as weak, moderate or strong. Since we studied entirely different biological aspects, and each of these aspects is extensively influenced by a large number of different predictors from different natures, it is unlikely to find a single linear correlation > 0.5 (> -0.5), since each parameter tends to be driven by multiple factors. Hence, in this particular case, according to the literature, a correlation > 0.4 is sufficient to be considered as a strong correlation (25–27), or at least moderate-to-strong.

The p-value for the linear correlations was lower and partial correlations were not considered to avoid incidental misinterpretative correlations. Analyses were performed using SAS 9.4 (SAS Institute, Inc., Cary, NC, USA).

Parameters that were independently influenced by OTS, as published in the EROS-DISRUPTORS study (24), were adjusted according to the level of influence of OTS, in order to homogenize the groups of athletes. The biological behaviors that were modified by the presence of OTS include: (1) cortisol 30 min after hypoglycemia, in response to an ITT (26.1% of influence by OTS); (2) cortisol increase during ITT (22.0%); (3) GH 30 min after hypoglycemia, in response to an ITT (23.0%); (4) testosterone-to-estradiol (T:E) ratio (30.7%); (5) neutrophils (13.8%); (6) neutrophil-to-lymphocyte ratio (13.6%) (7) Profile of Mood States (POMS) vigor subscale (83.6%); (8) POMS fatigue subscale (85.7%); (9) POMS tension subscale (42.8%); (10) muscle mass (33.7%); (11) body water (50.5%), and (12) visceral fat (38.2%).

Those parameters that were not modified by the presence of OTS did not require adjustments according to the population (if OTS-affected or if healthy athletes), since these markers behaved independently from OTS.

Correlations that were unlikely to have any biological plausibility were excluded.

Further information on the material, methods, individualized results, and the raw data are provided at a repository (https://osf.io/bhpq9/).

RESULTS

Primary Results

All sub-groups had similar age, BMI, and training patterns. As per selection criteria, all 34 parameters were significantly different between OTS-affected and healthy athletes. The primary results of these markers are detailed in **Table 2**.

From the primary findings, further statistical analyses were employed, including the analyses for predictions and linear correlations, which are presented in **Table 3** and **Figure 3** [when >0.40 ($p < 0.01$)], respectively.

Independent Predictors of Clinical, Metabolic, and Biochemical Biomarkers

None of the hormonal responses to the CST of the adrenal glands or the ITT predicted or was predicted by any of the other clinical or biochemical markers.

Conversely, among the basal hormones, the testosterone-to-estradiol (T:E) ratio, identified in the EROS study as a better predictor of performance and overall status than total testosterone or estradiol alone (21), positively predicted the measured-to-predicted BMR ratio (**Figures 4A,B**), as well as the chest-to-waist circumference ratio. Total testosterone was inversely predicted by fat mass (**Figures 4C,D**), and estradiol inversely predicted anger, both fat mass and anger mood were also influenced by the presence of OTS (24).

Body water content positively predicted fat oxidation (**Figures 4E,F**) and chest-to-waist circumference was inversely predicted by visceral fat (in addition to the T:E ratio)

TABLE 2 | Primary results of the markers selected for the present analysis.

Tests	OTS-affected athletes	Healthy athletes	Significance
BASAL BIOCHEMICAL TESTS			
Total testosterone (ng/dL)	422 (±173.2)	540.3 (±171.4)	$p = 0.008$
Estradiol (pg/mL)	40.1 (±10.8)	29.8 (±13.9)	$p = 0.001$
Total catecholamines (μg/12 h)	257 (±66)	175 (±69)	$p = 0.015$
Dopamine (μg/12 h)	227 (±159)	149 (±60)	$p = 0.01$
Salivary cortisol (ng/dL) 30 min after awakening	324 (±116)	500(±168)	$p = 0.005$
Lactate (nMol/L)	1.11(0.79 to 2.13)	0.78 (0.47-1.42)	$p = 0.003$
Neutrophils (mm^3)	2986 (±761)	3809 (±1431)	$p = 0.022$
Creatine kinase (CK, U/L)	569 (126 to 3012)	347 (92 to 780)	$p = 0.038$
Testosterone-to-oestradiol ratio	10.8 (±3.7)	20.8 (±9.9)	$p < 0.001$
Neutrophil-to-lymphocyte ratio	1.23(±0.34)	2.00 (±1.28)	$p = 0.008$
Lymphocyte (mm^3)*	2498 (±760)	2154 (±640)	*(NPAC = 2819±810) $p = 0.03$ (overall), $p = 0.018$ (NPAC x ATL) and $p = 0.224$ (NPAC x OTS)
Platelet-to-lymphocyte ratio*	104.1 (±34.2)	119.1 (±43.4)	*(NPAC = 82.4±19.5) $p = 0.017$ (overall), $p = 0.003$ (NPAC x ATL) and $p = 0.102$ (NPAC x OTS)
HORMONAL FUNCTIONAL TESTS			
Basal GH (μg/L)	0.1 (0.05 to 0.87)	0.26 (0.1 to 1.26)	$p = 0.007$
Basal prolactin (ng/mL)	9.2 (5.27 to 19.46)	12.1 (7.19 to 23.0)	$p = 0.048$
Cortisol during hypoglycaemia (μg/dL)	12.4 (±3.3)	15.9 (±5.3)	$p = 0.022$
GH during hypoglycaemia (μg/L)	0.4 (0.05 to 4.68)	2.5 (0.08 to 40.94)	$p = 0.012$
Prolactin during hypoglycaemia (ng/mL)	8.95 (4.72 to 47.22)	17.85 (10.0 to 63.39)	$p < 0.001$
ACTH 30 min after hypoglycemia (pg/mL)	30.3 (9.8–93.7)	59.9 (22.1 to 195.7)	$p < 0.001$
Cortisol 30 min after hypoglycemia (μg/dL)	17.9 (±2.9)	21.7 (±3.1)	$p < 0.001$
GH 30 min after hypoglycemia (μg/L)	1.28 (0.03 to 13.95)	12.73 (1.1 to 38.1)	$p < 0.001$
Prolactin 30 min after hypoglycemia (ng/mL)	11.35 (4.5 to 25.88)	24.3 (10.5 to 67.45)	$p < 0.001$
ACTH response to ITT (pg/mL)	9.7 (−14.4 to +64.4)	45.1 (22.1 to 195.7)	$p < 0.001$
CLINICAL PARAMETERS			
Self-reported libido (0–10)	6.2 (±2.1)	8.3 (±1.7)	$p = 0.004$
POMS questionnaire (Total score: −32 to +120)	+54.5 (−14.8 to +89.2)	−9.0 (−23.4 to +17.2)	$p < 0.001$
Anger subscale (0 to 48)	15.0 (4.0 to 21.0)	5.0 (0.2 to 15.0)	$p = 0.003$
Confusion subscale (0 to 28)	5.0 (1.6 to 17.1)	2.00 (0.0 to 5.0)	$p = 0.001$
Depression subscale (0 to 60)	7.5 (0.0 to 21.4)	0 (0.0 to 5.0)	$p = 0.008$
Vigor subscale (0 to 32)	9.5 (3.6 to 20.1)	26.0 (21.2 to 28.0)	$p < 0.001$
Fatigue subscale (0 to 28)	20.0 (9.3 to 26.7)	2.0 (0.0 to 4.8)	$p < 0.001$
Tension subscale (0 to 36)	16.5 (3.6 to 20.1)	6.0 (1.0 to 14.4)	$p < 0.001$
BODY PARAMETERS			
Measured-to-predicted basal metabolic rate (BMR, %)	102.6 (±8.3)	109.7 (±9.3)	$p = 0.012$
Percentage of fat burning compared to total BMR (%)	33.5 (±21.0)	58.7 (±18.7)	$p < 0.001$
Body fat percentage (%)	17.0 (±6.0)	10.8 (±4.2)	$p < 0.001$
Muscle mass percentage (%)	47.2 (±3.8)	50.5 (±2.3)	$p = 0.008$
Body water percentage (BW, %)	59.5 (±3.9)	64.7 (±2.7)	$p < 0.001$
Visceral fat (cm^2)	67.5 (±36.5)	35.7 (±20.6)	$p = 0.01$
Extracellular water compared to total BW (%)*	20.1 (±12.0)	21.8 (±11.8)	*(NPAC = 33.1±16.7) $p = 0.022$ (overall), $p = 0.019$ (NPAC x ATL) and $p = 0.083$ (NPAC × OTS)
Chest-to-waist circumference ratio*	1.276 (±0.068)	1.249 (±0.062)	*(NPAC = 1.157±0.069) $p = 0.001$ (overall), $p = 0.0005$ (NPAC × ATL) and $p = 0.002$ (NPAC × OTS)

These parameters were included because although p-value is above 0.05 between OTS-affected and healthy athletes, this is below 0.1, were significantly different between athletes (both groups) and sedentary ($p < 0.05$), and has potential physiopathological and clinical significance.

BW, Body Weight; POMS, Profile of mood states; BMR, Basal metabolic rate; T/E, Testoterone-to-estradiol ratio; OTS, Overtraining syndrome.

(**Figures 4G,H**), which was also predicted by OTS (24). None of the other psychological parameters, muscle mass, other biochemical parameters, or SCR independently predicted or were predicted by any of the other factors evaluated in this study.

Markers not listed in **Table 3** were not independent predictors of other clinical or biochemical behaviors.

Linear Correlations

Although none of the hormonal responses to the ITT predicted or was predicted by other parameters, both early and late growth hormone (GH), cortisol, and prolactin responses to the ITT were similar and had strong correlations (**Figures 5A–F**). These hormonal responses were positively correlated with vigor, fat

TABLE 3 | Clinical or biochemical parameters as independent predictors of other parameters (multivariate linear regression analysis).

Parameter	*p* of the influence of the modifiable variables	Level of influence by the modifiable variables*	Parameters with significant correlations (and *p*-value)	Equation for the estimation of the parameter level in male athletes
Total testosterone (ng/dL)	0.0415	8.4%	1. Fat mass (inverse) ($p = 0.0415$)	Testosterone (ng/dL) = 631.77–10.29 × (fat mass)
POMS anger subscore	0.0006	34.7%	1. Estradiol (inverse) ($p = 0.008$) 2. Presence of OTS (direct) ($p = 0.003$)	POMS anger subscore = 25.43–0.24 × (estradiol)−0.24(T:E ratio) + 6.97(if OTS)
Measured-to-predicted BMR (%)	0.026	10.9%	1. T:E ratio (direct) ($p = 0.026$)	BMR ratio (%) = 100.8 + 0.35(T:E ratio)
Fat oxidation (% of total BMR)	<0.0001 (together with extra-activities)	58.8%	1. Body water (%) (direct) ($p = 0.001$)	Fat oxidation = −66.96 + 2.30x(body water) + 0.51 × (T/E ratio)−4.99 × (extra activities)
Chest-to-waist circumference ratio	0.0003	37.8%	1. Visceral fat (inverse) ($p < 0.0001$) 2. T:E ratio (inverse) ($p = 0.038$)	Ratio = 1.362–0.012 × (visceral fat)−0.02 × (T:E ratio)

**Adjusted R-Square.*
POMS, Profile of mood states; BMR, Basal metabolic rate; T/E, Testosterone-to-estradiol ratio; OTS, Overtraining syndrome.

oxidation, the predicted-to-measured BMR ratio, and muscle mass, and they were inversely correlated with fatigue and fat mass. The correlations between late prolactin response to the ITT (30 min after hypoglycemia) and relative BMR (% of predicted), and between late cortisol response to the ITT (30 min after hypoglycemia) and body fat are shown in **Figures 6A,B**, respectively.

Salivary cortisol 30 min after awakening and the T:E ratio were inversely correlated with fatigue, whereas total testosterone was inversely correlated with the Profile of Mood States (POMS) total score (a negative score indicates a better mood), and it was directly correlated with sleep quality. Immunologic parameters were correlated with body composition: body fat was directly correlated with lymphocytes and inversely correlated with the platelet-to-lymphocyte ratio, whereas body water and muscle mass were correlated with these variables in the opposite directions.

Libido and sleep quality were directly correlated with vigor, while sleep quality was directly correlated with libido and inversely correlated with depression, fatigue, and overall mood. Vigor was directly correlated with body water and fat oxidation, and inversely correlated with body fat; conversely, tension was inversely correlated with body water, muscle mass, and fat oxidation, and directly correlated with body fat.

The predicted-to-measured BMR ratio and fat oxidation had a stronger positive correlation with muscle mass and body water, whereas an inverse correlation was found between fat oxidation and body fat. Even though body fat and muscle mass were strongly and inversely correlated ($r > -0.95$), they were not always correlated in the opposite direction. In addition to fat oxidation, body water was the only parameter directly correlated with muscle mass, and inversely correlated with body fat. Although muscle mass was directly correlated with late GH and cortisol responses to the ITT, extracellular water (i.e., the presence of edema) and body fat were directly correlated with the POMS total score and inversely correlated with vigor.

Though the chest-to-waist circumference ratio was directly correlated with body water, muscle mass, and fat oxidation, and inversely correlated with body fat, extracellular water was correlated with these variables in the opposite directions (**Figure 6C**). The parameters that are not mentioned in this report failed to show strong correlations (>0.40).

Linear correlations not presented in **Figure 3** were weaker than <0.40 ($p > 0.01$).

DISCUSSION

The EROS Study and the Present Analysis

The EROS study elucidated some of the physiological adaptive changes that occur in healthy athletes and how these changes are disrupted in OTS (18–24), as this study addressed the major methodological issues in studies of healthy and OTS-affected athletes, using concurrent comparisons between sex-, age-, and BMI-matched healthy athletes and non-athletes, and simultaneous comparisons of a broad range of aspects within the same participants. The prior results for the two groups analyzed here (healthy and OTS-affected athletes) showed multiple clinical, metabolic, and biochemical conditioning processes in the healthy athletes and a loss of 59.1% of these conditioning processes in the OTS-affected athletes (23), which was referred to as "a mix of paradoxical deconditioning processes."

The concomitant analysis of different biochemical, clinical, and metabolic aspects in the EROS study allowed us to explore the previously uninvestigated interactions, correlations, predictions, and synergistic actions between these parameters, using multivariate regression and other statistical techniques. Therefore, the EROS-CORRELATIONS study analyzed two major sorts of interactions among basal and stimulated hormonal, metabolic, immune, and muscular biomarkers, body composition and metabolism, and psychological patterns: independent predictors and linear correlations, as well as their mechanisms and outcomes.

Noteworthy, some markers previously hypothesized to be potential biomarkers for OTS yielded similar results between OTS and healthy athletes, including the testosterone-to-cortisol ratio (21) and the insulin growth factor-1 (IGF-1) (21). These

markers also failed to demonstrate any sort of relationship with other markers, as shown in the raw statistical analysis (https://osf.io/bhpq9).

Biochemical Responses as Predictors of Other Biochemical and Clinical Behaviors

Although fat mass is considered an independent suppressor of the GH response (28), this was not confirmed by our results, in that the body fat of male athletes was not important for the GH response. Although body fat did not have a negative effect on GH release, it reduced testosterone levels; however, the reduction was not due to increased aromatase activity, as the T:E ratio was not reduced by body fat or affected by any of the other markers. Conversely, the T:E ratio positively predicted the measured-to-predicted BMR ratio while neither testosterone nor estradiol had the same effect; the T:E ratio also predicted the chest-to-waist circumference ratio the measure of the torso's "V-shape." Our findings underscore the importance of evaluating the ratios of different hormones for the prediction of metabolic outcomes. Although an increase in estradiol without a concurrent analysis of testosterone does not necessarily suggest either a beneficial or a harmful outcome, in this study, testosterone level did not predict any of the parameters, such as body metabolism or composition, without the simultaneous analysis of estradiol. These findings are consistent with studies suggesting a simultaneous increase in testosterone and estradiol has synergistic positive effects, which include metabolic parameters (29–33). Conversely, fat mass reduced testosterone levels, which can be justified by exposure of testosterone to a more intense aromatase enzyme under higher body fat (31, 32).

Although estradiol alone did not predict body metabolism or composition parameters, it independently predicted lower anger. Although estradiol receptors are widely distributed in the brain (29, 33, 34), their effects on mood in males are still unclear. Body water content was the only predictor of fat oxidation, which supports the premise that good hydration status, particularly within the cells, is a key requirement for proper fat oxidation, as water is part of this pathway (35–37), while dehydration slightly impaired fat burning.

Linear Correlations

Strong inter-correlations were found among GH, prolactin, and cortisol for early and late responses to stimulation tests. Therefore, it was impossible to distinguish levels of responsiveness among the corticotropic, somatotropic, and lactotropic axes, as these hormones responded simultaneously and equally, which indicates a common, ubiquitous, and enhanced hypothalamic responsiveness in athletes, compared to sedentary controls. Although ACTH was not strictly correlated with the other hormones, its short half-life precluded drawing conclusions about its correlation with other hormones.

Late hormonal responses (30 min after hypoglycemia) were directly correlated with energy levels (higher vigor and lower fatigue levels), indicating a possible role for sustained hormonal release in response to stress in the prevention of burnout in chronically stressful situations. They were also correlated with increased muscle mass, lower body fat, and better hydration, without a distinction between the specific effects of each hormonal response (cortisol, GH, and prolactin). Acute cortisol and GH release promote fat oxidation and lipolysis, but chronic hypercortisolism may lead to the accumulation of visceral and central fat, even when it is mild.

Overall, the hormonal conditioning process that was found to occur in athletes, and identified by enhanced GH, prolactin and cortisol responses to exercise-independent stimulation tests, when compared to sex-, age-, and BMI-matched healthy sedentary controls, and adjusted for body composition, may be one of the underlying reasons for decreased extracellular water, decreased anger, fatigue, depression, confusion mood states, and indirect account for reduced fat, increased muscle, and better hydration (38). Indeed, under OTS, in which the optimization of the hormonal responses is lost (24), the concurrent benefits in response to intense exercise are also lost.

The inverse correlation between salivary cortisol 30 min after awakening and fatigue supported this parameter and the cortisol awakening response as markers of fatigue, as previously demonstrated by different studies (39–42). Indeed, cortisol awakening response (CAR), an indirect marker of cortisol 30 min after awakening, has been extensively used as a marker of fatigue (43–46). Nonetheless, they were not independent predictors of fatigue when analyzed using multivariate regression. Therefore, a lower increase in cortisol level between awakening and 30 min after awakening is unlikely to be the primary cause of fatigue, but rather a possible consequence of poor sleep quality leading to an impaired cortisol awakening response (47), with a blunted cortisol increase 30 min after awakening and a concurrent decrease in energy level (48–50), as well as other disruptions of the HPA axis (50). Thus, conclusions regarding adrenal function using these tools are inappropriate (51).

Testosterone level was directly correlated with sleep quality. Although this does not indicate a causal relationship, it supports the role of sleep quality in testosterone production because its' physiological peak also occurs in the early morning hours, which is affected by sleep quality (52–54).

The T:E ratio was also inversely correlated with fatigue, which implies that the ratio was a better predictor of energy level than either testosterone or estradiol alone. While estradiol alone was not linked to fatigue, its pathological increase, from an exacerbation of aromatase activity and consequent reduction of testosterone, may have increased fatigue, in accordance with the literature (32).

In the EROS study, although sleep duration did not have a role in psychological function, unlike previous studies, that strongly correlated sleep duration with mood states (49, 55, 56). Sleep quality was strongly correlated with better psychological outcomes, including lower depression and fatigue, and higher vigor, indicating that sleep quality led to a global improvement in mood, similarly to what has been unanimously observed (49, 55, 57–59). We speculate that sleep duration was not demonstrated to a major factor of mood states or any other characteristic in athletes because in higher quality sleep duration tends to play a less important role (60, 61).

Other moderate-to-strong linear correlations were identified. Body water was inversely correlated with lymphocyte and

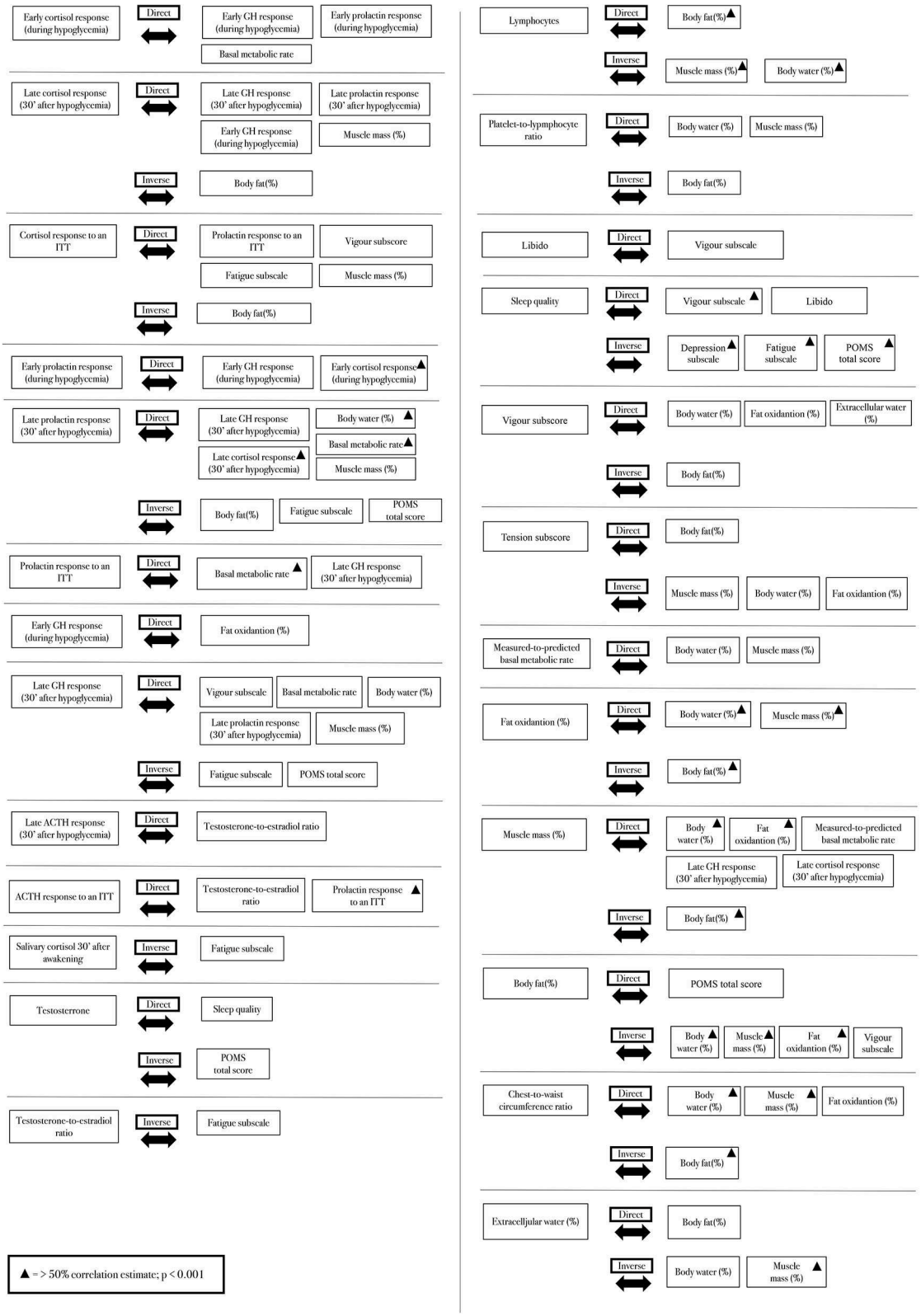

FIGURE 3 | Strict correlations (>0.40) between clinical, hormonal, psychological, and metabolic parameters.

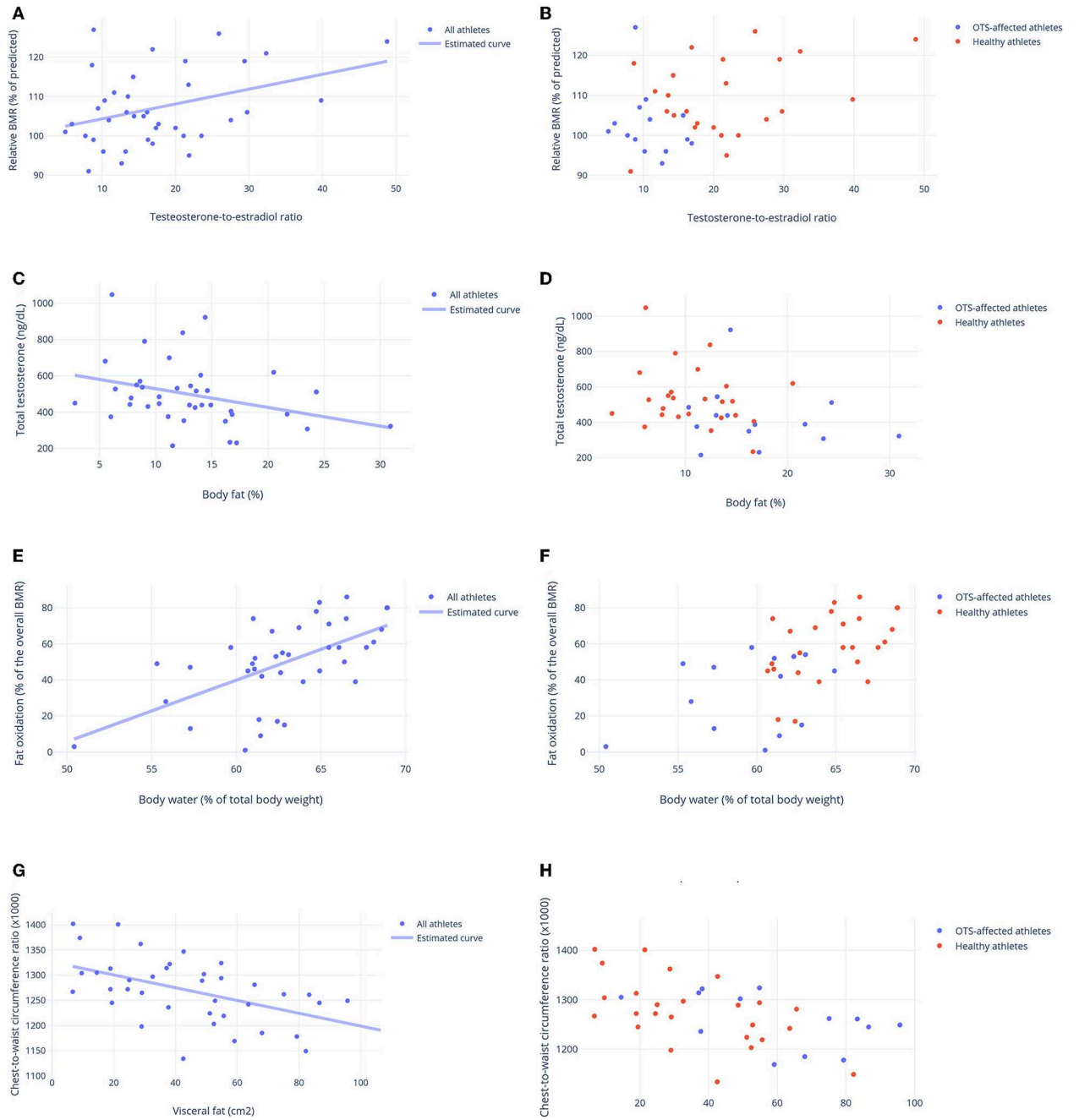

FIGURE 4 | Biological predictors of other clinical, metabolic and biochemical parameters. **(A,B)** Testosterone-to-estradiol (T:E) ratio as a predictor of basal metabolic ratio (BMR) (relative to the predicted BMR, in %). Estimated equation: BMR ratio (%) = 100.8 + 0.35(T:E ratio)–r = 0.33. **(C,D)** Body fat (%) as a predictor of total testosterone (ng/dL) Estimated equation: Testosterone (ng/dL) = 631.77–10.29 × (fat mass-%)–r = 0.29. **(E,F)** Level of hydration (body water, in % of body weight) as a predictor of fat oxidation (% of total BMR) Estimated equation: Fat oxidation = −66.96 + 2.30 × (body water) + 0.51 × (T/E ratio)−4.99 × (extra activities)–r = 0.77. **(G,H)** Visceral fat (cm^2) as a predictor of chest-to-waist circumference ratio. Estimated equation: Ratio = 1.362–0.012 × (visceral fat)−0.02 × (T:E ratio)–r = 0.62. **(A,C,E,G)** Estimated curve for all athletes, adjusted for OTS, when needed. **(B,D,F,H)** Results for athletes of OTS and healthy groups. Each point represents the result of one athlete.

directly correlated with platelet-to-lymphocyte ratio. However, little data has been identified in the literature, specifically for these correlations (62–69), since immune function and hydration has been more assessed in athletes, in response to exercises (67–69). Specific absolute and lymphocytes subpopulation counting have been assessed in athletes and in patients at high cardiovascular risk, with indirect but inconsistent correlations between lymphocytes and hydration

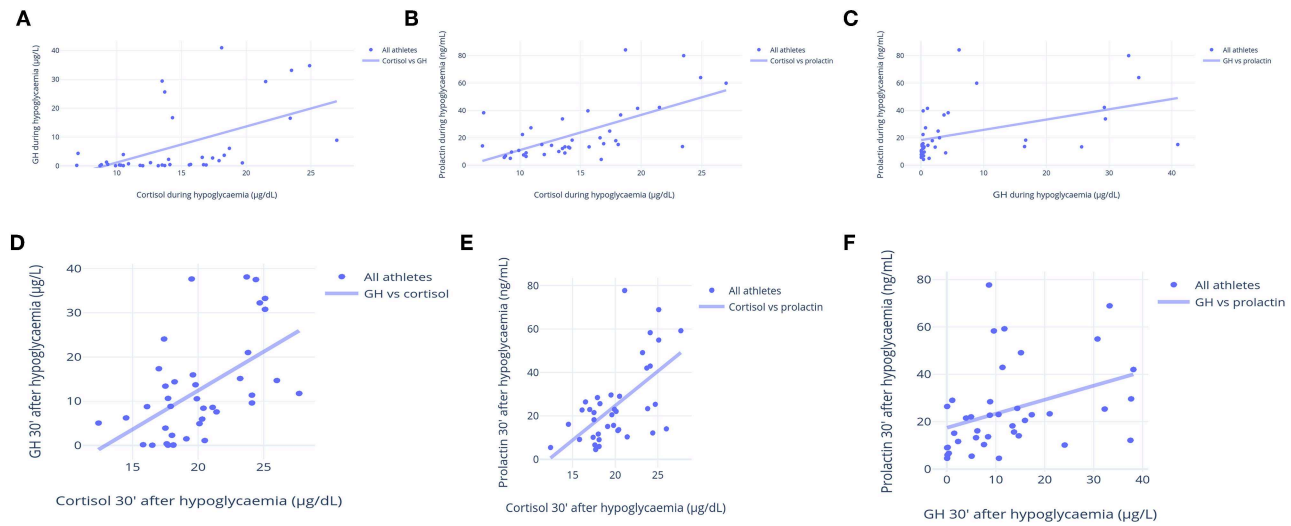

FIGURE 5 | Correlations between cortisol, GH and prolactin responses to an insulin tolerance test (ITT). **(A)** Early cortisol (µg/dL) and GH (µg/L) responses to ITT (during hypoglycemia) ($R = 0.72$). **(B)** Early cortisol (µg/dL) and prolactin (ng/mL) responses to ITT (during hypoglycemia) ($R = 0.80$). **(C)** Early GH (µg/L) and prolactin (ng/mL) responses to ITT (during hypoglycemia) ($R = 0.69$). **(D)** Late cortisol (µg/dL) and GH (µg/L) responses to ITT (during hypoglycemia) ($R = 0.77$). **(E)** Late cortisol (µg/dL) and prolactin (ng/mL) responses to ITT (during hypoglycemia) ($R = 0.76$). **(F)** Late GH (µg/L) and prolactin (ng/mL) responses to ITT (during hypoglycemia) ($R = 0.69$). Each point represents the result of one athlete.

status (70–75). Platelet-to-lymphocyte ratio has been proposed as an inflammatory marker of cardiovascular risk, acute pancreatitis and sarcopenia (76–80), with some speculations that higher ratio could indicate better hydration status, similarly to the direct linear correlation that we found. This occurs perhaps due to a direct correlation between platelet count and total body water (65, 66, 81), although this associations remain controversial (64–68).

Associations Between Improved Mood States and Body Composition and Metabolism

Vigor was directly correlated with better hydration, better fat oxidation, and less body fat, whereas libido was exclusively correlated with vigor, indicating improved overall body metabolism and composition, hydration, and fat oxidation. Since vigor has also been correlated with enhanced hormonal responses to stimulations and better sleep quality (24) in the present study, both of which have also been correlated with improved body metabolism and composition patterns, we hypothesize that vigor is an additional consequence of hormonal response and sleep quality, similarly to body composition and metabolism, and has no direct correlations of causal relationship with these parameters.

Conversely, while other moods were not significantly correlated with body metabolism or composition, tension had correlations in the opposite direction than those found for vigor. Oppositely to vigor, which unlikely leads to changes in hormonal responses, tension is likely the mood mostly correlated with disrupted hormonal responses (50). Once in our study tension was the mood state most strictly predicted by sleep quality, and that Impaired sleep independently leads to impaired muscle recovery (62), this may justify the correlations between vigor (positive) and tension (negative) and body muscle identified by the EROS study.

Also, although oxidative stress leads to muscle hypertrophy, chronic stress mediated by the HPA axis, leads to the opposite direction (82–85). Indeed, alterationsof the HPA axis disrupt the metabolism of the muscle tissue, toward a negative balance between protein generation and degradation, eventually leading to muscle mass loss (86–89). Conversely, impaired HPA axis also leads to concurrent independent body fat gain (84), as multiple mechanisms mediated by the HPA axis induce increase of fat cell size and induce a pro-inflammatory status irrespective of caloric balance, proportion of macronutrient intake, and sleeping patterns, since a post-receptor modification from cortisone to cortisol by enhanced activity of 11beta-hydroxysteroid dehydrogenase type 1(11beta-HSD1) (90, 91). In addition,disruption of the muscle metabolism, herein induced by altered cortisol regulation, has also demonstrated to have direct effects on the metabolism and accumulation of fat (92). Since the HPA axis is chronically and more severely enhanced by high tension levels, more than any other mood state, it would be expected, from this perspective, to find negative correlations between tension levels and body composition. Indeed, higher tension, which have been correlated with impaired hydration and fat oxidation, muscle catabolism, and increased body fat, are possibly due to the harmful metabolic effects of the chronic stimulation of the HPA axis (82–85), although direct effects of tension, depression, and anger on increased body fat has been observed (86).

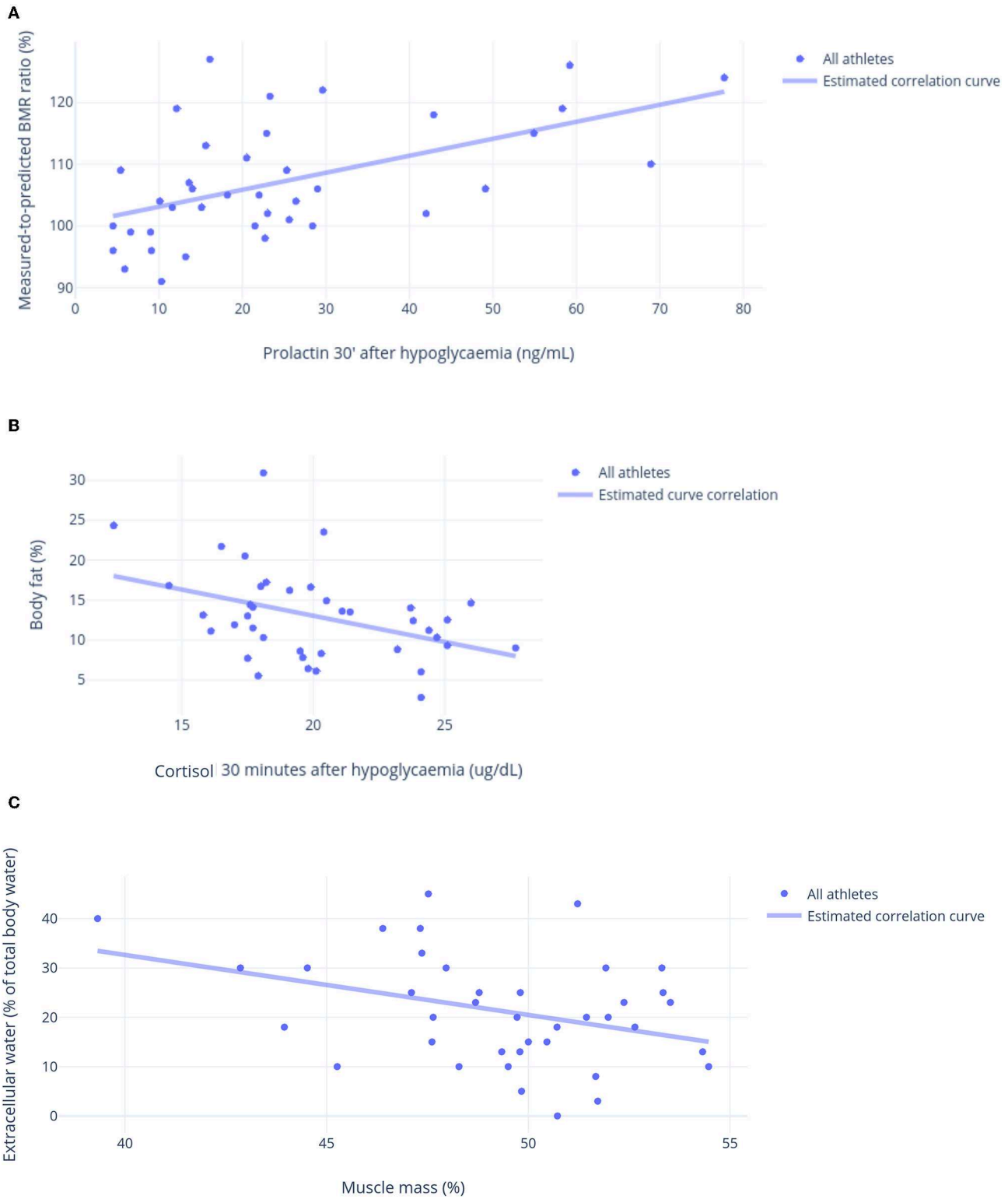

FIGURE 6 | Other linear correlations. **(A)** Prolactin 30 min after hypoglycemia in response to an insulin tolerance test (ITT) (ng/mL) and relative basal metabolic ratio (% of predicted) ($R = 0.71$). **(B)** Cortisol 30 min after hypoglycemia in response to an insulin tolerance test (ITT) (μg/dL) and body fat (%) ($R = 0.74$). **(C)** Muscle mass (%) and extracellular water (% of body water) ($R = 0.72$).

In additional, overall mood states can hypothetically be indirect signals of hydration status, although direct relationships are unlikely.

Hydration as a Key Characteristic for Athletes

Level of hydration is a key characteristic for the overall health status in humans, since water is a major participant of multiple reactions of metabolism and thermoregulation (37). Hydration, measured by the % of body water in relation to total body weight, was associated with multiple parameters (20, 22, 34), including both characteristics of body metabolism(the predicted-to-measured BMR ratio and fat oxidation), which is reasonable since water boosts fat metabolism (35, 36, 93, 94, 96) and overall metabolic rate (35, 94, 96–99), being considered as a potential thermogenic (35, 36, 93, 94, 96–98).

The need for a minimum amount of available water content for fat oxidation in catabolic states is conceivable in the context of a direct correlation between hydration and fat oxidation (35, 96). Conversely, the amount of body fat was inversely correlated with fat oxidation, which is expected, once more body fat may be a consequence of less fat utilization as source of energy, even under glycogen- and glucose-depleted circumstances, as observed by some studies (100, 101). Despite the previous reports on the correlation between body fat and fat oxidation, it is still unclear whether a larger fat mass resulted from reduced fat oxidation, or if a greater fat mass impaired fat oxidation through a non-classical inflammatory response that is enhanced in adipocytes when these are enlarged. Finally, since dehydration probably leads to lower fat oxidation, body water was expectedly found to be inversely correlated with body fat and the chest-to-waist circumference ratio.

Similar to the correlation between fat oxidation and body water, the measured-to-predicted BMR ratio was positively correlated with hydration status and muscle mass, which reinforces the extensive descriptions on the literature that body water content, hydration status, and muscle mass are the major components of the metabolic rate (94, 96–99, 102–104). Moreover, hydration has been correlated with vigor levels and other mood states at lesser extent, which is supported by the literature (105).

In regards with the location of the water—whether intra- or extracellular—extracellular water accounts for ∼40% of total body water (106), which was similar to that observed in the healthy sedentary of the EROS study. However, in healthy athletes extracellular water was shown to be reduced, possibly as a mechanism of facilitation for the optimization of intracellular metabolic pathways. Among which most reactions require water to occur. In the EROS study, in the groups of athletes ∼80% of the water was located intracellularly, which can be considered as an improvement of the water balance for metabolic purposes (107). Conversely, fat mass is inversely correlated with relative and absolute intracellular water, and consequently lower body water. Some authors speculate that this is possibly due to the fact that enlarged fat cells tend to become more hydrophobic and to contain less water. However, this is still a hypothesis to be further demonstrated.

We observed an inverse correlation between hydration status and extracellular water, i.e., better hydration was correlated with less edema, indicating that the amount of water consumed may redirect water toward inside cells (the preferred space), rather than interstitially (third space; extracellular water). However, external factors such as disturbed hormonal secretion, excessive sodium intake, and hypercaloric diets may lead to excessive extracellular water, which becomes the primary cause of dehydration: resulted from shifts in water compartment redistribution (106, 108). Indeed, dehydration may not only be a result from redistribution of water, but overall low water content can also induce further dehydration by its accumulation in the extracellular water, as a mechanism of protection against further water wasting, secondarily to vasopressin (ADH) and renin-angiotensin-aldosterone-system (RAAS) metabolism (109). In addition, interactions between water content, the RAAS, the HPA axis, the direct aldosterone actions and their relationship with chronic stress have been reported (105, 109).

In the EROS study, the major influences that drove the water destination were the metabolic environment, sleep quality (worse sleep leads to worse hydration and increased edema), the amount of muscle mass, eating patterns, and mood. Muscle mass was positively correlated with body water and it may have indirectly prevented edema, while body fat may have had the opposite effect, similarly to the observed in the literature (86, 98).

Implications of the Findings

The use of additional statistical techniques in this study facilitated the identification of independent predictors of linear correlations among clinical, metabolic, and biochemical parameters and other parameters, which has improved our understanding of hormonal and metabolic behaviors, and their multiple interactions and influences in athletes. They also yielded information to speculate on new potential markers and new understandings of current markers.

The summary of the findings and their possible implications are presented in **Table 3**. Hydration status, and, to a lesser extent, muscle mass, were the two major determinants of metabolic rate and fat oxidation (35, 93–99, 102–104). These results support the importance of adequate water intake and maintaining and building lean muscle for adequate metabolism and fat oxidation. The body fat effect on GH release was attenuated in athletes, while its effect on testosterone was maintained, suggesting that athletes with excessive body fat might not benefit from some of effects of exercise, which is a reason for sports professionals to have a good body shape.

Since the hormonal responses to an ITT were strictly correlated, i.e., the level of increase of GH, cortisol, ACTH, and prolactin was similar within each athlete (**Figure 5**), the level of hypothalamic responsiveness to stimulation seemed to be diffuse, rather than specific for certain axes. Energy levels were strongly correlated with hormonal status, including a prolonged optimization of hormonal responses and a better

cortisol response to awakening, although a causal relationship was undefined. In addition to energy levels, better hormonal responses were correlated with better body composition, of which the causal relationship remains uncertain.

Sleep quality seemed to be the most important factor in mood, rather than any other factor, such as hormonal levels or eating patterns, which has been demonstrated to play an essential role on overall cognitive and psychological functions (REF), in accordance with our findings. The level of hydration was inversely correlated with edema and better hydration was linked to less edema, depending on the location of the water in the body, regulated by external factors rather than the amount of water intake.

Testosterone, Estradiol, and Testosterone-To-Estradiol (T:E) Ratio

Testosterone, estradiol, and their ratio (T:E ratio) had different roles and influences. While testosterone was inversely related to body fat, positively linked to sleep quality, and indirectly linked to improved psychological outcomes, alone, it did not predict any of the parameters. Conversely, estradiol unexpectedly predicted anger, because of its actions on the male brains of the athletes. The T:E ratio had the most important roles in body metabolism and composition, and was linked to energy level. Hence, the balance between testosterone and estradiol might be more important than either testosterone or estradiol alone. This is extensively supported by the literature, since the T:E ratio predicts multiple outcomes, including cardiovascular changes (29, 30), while many of the neuroprotective and psychological effects of estradiol in males are mediated by testosterone (32–34), which requires a balance between these hormones to obtain the health benefits. The unaltered balance is more precisely assessed by the T:E ratio, rather than each hormone alone.

The T:E ratio is a significant ratio that has a promising role in the evaluation of athletes. It was found to be a better predictor of metabolic and psychological parameters than either testosterone or estradiol alone, supporting the hypothesis that it is a potential novel parameter. We based this hypothesis on a new understanding of the role of estradiol in males, and identified two types of estradiol increase: (1) a physiological increase, secondary to an increase in testosterone, when high testosterone levels are maintained and (2) a pathological increase, caused by an aberrant exacerbation of aromatase activity, leading to a decrease in testosterone. We found the most appropriate way to differentiate these situations objectively was to examine the T:E ratio. This ratio indicates whether an increase in estradiol is followed by an increase in testosterone; it should remain unaffected in the case of a physiological increase. These data were supported by a recent study showing that increased estradiol benefitted males in terms of increasing their libido, muscle mass, and bone mass, and reducing their fat mass, but only when accompanied by increased testosterone levels (26–28), which occurs when increased estradiol is actually desirable and the T:E ratio is unaffected (29). Conversely, the estradiol increase that we identified as a marker of OTS was due to a pathological conversion from testosterone, indicated by a substantial decrease in the T:E ratio, which was most likely a response to an anti-anabolic environment. For practical purposes, the T:E ratio should be above 13.7:1 (21).

Summary of the Findings

The EROS-CORRELATIONS study demonstrated that testosterone was predicted by fat mass, estradiol predicted anger, and the T:E ratio predicted the measured-to-predicted BMR ratio and chest-to-waist circumference, while hydration status predicted fat oxidation. Early and late somatotropic, corticotropic, and lactotropic responses were strong and strongly correlated, showing a diffuse hypothalamic rather than axis-specific response to stimulation. Late hormonal responses to stimulations, increased cortisol after awakening, and the T:E ratio was correlated with energy level. Sleep quality was the major factor correlated with most of the study's psychological measures, while fat oxidation, hydration, muscle mass, and body fat were highly inter-correlated, and edema was inversely correlated with hydration and muscle mass, and directly correlated with fat mass. The most remarkable findings are described in **Table 4**.

LIMITATIONS

The findings of the present study are valid only for male athletes that practice both endurance and strength sports, as basal hormone levels and responses to stimulations are highly sex-specific and may be sport-specific. Hence, different arms of the EROS study focused on purely strength, purely endurance, purely explosive, purely stop-and-go, and mixed sports, conducted with male and female participants, should provide data that are more specific. Given the unexpected findings regarding several hormones and other biochemical markers, we suggest additional parameters for further studies, including luteinizing hormone, follicle-stimulating hormone, sex hormone-binding globulin the tumor necrosis factor-alpha, interleukin-1 beta, lactate dehydrogenase (LDH), free thyroxin-4, and cortisol binding globulin. Longer stimulation tests, including thyrotrophic and gonadotrophic responses (given the unexpected response of the lactotropic axis), and an examination of the associations between exercise-dependent and exercise-independent tests should also be examined. Also, the estradiol levels in males may lose absolute precise using chemoiluminescence, compared to liquid chromatography mass spectrometry/tandem mass (LC/MS-MS/MS). However, the relative precision is highly accurate, which allows the in-between (pairwise) group comparisons as fully satisfactory (110–115).

FINAL DISCUSSION

We found multiple correlations and predictions between clinical, hormonal, biochemical markers, that occurred as a web of influences, as multiple and multi-directional chain-reactions, that allowed us speculate on several new mechanisms to occur in response to sports. The identification of a complex web of interactions among many different aspects allowed us to hypothesize that sports performance results from a combination of hormonal, energy, and water availability, and psychological and muscular status The predictions, correlations, and interactions revealed in the

TABLE 4 | Most remarkable findings of the EROS-CORRELATIONS study.

Parameter	Markers	Potential implication(s)
TESTOSTERONE, ESTRADIOL AND T:E RATIO		
Total testosterone	(1) Decreased body fat (P) (2) Better sleep quality (C)	1. Testosterone is blunted by body fat 2. Better sleep quality may boost testosterone production
Estradiol	(1) Lower anger levels (P)	1. Estradiol actions in the male brain improve anger levels
Testosterone-to-estradiol ratio	(1) Increased measured-to-predicted basal metabolic rate (P) (2) Increased chest-to-waist circumference ratio (P) (3) Lower fatigue levels (C)	1. The ratio between testosterone and estradiol is more important than testosterone or estradiol alone for body metabolism and composition
HORMONAL FUNCTIONAL TESTS		
GH, prolactin, and cortisol responses to an insulin tolerance test	(1) Positive (direct) inter-correlations between GH, prolactin, and cortisol in early responses (C) (2) Positive (direct) inter-correlations between GH, prolactin, and cortisol in late responses (C) (3) Lower body fat (C) (4) Higher fat oxidation (C) (5) Higher muscle mass (C) (6) Better hydration (C) (7) Lower fatigue levels (C)	1. Hypothalamic responsiveness to stimulations does not discriminate between different axes 2. Although causality is not confirmed, better hormonal responses are at least linked to more energy and to better body composition
SOCIAL AND PSYCHOLOGICAL ASPECTS		
Sleep quality	(1) Improved overall mood states (C) (2) Lower depression levels (C) (3) Less fatigue levels (C) (4) Higher vigor levels (C)	1. Sleep quality may be more important than hormonal levels or eating patterns for the psychological status of the athletes
Libido	(1) Higher vigor levels (C)	
Vigor	(1) Lower body fat (C) (2) Higher fat oxidation (C) (3) Better hydration (C) (4) Higher extracellular water (C)	1. Vigor is an indirect marker of less body fat, better fat oxidation, and lower edema
Tension	(1) Higher body fat (C) (2) Lower fat oxidation (C) (3) Worse hydration (C) (4) Lower muscle mass (C)	1. Tension is an indirect marker of lower muscle mass, increase of body fat, impaired fat oxidation, and less hydration
BODY METABOLISM AN COMPOSITION		
Measured-to-predicted basal metabolic ratio	(1) Higher testosterone-to-estradiol ratio (P) (2) Better hydration (P) (3) Higher muscle mass (C)	1. The balance between testosterone and estradiol, more than any hormone alone, is the major predictor of metabolic rate in male athletes 2. Together with the T:E ratio, body water and muscle mass are the two major contributors of the metabolic rate, which means that a minimum content of intracellular water is necessary for a proper metabolism
Fat oxidation	(1) Better hydration (C) (2) Higher muscle mass (C) (3) Lower body fat (C)	3. Body water and muscle mass play the most important roles for fat oxidation, the first as part of the pathway for fat oxidation, and the second as a possible signaller for the selective fat catabolism, over protein catabolism 4. Body fat and fat oxidation are inversely correlated; however, whether fat-induced inflammation leads to reduced fat oxidation, or higher body fat is a result from reduced fat oxidation, is unknown
Chest-to-waist circumference ratio	(1) Higher testosterone-to-estradiol ratio (P) (2) Lower visceral fat (P) (3) Higher muscle mass (C) (4) Higher fat oxidation (C) (5) Better hydration (C) (6) Lower body fat (C)	1. Similarly to other metabolic parameters, the T:E ratio is the most important direct predictor of the W:C ratio, leading to the popular "V-shape," highly correlated with an androgen phenotype. 2. Once body water is the intracellular water, mostly located within miocytes, rather than adipocytes, this contributes for a higher W/C ratio 3. Muscle mass and body fat are expectedly directly and inversely correlated with W/C ratio, respectively
Muscle mass	(1) Late GH response to stimulation (C) (2) Late cortisol response to stimulation (C) (3) Better hydration (C) (4) Higher fat oxidation (C) (5) Lower body fat (C)	1. Although the muscle mass is not the lean mass, i.e., the water within muscles are not accounted, the presence of body water helps provide a muscle anabolic environment, and predicts fat oxidation. 2. Late hormonal responses, although correlated with muscle mass, are probably two consequences of a same common factor.

(Continued)

TABLE 4 | Continued

Parameter	Markers	Potential implication(s)
Fat mass	(1) Improved overall mood states (C) (2) Higher vigor levels (C) (3) Decreased hydration (C) (4) Lower muscle mass (C) (5) Decreased fat oxidation (C)	1. Worse psychological moods may be indicators of less healthier environment, that naturally tends to save fat storage and catabolize muscle mass. 2. All correlated body composition parameters are accordingly.
Extracellular water (= edema)	(1) Worse hydration (C) (2) Lower muscle mass (C) (3) Increased fat mass (C)	1. The more proper hydration, the less edema; however, what determines the destination of the ingested water is the metabolic environment, not the amount of water intake 2. Fat mass, likely through inflammatory processes, may induce edema, although we did not find prediction relationship.

P, Prediction; C, Correlation; T:E, Testosterone-to-estradiol; W:C, Chest-to-waist circumference.

present study show that further studies should not evaluate each aspect separately, as this is unlikely to provide answers to important questions.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical committee of the Federal University of São Paulo (Approval Number: 1093965). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FC and CK developed the central idea of the present manuscript. FC performed the tests of the EROS study, compiled the data, analyzed the results, and participated in the discussions. CK actively participated in the discussion, supervised and reviewed the results, helped with the final version of the manuscript, and gave the last word before the submission. All authors have read and approved the manuscript.

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REVIEW
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Hypogonadism in Exercising Males: Dysfunction or Adaptive-Regulatory Adjustment?

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For decades researchers have reported men who engaged in intensive exercise training can develop low resting testosterone levels, alterations in their hypothalamic-pituitary-gonadal (HPG) axis, and display hypogonadism. Recently there is renewed interest in this topic since the International Olympic Committee (IOC) Medical Commission coined the term "Relative Energy Deficiency in Sports" (RED-S) as clinical terminology to address both the female-male occurrences of reproductive system health disruptions associated with exercise. This IOC Commission action attempted to move beyond the sex-specific terminology of the "Female Athlete Triad" (Triad) and heighten awareness/realization that some athletic men do have reproductive related physiologic disturbances such as lowered sex hormone levels, HPG regulatory axis alterations, and low bone mineral density similar to Triad women. There are elements in the development and symptomology of exercise-related male hypogonadism that mirror closely that of women experiencing the Triad/RED-S, but evidence also exists that dissimilarities exist between the sexes on this issue. Our research group postulates that the inconsistency and differences in the male findings in relation to women with Triad/RED-S are not just due to sex dimorphism, but that there are varying forms of exercise-related reproductive disruptions existing in athletic men resulting in them displaying a relative hypogonadism condition. Specifically, such conditions in men may derive acutely and be associated with low energy availability (Triad/RED-S) or excessive training load (overtraining) and appear transient in nature, and resolve with appropriate clinical interventions. However, manifestations of a more chronic based hypogonadism that persists on a more permanent basis (years) exist and is termed the "Exercise Hypogonadal Male Condition." This article presents an up-to-date overview of the various types of acute and chronic relative hypogonadism found in athletic, exercising men and proposes mechanistic models of how these various forms of exercise relative hypogonadism develop.

Keywords: testosterone, sport, androgens, athletes, impairment, sex

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INTRODUCTION

Many national and international organizations have touted the health benefits of being physically activity and engaging in exercise training (1, 2). Research evidence is overwhelmingly supportive that an active lifestyle leads to improved quality and quantity of life for individuals (3, 4). For this reason, many public health professionals are promoting and encouraging the populations within

their respective countries to adopt behaviors that incorporate more physical activity into their daily living. To this end, the concept of using physical activity and exercise training as a preventative healthcare adjunctive therapy has become a popular contemporary theme. Furthermore, this is sound medical policy as preventative steps in promoting improved health are typically far most cost-effective and successful than interventional alternatives (5).

However, exercise is not a panacea for all human afflictions and ills and in and of itself can induce health complications (*N.B.,* for convenience in this paper the term *exercise* is used to refer to both physical activity and exercise). Most healthy individuals recognize that by doing more exercise the risk for musculoskeletal injury increases; but, what most do not know, however, is other complications can present themselves with exercise. In particular many in the general public are unaware of how increasing levels of exercise can precipitate endocrine dysfunction by promoting changes in circulating hormone levels (the term *dysfunction* and *disorder* are used interchangeably by researchers, this article uses the term dysfunction). Although it is important to note, such occurrences are primarily associated with individuals who perform exercise at levels beyond the recommendations for health and physical fitness improvement (6). That is, specifically, men and women who are conducting exercise training at levels to allow themselves to be highly competitive in sporting events are more at risk.

Perhaps the most notable endocrine dysfunction linked to exercise training is that which involves disrupts in a woman's reproductive system leading to the development of secondary amenorrhea—what was originally referred to as "athletic amenorrhea." This occurrence is now recognized as part of the consequences of the medical condition known as the Female Athletic Triad (Triad) which is associated with increased risk for infertility, bone mineral loss, potentially disordered eating behaviors as well as reduced reproductive hormone levels (7). In the 1970's medical researchers began to understand that exercise training could have these negative consequences in women. Landmark research studies by scientists such as Drs. Anne Loucks, Constance Lebrun, Naama Constantini, Michelle Warren, and the late Barbara Drinkwater, to name just a few, laid the groundwork for this important medical finding.

Less familiar to the public is the influence of exercise training on the reproductive endocrinology of men. For many years researchers assumed the male reproductive system was robust enough to tolerate the stress of demanding levels of exercise training and was thus unaffected. Today we know that is not the case and in fact, there are many similarities in the aspects of the reproductive dysfunctions that develop in women and men. The degree and scope of the research on men are far more limited than that in women; and, perhaps rightly so due to the prevalence and severity of the health consequences found in women with the Triad.

The research addressing reproductive dysfunctions in men began later than that involving women and was pursued by a very limited number of researchers for many years. Today the number of researchers and studies addressing men on this issue has grown dramatically; and, now more attention is being focused than ever before on the negative reproductive health consequences suffered by men engaged in exercise training.

The growth and expansion of interest in the male reproductive system as an exercise research topic is long overdue and it is exciting to see many new researchers now pursuing this line of work. But, the rapid expansion of interests in this topic has led to some misconceptions and misunderstandings by the general public as well as some in the research community concerning male endocrinology and the reproductive hormonal anomalies associated with exercise training. These occurrences have developed for several reasons: (1) misinformation or overly simplified information presented on internet exercise websites; (2) lack of general familiarity with the nearly three-plus decades of prior research already done on men and reproductive dysfunction; (3) faulty assumptions that all exercise reproductive dysfunction in men are of one causation—i.e., the "one size fits all" explanation, and (4) the application of finding on reproductive dysfunction in women being directly translated and applied to men.

This review article intends to clarify some of these misconceptions and misunderstandings and provide historical background and physiological overview of reproductive dysfunctions found in men engaged in exercise training—specifically, focusing on the development of exercise relative hypogonadism (i.e., low testosterone). This article is organized into several sections addressing specific questions related to the topic: (1) How is hypogonadism defined? (2) What is normal testosterone levels in men? (3) Why is testosterone so critical to athletes-exercisers? (3) What are situations inducing exercise hypogonadism? (4) Dysfunction or adaption-regulatory adjustment? (5) What are actions to deal with low testosterone in athletes-exercisers? and (6) Summary, conclusions and perspective.

HOW IS HYPOGONADISM DEFINED?

Hypogonadism is the medical term for decreased functional activity of the gonads. Male hypogonadism is characterized by a deficiency in the production of the critical male reproductive hormone testosterone from the testicles (8–10).

Testosterone production is regulated by the hypothalamic-pituitary-gonadal (HPG) axis which involves the hypothalamic hormone gonadotrophin-releasing hormone (GnRH), and the pituitary hormones luteinizing hormones (LH), and follicle-stimulating hormones (FSH) (see **Figure 1**). As such, the low testosterone levels of hypogonadism may be due to testicular production or abnormalities in the HPG regulatory axis (11).

Specifically, two basic clinical types of male hypogonadism exist (9):

- *Primary*—This type of hypogonadism—also known as primary testicular failure—originates from a problem in the testicles. This can lead to what is termed hypergonadotropic hypogonadism, an impaired response of the gonads to GnRH, or LH and FSH stimuli (10).
- *Secondary*—This type of hypogonadism indicates a problem in the hypothalamus or the pituitary gland—which signals

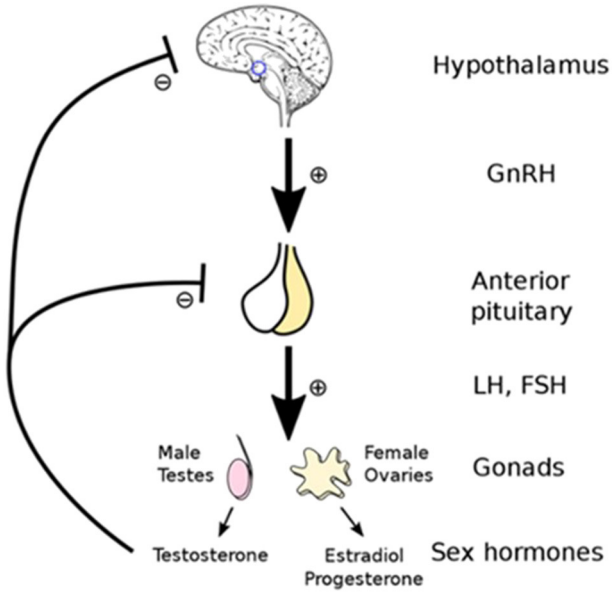

FIGURE 1 | Testosterone production is controlled by the hypothalamic -pituitary-gonadal (HPG) regulatory axis which involves the hormones gonadotrophin-releasing hormone (GnRH), luteinizing hormones (LH), and follicle-stimulating hormones (FSH). Reprinted with permission: Artoria2e5 [CC BY 3.0 (https://creativecommons.org/licenses/by/3.0)].

the testicles to produce testosterone. That is, within the HPG regulatory axis GnRH or LH and FSH are not produced adequately. In secondary hypogonadism, the testicles are generally normal in function. Another term used for this hypogonadism form is hypogonadotropic hypogonadism (10).

Either type of hypogonadism may be caused by an inherited (congenital) trait or something that occurs during a persons' lifespan (acquired). Relative to the discussion of this article, exercise hypogonadism would be viewed as acquired. **Table 1** presents some of the major health-related clinical conditions associated with primary and secondary hypogonadism development (9, 12, 13).

TABLE 1 | The major clinical conditions associated with the development of primary and secondary hypogonadism in men (9, 12).

Primary hypogonadism conditions
Klinefelter's syndrome
Undescended testicles
Mumps orchitis
Hemochromatosis
Injury to the testicles
Cancer treatment
Normal aging (*andropause*)
Secondary hypogonadism conditions
Kallmann syndrome
Pituitary disorders
Inflammatory disease
HIV/AIDS
Medications/pharmaceuticals
Obesity
Stress-induced hypogonadism

TABLE 2 | The reference range for clinical assessment of testosterone from select sources for non-obese men (i.e., Body Mass Index [BMI] < 30 kg•m^2).

Source	Total testosterone**	Free testosterone
Mayo Clinical Laboratories (14)	17–18 years: 300–1,200 ng/dl ≥ 19 years: 240-950 ng/dl	20<25 years: 5.25–20.7 ng/dl 25<30 years: 5.05–19.8 ng/dl 30<35 years: 4.85–19.0 ng/d 35<40 years: 4.65–18.1 ng/dl 40<45 years: 4.46–17.1 ng/dl 45<50 years: 4.26–16.4 ng/dl 50<55 years: 4.06–15.6 ng/dl 55<60 years: 3.87–14.7 ng/dl 60<65 years: 3.67–13.9 ng/dl
Travison et al. (15)	19–39 years: 304–850 ng/dl* 40–49 years: 273–839 ng/dl 50–59 years: 256–839 ng/dl 60–69 years: 254–839 ng/dl	

*(5th−95th percentile).
**Total testosterone encompasses the free and carrier-protein bound levels of the hormone, while free refers only to that portion not bound to a carrier-protein in the circulation.

WHAT IS NORMAL TESTOSTERONE LEVELS IN MEN?

The clinical reference range for normal testosterone levels in healthy, non-obese human males varies slightly based upon which scientific source is examined, and is relative to the age of the males. For example, **Table 2** presents the reference values reported by the Mayo Clinic (14), as well as from the innovative study by Travison et al. which attempted to develop harmonized reference values of testosterone for wide clinical use (15). The values presented from these two sources are similar and overlapping but are not exactly the same.

As noted in **Table 2**, testosterone can be expressed as either in total or free forms. The free, unbound form represents typically 1.5–2.0% (males) of the total hormonal amount circulating in the blood. The remainder is bound to carrier proteins; about 65% to sex hormone-binding globulin (SHBG) and 33% bound weakly to albumin (9, 10, 12, 16). The free and albumin-bound forms of testosterone constitute what is referred to as bioavailable testosterone (i.e., able to interact with androgenic receptors at target tissues). As males age, the amount of total and free forms of testosterone in the circulation change as does SHBG (see **Figure 2**) leading to a gradual overall reduction in the hormone forms in the blood; see subsequent section for discussion on the phenomena of andropause in males.

What About Exercising Men?

Perhaps more pertinent to exercising or athletic males are the recent findings reported by Handelsman et al. in *Endocrine Reviews* (16). These authors did an exhaustive examination of the available research literature as well as the extensive database from the International Association of Athletics Federation (IAAF) on athletes who have competed over many years at elite levels in track and field (i.e., athletics).

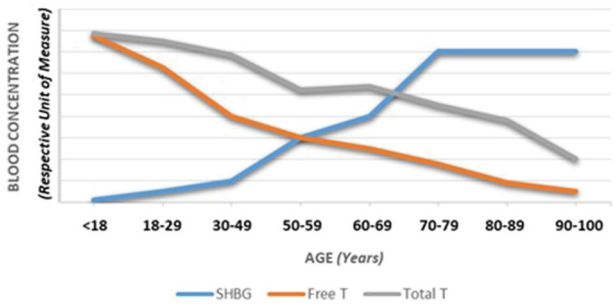

FIGURE 2 | A depiction of the typical changes observed in total and free testosterone as well as sex hormone-binding globulin over the course of a male lifespan. Adapted from information provided in references 16, 28, and 49.

TABLE 3 | Testosterone threshold levels for diagnosis of hypogonadism and, or androgen deficiency (also called testosterone deficiency) (18).

Organization	Total testosterone	Free testosterone
European academy of andrology International society of andrology International society for the study of the aging male (2009)	<350 ng/dl (12.1 nmol/l)	<65 pg/ml (<225 pmol/l)
The endocrine society (2010)	<300 ng/dl (<10.4 nmol/l)	<50–90 pg/ml (173–312 pmol/l)
European association of urology (2012)	<350 ng/dl (12.1 nmol/l)	<84 pg/ml (<243 pmol/l)
Expert opinion (2014)	<400 ng/dl (13.9 nmol/l)	80–100 pg/ml (277–347 pmol/l)

They concluded that a reference range (95%) of 223–849 ng/dl (7.7–29.4 nmol/L) existed in healthy adult athletic men, and 0–144 ng/dl (0–5.0 nmol/L) in athletic women. To this last point, the Handelsman et al. reference range, however, is an issue of some contention as it has been challenged by the legal team involved with the Caster Semenya vs. IAAF case at the Tribunal Arbitral du Sport (Court of Arbitration for Sport) concerning male and female categorical standards for acceptable gender-based testosterone levels (16, 17).

Nevertheless, and importantly though, the universal agreement does not currently exist in the world-wide medical community on what is precisely normal testosterone levels. Furthermore, the clinical definition of what constituents "low testosterone" and the diagnostic threshold for hypogonadism diagnosis varies too. To this last point, **Table 3** illustrates this lack of agreement as it displays what might constitute hypogonadism based upon testosterone levels as defined by several medical organizations (18).

It should be noted, for some clinicians and medical professional groups, hypogonadism is characterized by not just low testosterone but includes at least one clinical sign or symptom (9). Overt signs of hypogonadism include absence or regression of secondary sex characteristics, anemia, muscle wasting, reduced bone mass or bone mineral density, oligospermia, and abdominal adiposity. Symptoms include sexual dysfunction (e.g., erectile dysfunction, reduced libido, diminished penile sensation, difficulty attaining orgasm, and reduced ejaculate), reduced energy, and stamina, depressed mood, increased irritability, difficulty concentrating, changes in cholesterol levels, anemia, osteoporosis, and hot flushes (9, 12, 13).

In the absence of any of the clinical signs or symptoms, the presence of low testosterone alone may lead to a diagnosis of "androgen deficiency" (also called testosterone deficiency) and not definitively hypogonadism. That said, nonetheless, many leading medical resources define hypogonadism based solely on the presence of low circulating testosterone (9, 12).

How is Exercise Hypogonadism Defined?

The term exercise hypogonadism has been applied in a number of exercise studies in which low testosterone levels are reported, but in doing so investigators have seldom applied the criteria as outlined in **Table 3** for their defining of hypogonadism. In fact, other criteria have been used, for example:

- If the study was cross-sectional in design there has typically been a matched-control group (sedentary) to whom the exercising males are compared to determine if testosterone status is low or reduced;
- If the study research design was prospective, or longitudinal in approach the exercising males are usually compared to themselves at some point in time before training when their testosterone was not affected; and,
- In some studies, the testosterone levels of exercising males have been compared to a clinical reference range set of values to determine testosterone status.

Additionally, some research groups have been hesitant to use the term hypogonadism altogether, and have referred to the exercising males as having states of "low testosterone," "testosterone deficiency," or "androgen deficiency" (6, 19–22). Although, again what constitutes a *low* or *deficiency* level has not been clearly defined or have used endocrine standards per professional organization guidelines as noted in see **Table 3**. And, while not using the term hypogonadism strictly some published exercise reports have alluded to consequences associated with hypogonadal states from there testosterone findings.

In short, there is a lack of consistency in the exercise literature determining what exactly constitutes exercise hypogonadism. Additionally, few investigators have attempted to set or use a threshold, or cut-point value to denote when testosterone levels are reduced enough to use the "exercise hypogonadism" distinction. Regardless of the terms used to refer to testosterone levels in exercising men, it is important to note that even were testosterone is reduced, for many of these individuals it is low but within the normal range and seldom found to reach clinical definitions of hypogonadism (**Table 3**). Although, reports of sub-clinical findings and testosterone levels well below those established for clinical hypogonadism exist (23–25).

Notably, in 2005 Hackney and associates did outline criteria for the level of testosterone reduction necessary to denote an athlete having what they termed the "Exercise Hypogonadal Male Condition" (see later discussion) (19, 26). These investigators suggested persistent reductions of 25–50% or greater in testosterone were necessary for this distinction as a relative form of hypogonadism.

WHY IS TESTOSTERONE SO CRITICAL TO ATHLETES-EXERCISERS?

Throughout the male lifespan, testosterone plays a critical role in sexual, cognitive, and body morphology development. The most visible effects of rising testosterone levels begin in the pre-pubertal stage for males. During this time a multitude of physiological changes occur; e.g., body odor develops, oiliness of the skin and hair increase, acne develops, accelerated growth spurts occur, and pubic, early facial, and axillary hair grow. The pubertal effects also include enlargement of the sebaceous glands, penis enlargement, increased libido, increased frequency of erections, increased muscle mass development, deepening of the voice, increased height, bone maturation, loss of scalp hair, and growth of facial, chest, leg, and axillary hair. Several, but not all of these essential effects and influences continue into adulthood (27, 28).

Many aspects of the above influences affect the male physiology advantageous for sporting performance. Perhaps the most striking being the anabolic action of testosterone on protein turnover and the potential to develop muscle accretion (16, 29, 30). Although, the process is not solely dependent upon anabolic hormones such as testosterone (31). With proper exercise training regimens, such muscular development can lead to enhanced strength and power. Additionally, testosterone exhibits positive effects on erythropoiesis and hemoglobin concentrations (16). The latter in turn can facilitate the oxygen content capacity of the blood and maximal aerobic capacity (VO_{2max}) (16, 32). All of these components, strength-power-oxygen content-VO_{2max}, are critical factors in the performance of a multitude of sporting activities and essential elements in the exercise training adaptation process (16, 32, 33).

Unlike women who experience a rapid decline in sex hormone levels during menopause, men experience a slow, continuous decline in testosterone levels over time (see **Figure 2**). The term "andropause" is sometimes used to denote this hormonal change. As testosterone levels slowly reflect this decline with aging, a form of hypogonadism can develop and is sometimes referred to as the partial androgen deficiency of the aging male (PADAM) (34). In older athletic men who display reduced levels of testosterone, this aging event could be a partial contributor to hormonal change. But, research examining older men who are exercisers with low testosterone compared to sedentary controls still show reductions in their testosterone levels compared to age-matched controls, although the amount of research on this topic is extremely limited (35).

When male athletes develop low testosterone-hypogonadism the physiological and psychological consequences and side effects are variable. Some studies report serious negative consequences and other studies reporting no negative effects whatsoever (21, 23, 25, 36–38). This lack of consistency in studies may relate to the degree of reduction in testosterone observed and, or the scope of health-related outcomes monitored within these studies (39). Examples of the negative psychophysiological consequences typically reported are given in **Table 4** (39).

TABLE 4 | Signs and symptoms of low testosterone and hypogonadism typically reported by men, non-athletes as well as athletes (39).

Low testosterone—hypogonadism consequences
Decreasing physical performance
Sleep disturbances
Lethargy
Decreased motivation
Decreased libido
Sexual dysfunction
Spermatogenesis abnormalities
Muscle mass loss
Sperm abnormalities
Bone mineral density loss
Depression

WHAT ARE SITUATIONS INDUCING EXERCISE HYPOGONADISM?

Background

The systematic and scientific study of the influence of exercise on testosterone levels in human males began in the 1970's. Animal-based research had pre-dated this period considerably, and human anabolic steroid "doping experiments" by athletes-coaches also occurred before this period. Although the evidence of the latter actually occurring was withheld from public and scientific scrutiny due to legality and ethical violation issues for many decades. Perhaps the first systematic exercise study on humans was performed by the late Dr. John Sutton of Australia in the 1970's. He and his associates published an article on the testosterone response in men and women to acute submaximal and maximal exercise sessions (40). They reported that maximal exercise increased testosterone levels, and with this finding, a cornucopia of studies was begun by the scientific community examining testosterone, exercise, and training adaptations.

By the mid-to-late 1980's, several key studies were published which reported men involved with endurance exercise training had substantially lower resting testosterone levels (41–44), and or HPG axis disruptions [potentially affecting testosterone levels (historically the vast majority of these studies have examined total testosterone; although, a few research groups have addressed free testosterone too and found both total and free to be reduced)] (45). These studies involved distance runners, and at the time these investigators did not speculate on the causation of the low resting testosterone. Nonetheless, these studies served as the basis for subsequent work which did attempt to examine causality (see following discussions).

In the context of exercise endocrinology, it is important to understand the distinction between the effects of an acute exercise session on hormones, and the more chronic effect of

exercise training on hormones. In the acute scenario, nearly all forms of exercise provoke changes in circulating hormone concentrations—almost universally being increased levels, which tend to be proportional to the intensity at which the exercise is conducted and, or the extent of the exercise duration. Although the mode of exercise utilized creates some variance in the degree of response (e.g., swimming vs. running, vs. weight lifting) (46–48). Additionally, some hormones do display a "threshold" level of exercise volume (i.e., intensity X duration of exercise sessions) be achieved before a response is detected in the blood (49). These acute exercise-induced changes abate relatively quickly during the recovery period unless the exercise session is extremely excessive (e.g., hours) in duration (49). **Table 5** provides a basic summary of the generalized effects of exercise on the major hormones associated with research-clinical interests in the area of sports physiology and exercise.

Conversely, when examining the chronic effect of exercise one can examine resting (basal) effects and, or responses to a subsequent exercise session after some period of training has been performed. Resting, basal hormone levels after substantial exercise training are commonly unchanged, increased slightly or perhaps reduced slightly. Relative to the latter, the "basement effect" phenomena prevent some aspects of detectable reductions being observed; that is a hormone value near zero cannot be reduced substantial further (50). In response to performing an acute exercise session following chronic exercise training, many hormone responses are reduced when compared to performing a similar exercise session before the training intervention; although the direction (↑ or ↓) of the hormonal change remains the same. These reduced responses tend to be a function of reduced stress reactivity to any given exercise bout and due to improved target tissue sensitivity as a training adaptation (51, 52). In general, these acute-chronic exercise endocrine principles for hormonal response hold true for the reproductive and non-reproductive hormones (52). Finally, and importantly to the present discussion, in most clinical diagnosis settings, much of the assessment and detection of reproductive dysfunction relies on evaluating hormonal status in a resting, basal condition and not in response to an exercise session (53). In such assessments, the gold standard, biological fluid for measurement is blood serum or plasma. Other fluids are occasionally assessed such as saliva or sweat; but, these fluids can produce a variance in outcomes. For example, Adebero and associates compared salivary and serum concentrations of testosterone and cortisol at rest and in response to intense exercise in boys and men; and, found testosterone was reduced post-exercise in serum but not in saliva (54). VanBruggen and colleagues have attributed such discrepancy in blood-saliva findings as being due to changes in hormonal diffusion rates into the salivary gland-saliva being effected by the physiological consequences of exercise (e.g., plasma volumes shifts, changing hormonal concentration gradients) (55).

Overtraining Syndrome

In their extensive review, Kuiper and Keizer, provide a thorough historical background on the use of the term overtraining, and commentary on the early research in the topic. Many coaches and exercise scientists would be surprised to find that this topic has been recognized and discussed for nearly 100 years (56). That said, there have been attempts to change the language and nomenclature used in describing the issue and shift the explanations to some degree in the operational definitions of the terms associated with it over the decades (57). For example, in their recent innovative EROS study (Endocrine and Metabolic Responses on Overtraining Syndrome), Cadegiani and Kater proposed a new designation of "Paradoxical Deconditioning Syndrome" rather than Overtraining Syndrome (58, 59). Nevertheless, regardless of what is called, for the most part, the indicators of the condition are essentially the same topical area as when first mentioned in a 1939 sports medicine article by Jezler (60). To aid the reader, with what constituents the progression from normal and appropriate levels of training to overtraining **Figure 3** (61) is provided and references 56 and 61 are recommended reading.

Because of testosterone's critical physiological role, early in the pursue of exercise adaptation research investigators began proposing the question—"Can monitoring of circulating testosterone changes serve as a viable biomarker of training adaptation?". Research work in the late 1970's and early 1980's by groups of various Scandinavian and Baltic researchers reported intensive exercise sessions and training loads resulted in substantial reductions in blood testosterone (62–67). These numerous findings led to Aldercreutz and associates in 1986 releasing their seminal paper suggesting that testosterone, cortisol and, or the ratio of the two (T:C ratio) could be used as a means of accessing "overstrain" (i.e., overtraining) in an athlete and monitoring whether their training was progressing advantageously (68). Shortly thereafter, reports began appearing of overtrained athletes having low testosterone, and in some cases elevated cortisol which was associated with the testosterone reductions (69–73).

To that end, over the next 30 years, a great number of studies reported with increasingly heavy training loads testosterone becomes reduced and this typically coincides with performance stagnation or declines in athletes as they become overtrained (i.e., primarily males; see review articles—references (74–76)); although, this is not a universal finding (25). **Table 6** displays some of the signs, symptoms and health consequences of athletes diagnosed as having the Overtraining Syndrome. The syndrome results in a chronic under-performance, negative health consequences (see **Table 6**), and typically can end or curtail an athlete's competitive season (56, 57, 77). The development of the Overtraining Syndrome has been reported in a multitude of sports, regardless of the emphasis on training modality employed (e.g., runners vs. weight lifters vs. tennis players) although the specific symptoms and frequency of select symptoms can be somewhat sports specific (74, 75).

Researchers have proposed two major rationales and mechanisms for testosterone reductions observed with overtraining; (1) testosterone production being disrupted by inhibitory factors such as other hormones in a stress response cascade; and, (2) inadequate energy intake disruption of the HPG axis regulatory function.

Relative to the first mechanism, Doerr and Pirke, as well as Cummings and associates, demonstrated blood cortisol elevations disrupt testosterone production peripherally at the

TABLE 5 | The generalized hormonal responses to exercise (e.g., resting-basal levels compared to after an exercise session [∼immediately] of the respective exercise type).

Hormone	Physiological actions	Exercise type—response		
		High intensity (e.g., HIIT)	Endurance exercise (>60 min)	Resistance exercise
ACTH	Adrendo-regulatory	↑	↑	↑
ADH	Hydration, fluid balance	↑	↑	↑, ↓, ↔
Aldosterone	Hydration, fluid balance	↑	↑	↑
Catecholamines (adrenaline, noradrenaline)	Catabolic (e.g., lipolysis, glycogenolysis), cardio-regulatory	↑	↑	↑
Cortisol	Catabolic (e.g., lipolysis, gluconeogenesis), stress reactivity	↑ >60%VO_{2max}	↑ >60%VO_{2max}	↑
DHEA	Anabolic	↑	↑	↑
Estradiol-β-17	Bone metabolism, catabolic (e.g., lipolysis), reproductive function	↑	↑ ↓ if excessive	↑
FSH—LH	Reproductive function	↑, ↓, ↔	↑, ↓, ↔	↑, ↓, ↔
Glucagon	Glucoregulatory	↑	↑	↑
Growth Hormone	Anabolic (e.g., myoplasticity), Catabolic (e.g., lipolysis)	↑	↑	↑
Insulin	Glucoregulatory, anabolic	↓	↓	↑, ↓, ↔
IGF-1	Anabolic	↑, ↔	↑, ↔	↑, ↔
Leptin	Satiety, reproductive function	↑, ↓, ↔	↑, ↓, ↔	↑, ↓, ↔
Parathyroid	Calcium metabolism	↑	↑	↔
Prolactin	Immune function, stress reactivity	↑	↑	↑
Progesterone	Reproductive function	↑	↑	↑
Testosterone	Anabolic (e.g., myoplasticity), reproductive function	↑	↑ ↓ if excessive	↑
T_4–T_3	Calorigenesis, endo-permissive actions	↑, ↓, ↔	↑, ↓, ↔	↑, ↓, ↔
TSH	Thyroid-regulatory	↑, ↓, ↔	↑, ↓, ↔	↑, ↓, ↔
Vitamin D	Calcium metabolism	↔, ?	↑	↔, ?

HIIT, high intensity interval training exercise; ACTH, adrenocorticotropic hormone; ADH, antidiuretic hormone (vasopressin); DHEA, dehydroepiandrosterone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; T_4, thyroxine; T_3, triiodothyronine; TSH, thyroid-stimulating hormone; VO_{2max}, maximal oxygen uptake; ↑ = increase; ↓ = decrease; ↔ = no change; ? = unknown.

gonad (testes) when cortisol levels were elevated (78, 79). There are numerous research studies reporting findings of exercise-induced short-term increases in cortisol levels (see review articles—references (74, 78)), as well as these acute elevations in cortisol from an exercise session being associated with decreases in testosterone (72, 80, 81). Furthermore, evidence exists for circulating testosterone and cortisol to be negatively associated with athletes even in the resting, basal state (82). In these scenarios the inhibitory effect of cortisol appears twofold; i.e., to impact LH and FSH via GnRH suppression as well as a compromise of Leydig cell function via direct steroidogenesis inhibition (79, 83). Prolactin is another hormone that can induce reductions in testosterone levels, and this hormone's release is also stimulated by exercise (see review article—(84)). The evidence convincingly shows elevated prolactin concentrations inhibit the secretion of GnRH, thereby decreasing the secretion of gonadotropins (LH, FSH) and affecting the central aspects of the HPG axis (85). Additionally, prolactin may also inhibit the action of gonadotropins on the gonads directly (86). Acute exercise-induced elevations in prolactin have been associated with testosterone reductions (87), as have training-induced increases in resting, basal prolactin associated with testosterone reductions (73, 88); but the latter is not universally reported (41, 89).

Nevertheless, resting hypercortisolemic or hyperprolactinemic states are not frequently found in athletes, but consistent daily exercise sessions could create frequent transient periods of such hyper-exposure during an actual exercise session as well as for extended periods in the recovery from such exercise sessions (80, 84, 90, 91).

In the case of the second proposed mechanism, several researchers' decades ago demonstrated short- and long-term caloric deficient results in testosterone reductions in men (92–94). It is well-recognized that a common finding is overtrained athletics is weight loss and suppressed appetite/anorexic tendencies (56, 61). The effect of inadequate caloric intake on testosterone seems more related to central HPG axis suppression than direct action at the testes as both LH and FSH levels

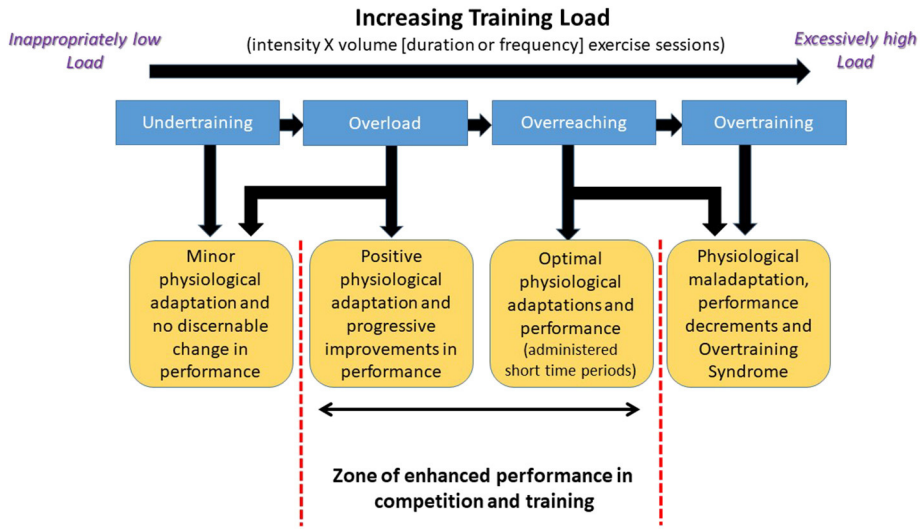

FIGURE 3 | Schematic representation of the progression in exercise training load that leads to the development of the Overtraining Syndrome in athletes. Adapted from information provided in reference (61). Used with permission.

TABLE 6 | Symptoms and characteristics displayed by athletes (male) who are overtrained (74, 75).

Parasympathetic alterations[a]	Sympathetic alterations[b]	Other-combined[c]
Fatigue	Insomnia	Declining performance
Depression	Irritability	Anorexia—weight loss
Bradycardia	Agitation	Lack of mental concentration
Loss of motivation	Tachycardia	Heavy, sore stiff muscles
Hypotension	Hypertension	Anxiety
Abnormal heart rate during recovery	Restlessness	Awaking unrefreshed
	Increased basal metabolic rate	Endocrine abnormalities (e.g., low testosterone, elevated cortisol, low thyroid hormones)

[a] *Typically symptoms more associated with more endurance-based sports.*
[b] *Typically symptoms more associated with strength-power based sports.*
[c] *Symptoms common to either form of sports activities.*

become reduced in such scenarios. For example, Bergendahl et al. (95) found such gonadotrophin reductions were driven by suppressed GnRH release by the hypothalamus. Recently, Wong and associates (96) propose this dysfunction likely involves hypothalamic suppression due to dysregulation of leptin, ghrelin, and pro-inflammatory cytokines. The gonadal axis suppression transient and the axis functional, as the effect, can be reversible with weight gain; although the rate of testosterone returning to normal seems highly individualistic (96–98).

Weight Restricted Sports

Historically, one of the exercise activities, where dramatic testosterone reductions were first reported in athletes, involved the sport of wrestling (i.e., Olympic free-style, Greco-Roman, and or American scholastic-collegiate forms). For example, nearly 40 years ago researchers described substantial testosterone reductions in adult male wrestlers during their competitive season compared to their off-season period (99). Subsequent reports by numerous other investigators substantiated these findings not only in wrestlers but other weight-restricted sports too (100–104).

Mechanistically the reason for this reduction in testosterone most likely is related to the practice of many athletes in these sports to use extreme weight loss tactics (e.g., semi-starvation) in attempting to reach a specific competitive weigh category. That is, their reduced caloric intakes plus high exercise expenditures lead to extreme negative energy balances and an HPG axis suppression—specifically, a hypogonadotropic hypogonadism state development—see preceding section discussion (105). Although this occurrence also seems highly reversible as a resumption of appropriate caloric intake reverts the HPG axis function relatively quickly (96, 98, 105).

Contact—Combative Sports

It is well-known traumatic brain injuries (TBI), such as concussions, can result in the development of low testosterone; specifically, a secondary hypogonadism usually develops due to a pituitary dysfunction (106, 107). A great deal of contemporary research has focused on American football and these type injuries as investigations on professional and collegiate athletes who have experienced multiple concussions show serious long term negative health consequences of such repeated head traumas (108). But, there are a number of sporting activities which results in participants being at an increased risk for the development of some form of TBI. Sporting activities categorized as "contact sports" (some of which are also referred to as combative sports) present the greatest risk—boxing, kickboxing, karate, taekwondo, aikido, jujitsu, judo, rugby, and Australian football. While sporting activities such as these have a greater risk for TBI exposure, a multitude of sports even if not specifically categorized

as a contact-combative can result in an athlete developing a TBI (e.g., wrestling discussed in prior section or football [soccer]). It is important for clinicians to examine an athlete's medical history for TBI events if they detect the presence of low testosterone.

Male Triad/RED-S

The Female Athlete Triad refers to a medical condition that is a constellation of three clinical entities: menstrual dysfunction, low energy availability (with or without an eating disorder), and decreased bone mineral density (7). The Triad term for this disorder was first coined by the American College of Sports Medicine in 1992 after many experts in the field had noticed a pattern among adolescent and young adult female athletes. Evidence from landmark work by Dr. Anne Loucks demonstrated that the etiological cause of the Triad in women was a persistent state of low energy availability (109).

Relative to this discussion, it is important to define the term "energy availability". Energy availability refers to the amount of energy leftover and available for your body's functions after the energy expended for daily exercise training is subtracted from the energy taken in from daily caloric intake from food. In other words, in its most basic form:

$$\text{Energy Availability} = \text{Dietary Energy Intake (food)} - \text{Exercise Energy Expenditure}$$

Extensive research in females has identified low energy availability cut points indicative of risk level for the development of physiological and performance disturbances associated with the Triad. These cut-points are: at risk = ≤30 kcal/kg lean body mass (LBM); moderate risk = 30–45 kcal/kg LBM; and no risk = ≥45 kcal/kg LBM (109). Whether male athletes share the same risk factor cut points is currently unknown, and is an issue of debate (109).

Recently, DeSouza and associates have proposed an expansion of the scope of the Triad condition and use of the term to encompass not only the historic population of women but also males (110). Interestingly, earlier researchers had drawn an analogy between the development of menstrual disruptions in exercising women and the observation of low testosterone in men but had never applied the Triad terminology to men (111, 112).

While the state of low energy availability (LEA) produces a myriad of physiological consequences in women and supposedly men, it is associated specifically with the development of low testosterone in men (110). The mechanism for such a change appears consistent with earlier work supporting the development of hypogonadotropic hypogonadism as with extensive caloric deficient, weight loss and restricted food intake (see prior discussions). Historically the idea of caloric intake and energy status as being associated with the low testosterone in exercising men was alluded to in the 1980's but a systematic examination of the concept was not thoroughly pursued until recent times (44, 101).

It is now recognized that a state of LEA not only can lead to the Triad condition but also the "Reduced Energy Deficiency in Sports" [RED-S] condition. RED-S was designated as a separate entity from the Triad by an International Olympic Committee medical commission group of clinicians; and, is found in men as well as women. RED-S is different from the Triad as it is viewed as more broad in scope. It is defined as impaired physiological function including but not limited to, metabolic rate, menstrual function, bone health, immunity, protein synthesis, and cardiovascular health caused by relative energy deficiency brought on by a state of LEA (113).

The common etiology and a certain degree of overlapping symptomology of the Triad/RED-S have caused some to question whether they truly represent two distinct conditions (114). That difference of opinion requires more research to be fully resolved. What is clear is a state of LEA can lead to low testosterone levels in men. Hooper and associates show this clearly in their cross-section studies where LEA was linked to low testosterone in distance runners and triathletes (115, 116). For a full discussion of the endocrinological impact of RED-S the reader is direct to the recent review article by Elliot-Sale and associates (117).

Exercise Hypogonadal Male Condition

In 2005 Hackney and associates proposed the use of the term "Exercise Hypogonadal Male Condition" (EHMC) for exercise-trained men who showed lowered testosterone (19, 26). They based this recommendation upon work by their own and other research groups from the 1980's and 90's. This recommended terminology was targeted to exercising men who displayed functional hypogonadotropic hypogonadism and met certain criteria and was not intended for universal application to all exercising men with low testosterone. The key characteristics and traits of EHMC laid out by this research group were (19, 26):

- These men had testosterone levels at least 25% to 50% lower than expected for their age.
- The lowered testosterone levels did not appear to be a transient phenomenon related to the acute stress-strain of exercise training.
- The men were not experiencing a performance decrement or lack of motivation (i.e., overtrained).
- They had not experienced a major bodyweight loss in recent months.
- The men had a history of early involvement in sports resulting in them have many years of nearly daily exercise activity.
- The modality of exercise and training most frequency associated involved high volume endurance activities such as running, triathlons, cycling cross-country skiing, and race walking.

Regrettably, there has been some confusion in the research community concerning the EHMC terminology. That is, many researchers have assumed that the EHMC connotation was the same as exercising men displaying overtraining or Triad/RED-S (… *etcetera*) related to the lowered testosterone. EHMC as originally proposed over 15 years ago was for a different condition and one representing a potential adaptive response in the reproductive system HPG axis from chronic, long-term exercise exposure (see the following section). This point seems to have been overlooked and as such use of the EHMC term has been applied incorrectly, or entirely ignored altogether as

a categorical distinction for exercising men with persistent low, resting testosterone.

Special Considerations

Regrettably, it is nearly impossible to address the topic of testosterone and sporting activities without mentioning anabolic-androgenic steroids (AAS) and doping by athletes. AAS, which are the synthetically produced variants of naturally occurring testosterone, have been associated with certain sports for decades. While these products have valid and legitimate medical uses they are banned or prohibited by sports governing bodies for creating an unfair physiologic advantage (16, 21, 52). There are a great number of side-effects of AAS use, and the complications are variable and individually specific; but, one common outcome is a variant of hypogonadism developing (118). The hypogonadism in this situation can be during active AAS use as well as a long-term side effect once usage has ceased (118). It is advisable when considering some of the potential causes of hypogonadism in athletes, as discussed in prior sections that researchers and clinicians rule out AAS use as likely causative factor.

DYSFUNCTION OR ADAPTATION-REGULATORY ADJUSTMENT?

Much of the current contemporary research focuses on the role of energy balance and energy availability on the development of exercise relative hypogonadism. Ample evidence points to a negative energy balance, caloric restriction or a state of LEA leading to low testosterone development. This form of exercise hypogonadism-low testosterone is a transient phenomenon that can be abated with appropriate interventions (see the following section). As noted though, it has been proposed that not all formed of exercise hypogonadism-low testosterone fall into this category (119). Specifically for some men, this occurrence may represent an adaptation within the reproductive system due to their persistent and chronic exposure to large volumes of exercise training regularly; which has been termed the EHMC state.

Evidence supports that the reduction in testosterone inducing a form of exercise relative hypogonadism is detrimental in the case of men experiencing the overtraining and, or Triad/RED-S. These individuals have compromised health and physical performance that results in an inability to compete at their maximal potential, optimal level. These individuals are experiencing a classic endocrine dysfunction.

Conversely, men denoted as experiencing EHMC does not show the same compromised health and performance issues; and report no overt adverse signs or symptoms of poor health (although, granted not all studies examining EHMC men have thoroughly examined all aspects of their subject's health profile). These individuals do not appear to be experiencing an endocrine dysfunction, but it is hypothesized their condition reflects an adaptation-regulatory adjustment in the HPG axis in which a new set-point for what is a "normal" level of testosterone develops due to their chronic, regular exercise training, a view speculated on as well by other research groups (120).

Such a premise is in line with anthropological research and the energy constraint model as outlined by Pontzer (121). This model of Pontzer posits that total energy expenditure (TEE) is maintained within a narrow range. As daily physical activity increases, other components of daily energy expenditure are reduced to keep TEE in check. Non-essential expenditure would be expected to decrease first; essential activity would be spared unless physical activity workload becomes too excessive. Subsequently, moving from a sedentary to a chronic active lifestyle leads to a persistent downregulation of non-essential expenditures including reduced inflammation, reduced hypothalamic-pituitary-adrenal axis, and sympathetic nervous system reactivity, as well as reduced reproductive hormone levels and HPG axis function. Collectively these reductions lower the risk for a broad range of chronic diseases (e.g., cardiovascular disease; T2D, Type 2 diabetes) (121). In support of this model and the effect on reproductive function, Raichlen associates (122) found the Hadza, a hunter-gatherer population in northern Tanzania, where men accumulate nearly 2 h of moderate and vigorous physical activity daily, have testosterone concentrations roughly 50% lower to those in comparable North American men. Likewise, Trumble et al. found the Tsimane men, Bolivian foragers-farmers with high levels of daily physical activity, display similar testosterone reduction (30–35% lower) (123). Furthermore, generally resting testosterone is also lower among men in physically active non-industrial populations compared with those in less active, industrialized countries (124). Collectively these studies did not report their populations to be in high-stress situations (e.g., famine, warfare) or having insufficient food-caloric availability; hence, these hormonal changes seemed adaptive consequences of their lifestyle (121). Similar long term reproductive hormonal adjustments could be occurring in men designated as experiencing EHMC.

In support of this persistent downregulation phenomena as proposed by Pontzer, as a more chronic and regular physically active life-style develops, are the data presented in **Figure 4** (24, 35). This figure illustrates that the longer an endurance athlete (i.e., runner) is engaged in consistent and chronic endurance training, the lower their resting testosterone becomes. These data are from a cross-sectional, longitudinal case-control study ($n = 196$) in which the result suggests the level of reductions plateaus at approximately 30–35%. In this study, and all runners met the criteria for EHMC as noted earlier. One could argue that these are perhaps LEA related occurrence, but it seems unlikely that chronic LEA over years would not precipitate a myriad of health problems associated with that condition and prevent these athletes from training, competing and being in a good physical condition/health (which was reported by all the participants). Furthermore, earlier work by our research group demonstrated that both pituitary and testicular responsiveness—sensitivity to drug challenges is attenuated in EHMC men and was substantially less than matched, sedentary control men (125, 126). This is inline and supported by the findings of Bobbert et al. who show hypothalamic-pituitary regulatory sensitivity is adjusted with exposure to endurance exercise training (127).

Granted this premise is postulated on limited evidence and research findings and as such the proposed etiology for EHMC development is a "working hypothesis;" but to that end, the entire scope of available research dealing directly with male exercise-related hypogonadism is extremely small in its totality and an

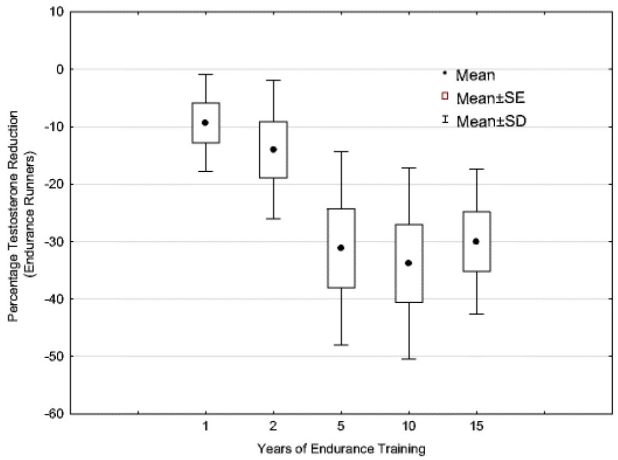

FIGURE 4 | Testosterone levels of endurance-trained runners (age = 18–57 years) expressed as a percentage decrease of the non-exercising matched control subjects ($n = 196$). For years training: 1 year, $n = 49$; 2 years, $n = 28$; 5 years, $n = 52$; 10 years, $n = 40$; 15+ years, $n = 27$ ($N = 196$). Adapted from information provided in reference (35). Used with permission.

evolving field of study. As stated by Sansone and associates, *"whether testosterone suppression is the result of a physiological adaptation to stress or an undesirable side effect of excessive training is a matter still open to debate"* and hence addition research on this important question needs to be pursued (128). That is, specifically researchers and clinicians need to address the questions within this statement and discern whether:

- The reduction in testosterone levels (and hypogonadism) are occurring as an undesirable side effect of exercise training, which suggests there are potentially harmful effects on the human physiology from performing chronic physical activity (*N.B.,* a line of thinking rarely discussed or mentioned in the exercise literature or media portrayal); or,
- If low testosterone (and hypogonadism) occurs as an adaptation response to the stress-stimulus of exercise training, would it be beneficial to leave such a condition untreated medically while athletes are training/competing? Or, would treatment of exercise-induced hypogonadism improve the relevant symptoms and overall health of the athlete (see **Table 4**)? (see the following section on treatment options).

These questions are open to discussion and future debate in the scientific and medical healthcare community.

WHAT ARE ACTIONS TO DEAL WITH LOW TESTOSTERONE IN ATHLETES-EXERCISERS?

Normally, the medical standard of care for treatment of male hypogonadism typically centers on the use of pharmaceutical agents to address the existing low serum testosterone, either through exogenous testosterone administration or drugs to stimulate the production of testosterone via the HPG axis. However, athletes who are competing may not use such means according to the World Anti-Doping Agency (WADA; international agency regulating and monitoring doping in sports). Endogenous testosterone and gonadotropin stimulator agents (acting on the HPG axis) fall into the WADA "List of Prohibited Substances and Methods" (categories: S1 *Anabolic agents*; S2, *Peptide hormones, growth factors related substances, and mimetics*) which if used constitutes a doping violation by the athlete (129). WADA does have Therapeutic Use Exception (TUE) options which would allow for pharmacological intervention and treatment for health reasons, but the scenario by which hypogonadism-low testosterone occurs in men as a consequence of exercise training does not fit into the circumstances by which WADA would grant a TUE to an athlete (21). That is, in athletes hypogonadism-low testosterone develops due to the consequences of exercise training, and is not a preexisting medical condition, or considered an acquired disease outcome.

This leaves the athlete with more behavioral related options for treatment of their condition; i.e., if they choose to treat it. In the case of the overtraining-Triad/RED-S treatment seems warranted and advised, but in the case of weight-restricted sporting activities or EHMC scenarios, such actions may not always be chosen by the athlete. In 2018 Hooper and colleagues presented in *The Physician and Sportsmedicine* a thorough overview of treatment approaches. In short, they recommended treatment be centered on non-pharmacological strategies including nutritional intervention, and modifications in training volume to improve energy availability and support the normal hormonal function of the HPG axis in male athletes (21).

Even though testosterone or anabolic stimulator agents are not permitted by WADA, if the athlete is suffering from low body mineral density, bisphosphonates (also called diphosphonates; e.g., Fosamax®) can be a viable option as they are permitted as a treatment by WADA. Some research findings support an increase in total or free testosterone concentrations through legal supplements (for example; such as D-aspartic acid and fenugreek [*Trigonella foenum-graecum*]) (130, 131). But, the reported outcomes from such supplements are not substantial and as such is seldom recommended.

Copious internet sites advertise for male sexual performance enhancer supplements, which supposedly promote testosterone elevations (and increase libido). These sites are typically vague in what is the physiological mechanism for such actions, proprietary as to what are their "secret ingredients," and heavy in testimonial accounts of efficacy; but lacking in scientific evidence. Furthermore, cases of such supplements containing substances that are banned by WADA have been reported; and ignorance of the contents of the supplement used by an athlete is not viewed as a viable excuse by WADA (132). Therefore, the athlete is not advised to experiment with supplements from such sites if they are actively competing and could be screened for doping violations.

Essentially, athletes and the clinicians working with them are left with few viable options for dealing with exercise-related hypogonadism and the consequences of the condition if they wish to stay within WADA guidelines. A review of the symptomology of hypogonadism, **Table 4**, clearly

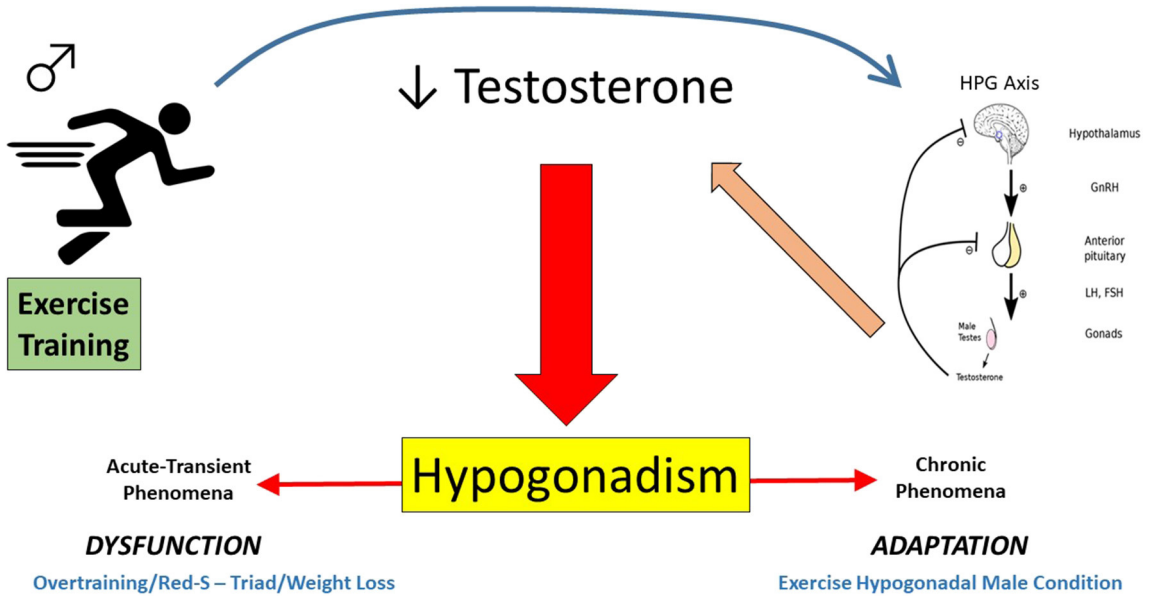

FIGURE 5 | Pictorial depiction of the proposed continuum of exercise-related hypogonadism-low testosterone in exercising men (Acute-Transient = affect lasting days/weeks/months, whereas Chronic = more persistent affect displayed for years). This excludes trauma-related or anabolic androgenic steroid induced hypogonadism.

demonstrates that such individuals (athlete or non-athlete) would be compromised in many aspects of daily life and function.

Interestingly, much of the current, contemporary medical emphasis related to low testosterone and hypogonadism in exercising men has focused on bone health. This is a critically important concern, but the other consequences as noted can also substantially impact on overall health and quality of life in an individual, and as such should not be ignored by healthcare providers.

SUMMARY, CONCLUSIONS, AND PERSPECTIVE

The renewed interest and explosion of new research on exercising men and hypogonadism development seems long overdue; as the topic has *flown under the rad*ar for many years. That said, investigators must approach this topic with a grasp of the scope of what has been done, what is known, and what needs to be addressed. This review was written with that intent.

The evidence clearly indicates that exercise training can result in the development of low testosterone in men, and at times the level of reductions reaches the clinical definition of hypogonadism. That said, some researchers support the use of terminology noting the existence of an exercise relative hypogonadism. The vast majority of the publishing findings, however, suggest the testosterone reductions found with training are in the normal clinical range (healthy, non-obese men), but frequently at the low end of the range.

It is proposed herein, that the development of exercise relative hypogonadism from training can be generalized into one of two categories; an acute, transient phenomenon (overtraining, Triad/RED-S … *etcetera*) or a more chronic phenomenon reflective of a training-induced adaptation (EHMC). **Figure 5** presents a schematic representation of the conceptual framework for the forms of exercise relative hypogonadism proposed, unrelated to trauma events or AAS use.

The physiological mechanisms by which low testosterone-hypogonadism develops presently unresolved, but theories revolve around either peripheral or central disruption of the HPG axis resulting in hypogonadotropic hypogonadism. Specifically involving either stress hormone interference or caloric deficient/energy availability compromise of the axis function. Most current contemporary research work has focused on the latter, and almost explicitly on the role of LEA associated axis disruption. Although it is important to remember that low testosterone-hypogonadism can exist in athletes-exercisers due to other scenarios such as TBI events or AAS use, and should always be ruled-out before assuming other causalities.

In looking to the future, it is important to recognize the available research literature is limited in number and need for expansion. Also, there is a need to have more replication of existing findings. Furthermore, many of the existing studies are of a retrospective, cross-sectional approach and involve small sample sizes. These types of studies are informative but more prospective, experimental research designed is needed where variables are manipulated which allows addressing of cause and effect issues. Granted, such approaches are desirable in executing the scientific method, but problematic in logistics, ethics and demanding financially. Nonetheless, they are needed.

Clinical attention is sorely needed for the male athlete-exerciser suffering from the debilitating aspects of the Overtraining Syndrome and, or Triad/RED-S conditions. First and foremost they should be the ones to be aided by future research endeavors as their health, and in some cases, livelihood is being adversely affected by their conditions. Furthermore, these individuals may suffer long-term, delayed health consequences we are currently unaware of; future researchers should examine this issue too. As to the EHMC individuals who displayed an exercise relative hypogonadism (proposed due to an adjustment in the HPG regulatory axis; i.e., allowing for a new set-point lowering of testosterone levels), it is entirely unclear is a clinical intervention is warranted (or desired) since negative health consequences are not reported. Nonetheless, more expansive healthcare assessments and evaluations based studies are recommended to ensure there are not some insidious consequences thus far undetected in such men.

Finally, it is recommended that exercise physiologists who study hormones and clinical endocrinologists who are interested in exercise attempt to work together more closely in a cooperative fashion on this issue—this has not always been the case in the past (133, 134). This type of collective team approach will most surely lead to a more clear and precise understanding of how exercise and the training process influence the reproductive system in women and men.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Growth Hormone(s), Testosterone, Insulin-Like Growth Factors, and Cortisol: Roles and Integration for Cellular Development and Growth With Exercise

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Hormones are largely responsible for the integrated communication of several physiological systems responsible for modulating cellular growth and development. Although the specific hormonal influence must be considered within the context of the entire endocrine system and its relationship with other physiological systems, three key hormones are considered the "anabolic giants" in cellular growth and repair: testosterone, the growth hormone superfamily, and the insulin-like growth factor (IGF) superfamily. In addition to these anabolic hormones, glucocorticoids, mainly cortisol must also be considered because of their profound opposing influence on human skeletal muscle anabolism in many instances. This review presents emerging research on: (1) Testosterone signaling pathways, responses, and adaptations to resistance training; (2) Growth hormone: presents new complexity with exercise stress; (3) Current perspectives on IGF-I and physiological adaptations and complexity these hormones as related to training; and (4) Glucocorticoid roles in integrated communication for anabolic/catabolic signaling. Specifically, the review describes (1) Testosterone as the primary anabolic hormone, with an anabolic influence largely dictated primarily by genomic and possible non-genomic signaling, satellite cell activation, interaction with other anabolic signaling pathways, upregulation or downregulation of the androgen receptor, and potential roles in co-activators and transcriptional activity; (2) Differential influences of growth hormones depending on the "type" of the hormone being assayed and the magnitude of the physiological stress; (3) The exquisite regulation of IGF-1 by a family of binding proteins (IGFBPs 1–6), which can either stimulate or inhibit biological action depending on binding; and (4) Circadian patterning and newly discovered variants of glucocorticoid isoforms largely dictating glucocorticoid sensitivity and catabolic, muscle sparing, or pathological influence. The downstream integrated anabolic and catabolic mechanisms of these hormones not only affect the ability of skeletal muscle to generate force; they also have

implications for pharmaceutical treatments, aging, and prevalent chronic conditions such as metabolic syndrome, insulin resistance, and hypertension. Thus, advances in our understanding of hormones that impact anabolic: catabolic processes have relevance for athletes and the general population, alike.

Keywords: anabolic, catabolic, protein synthesis, skeletal muscle, endocrine, glucocorticoid, androgen, signaling

INTRODUCTION: THE COMPLEXITY OF HORMONE SIGNALING

Cell signaling may be described as a critical part of communication that governs basic activities of cells and coordinates all cellular actions. Hormonal signaling is part of a complex system involving a plethora of molecules. The actions and potency of any hormone will be affected by all components of the signaling chain (**Figure 1**). Depending on the cellular environment and negative feedback control, some components of the chain may be more proactive in eliciting a response or adaptation. To make a simple analogy, hormone signaling is analogous to playing a team sport. All players on the team have distinct roles and responsibilities during each play. Success depends on how well the team executes and communicates in an integrative manner to carry out team objectives. Hormones work in a similar manner. All stages from production, release, transportation, tissue uptake, and intracellular signaling must be considered in an integrative manner to accurately portray the effects of the hormone-receptor interaction (1). Thus, viewing only a fraction of the signaling chain may underrepresent the entirety of the hormonal actions. Science has shown the great complexity of hormonal signaling as strides have been made in cell biology and biochemistry.

TESTOSTERONE SIGNALING PATHWAYS, RESPONSES, AND ADAPTATIONS TO RESISTANCE TRAINING

Testosterone (T) is an anabolic-androgenic steroid hormone that primarily interacts with androgen receptors (AR) in skeletal

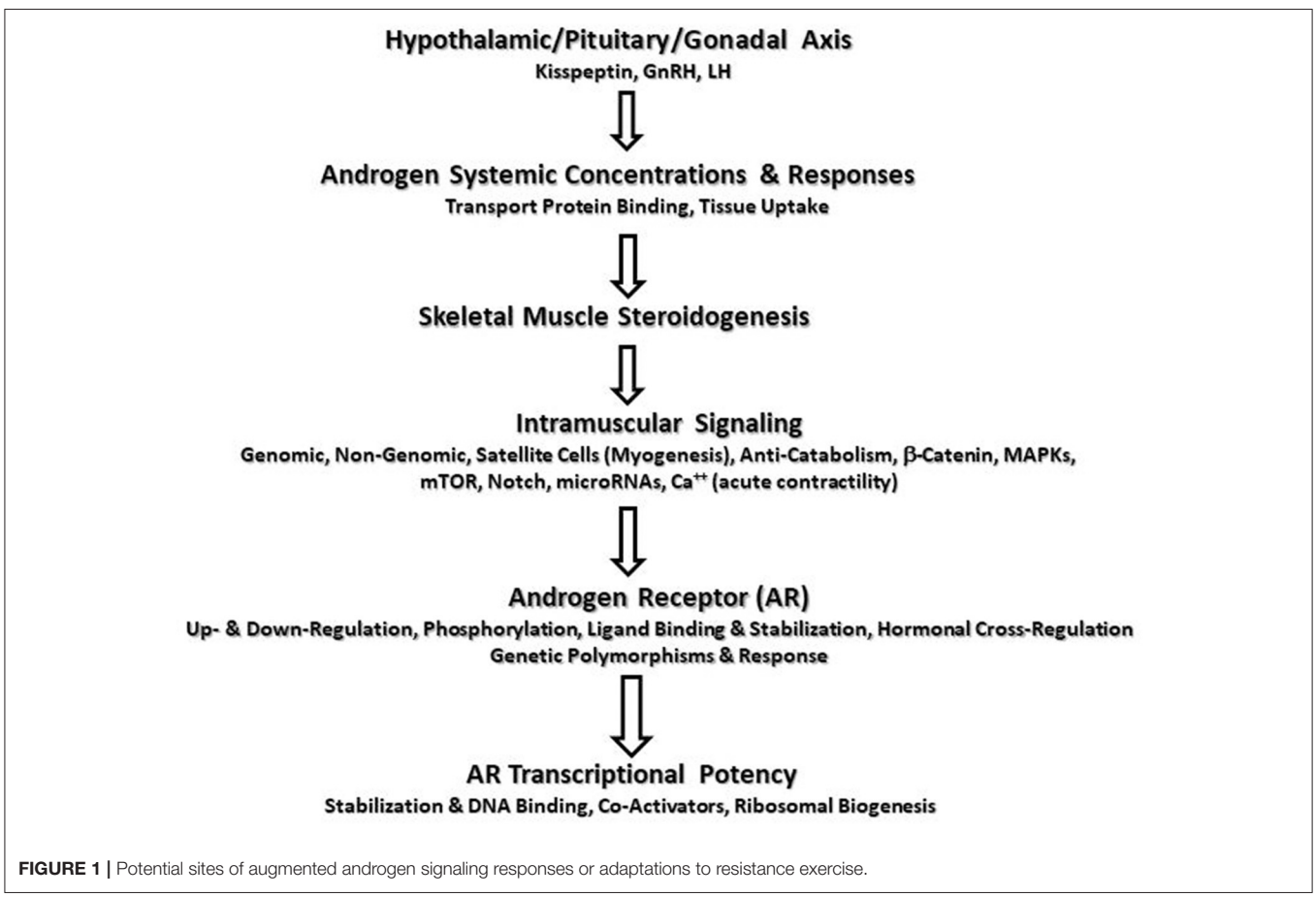

FIGURE 1 | Potential sites of augmented androgen signaling responses or adaptations to resistance exercise.

muscle whereas the more-potent dihydrotestosterone (DHT) primarily acts within sex-linked tissues with a possible secondary role in skeletal muscle (2) Although skeletal muscle content of DHT has been correlated to muscle strength and power (3), T replacement with and without dutasteride or finasteride (5α-reductase inhibitors) produces similar increases in lean tissue mass and muscle strength (4, 5). Thus, it is currently unclear if DHT is more anabolic in skeletal muscle than T alone. Testosterone performs a multitude of ergogenic, anabolic, and anti-catabolic functions in skeletal muscle and neuronal tissue leading to increased muscle strength, power, endurance, and hypertrophy in a dose-dependent manner (1). Genomic androgen/AR binding may alter the expression of more than 90 genes, several of which are involved in the regulation of skeletal muscle structure, fiber types, metabolism, and transcription (6). Studies show androgens increase protein synthesis rates, and reduce protein catabolism and autophagy (7). Castration reduces several markers of ribosome biogenesis that may only be partially restored by androgen treatment coupled with castration (8). In addition, evidence indicates that androgens may play a role in stimulating physical activity in males (9). Thus, androgens play important roles, in part, in mediating skeletal muscle protein synthesis and adaptations to resistance training (RT).

The primary androgen, T, is synthesized from cholesterol and other precursors in the Leydig cells of the testes (>95% in men with some adrenal contributions) under control of the hypothalamic-anterior pituitary-gonadal axis where gonadotropin releasing hormone (GnRH) stimulates the release of luteinizing hormone (LH) from gonadotrophs. GnRH functions under the control of hypothalamic neuropeptides such as kisspeptins, neurokinin-B, dynorphin-A, and phoenixins (10, 11). Kisspeptin (a 54 amino acid peptide) is encoded from the *KISS1* gene and is released from neurons within the arcuate nucleus and anteroventral periventricular nucleus of the hypothalamus as well as other tissues outside of the CNS. Kisspeptin binds to KISS1R (GPR54) receptors on GnRH neurons and causes the release of GnRH (via a G-protein 2nd messenger system). Hypothalamic neuropeptide expression is dependent on metabolic status (12); however, little is known regarding exercise responses. Khajehnasiri et al. (13) examined moderate vs. intense treadmill training for 6 months in rats and showed intense exercise (but not moderate) led to decreased GnRH mRNA and serum total T (TT) and LH. No differences were seen in kisspeptin mRNA although some differences were seen neurokinin-B and pro-dynorphin mRNA. Short-term administration of kisspeptin (Kp-54) or kisspeptin analogs (i.e., Kp-10) increase LH and TT in a dose-dependent manner in men with increases in LH but little change in TT in women (11, 14).

In women, ovarian and adrenal production of androgens are major sources (15). Skeletal muscle contains the enzymes and produces small amounts of androgens (16, 17). Testosterone is released into circulation and transported mostly by sex hormone-binding globulin (SHBG) (44–60%) and loosely-bound to albumin or other proteins. Free (unbound, up to 2% in circulation) T (FT) is taken up by tissues for binding to membrane-bound or cytoplasmic ARs and subsequent cellular signaling. However, some evidence suggests the possibility of an alternative mechanism to the "free hormone" hypothesis where membrane-bound receptor proteins (e.g., megalin—a low-density lipoprotein endocytic receptor) have been identified in multiple tissues including skeletal muscle, although the ability to internalize the bound steroid hormone complex and enable uptake via endocytosis still remains to be elucidated (18, 19). Nevertheless, SHBG concentrations influence T binding capacity and FT available for diffusion across the cell membrane. The presence of G-protein coupled receptors for SHBG in skeletal muscle membranes has been suggested to influence (i.e., inhibit or stimulate) non-genomic androgen signaling via modulation of adenylate-cyclase with cAMP synthesis and activation of protein kinase A (20), although it is currently unclear as to the magnitude of, if any, impact it may have during androgen signaling. Some T is converted to the more potent DHT via 5α reductase. This enzyme is present in skeletal muscle and circulating DHT can diffuse into muscle cells and bind to ARs with higher affinity than T. Some T is aromatized to estrogens, and final metabolism of T occurs in the liver and kidneys where inactivated metabolites are excreted in urine.

The responses of T to RT in men and women have been extensively reviewed (2, 21). Most studies show significant elevations of TT and FT in men through 30 min into recovery with few changes in resting baseline concentrations. In women, studies show no or limited acute elevations. The magnitude of the acute responses is affected by many factors including the demands of the protocol, nutritional intake, training experience but mostly due to plasma volume reductions and reduced clearance (1). The ramifications of acute elevations during RT are unclear but appear to be part of the macro-signaling cascade affecting, in part, muscular adaptations. Some studies indicate relationships between T elevation and AR up-regulation, strength and hypertrophy enhancement (22–25) whereas other reports indicated no such relationships (26). These conflicting results demonstrate the complexity of hormonal responses and the likelihood several factors are contributing to the response. Acute T responses must be viewed within the context of multiple skeletal muscle signaling pathway adaptations as well the well-known interaction between T signaling and other hormone signaling pathways involving the GH isoforms and aggregates, IGF-I and mechano-growth factor (MGF), insulin, and cortisol responses (27–29).

SKELETAL MUSCLE STEROIDOGENESIS

Skeletal muscle steroidogenesis from DHEA is another potential source of T (16). Steroidogenic enzyme content and T concentrations in skeletal muscle are similar between men and women (17). In older men, 12 weeks of RT increases skeletal muscle DHEA, FT, DHT, 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), 5α-reductase type I content, and AR protein content (30). The increased DHT and FT were related to increased isokinetic strength, muscle CSA, and power (30). However, RT studies in younger men and women show no changes in muscle T or steroidogenic enzymes (17, 31). However, responders to RT were

shown to increase 5α-reductase (31). It is possible that increased muscle steroidogenesis may be a mechanism to help counteract T reductions in older men undergoing RT but less likely in healthy, young men.

ANDROGEN SIGNALING PATHWAYS

Historically, androgen signaling was thought to be governed predominately by classical genomic signaling common to steroids and steroid receptors. FT or DHT (or other synthetic anabolic steroid) binds to a cytoplasmic AR, dissociates from heat shock proteins, and the complex translocates to the nucleus to bind to specific androgen response elements on DNA. The androgen/AR complex serves as a transcription factor leading to protein synthesis with the help of co-activator proteins. Prior to androgen stimulation of skeletal muscle tissue, higher order muscle tissue activation is needed. Increased muscle strength, power, endurance, and hypertrophy resulting from RT begins with neural stimulation and the optimal recruitment of motor units based on the size principle. Androgen signaling increases neural transmission, neurotransmitter release, motoneuron cell body and dendrite size, and regrowth of damaged peripheral nerves (32). Androgen signaling in cerebral neurons is needed to maintain muscle mass in fast-twitch muscles despite elevations in circulating T (33). This may be regulated by reduced spontaneous locomotor activity. Thus, the RT stimulus is critical to activation of muscle tissue and the role of androgens in enhanced neural drive warrants further study. Genomic signaling accounts for a large magnitude of androgen actions; however, a number of other signaling pathways have been identified demonstrating the complexity of androgen signaling its impact on skeletal muscle development.

Non-genomic AR signaling has been a topic of interest and debate in recent times. Non-genomic actions are rapid with short latency periods acting independently (mostly at the cell membrane and cytoplasmic levels) of nuclear receptors (20). Non-genomic effects are thought to be mediated by direct binding to a target molecule, through intracellular AR activation (i.e., Src kinase), through a transmembrane AR receptor, or via changes in membrane fluidity (20). Non-genomic signaling may involve G-protein 2nd messenger system signaling. Non-genomic signaling may increase intracellular calcium concentrations (possibly affecting contractile properties) (34), stimulate activation of MAPK signaling (35), and mammalian target of rapamycin (mTOR) pathway signaling (36). Binding of bound or unbound T to ARs activate G-protein-linked receptor that activates PI3K and phospholipase C, increases IP_3 which binds to receptors on the sarcoplasmic reticulum to liberate calcium. Calcium may activate Ras/ERK1/2 pathway signaling (34). Castration reduces Akt/mTORC1 signaling and AR protein expression whereas nandrolone decanoate administration has the opposite effect (37). Basulato-Alarcon et al. (36) showed T increased MTORC1/S6K1 pathway signaling possibly through PI3K activation of Akt. Zeng et al. (38) reported DHT implantation plus exercise in rats for 10 days increased AR and IGF-I mRNA and AR phosphorylation (Ser210). They reported greater muscle hypertrophy via mTOR signaling and suggested cross-talk between IGF-I signaling and non-genomic AR signaling was critical to the augmented combined effects. Non-genomic signaling occurs rapidly within seconds to minutes, much faster than classic genomic signaling which takes hours, and requires constant presence of androgens to maintain intracellular signaling. It is unclear if the increased intracellular calcium enhances force production (35). The impact of non-genomic signaling to training-related adaptations remains unclear; however, it appears the interaction between genomic and non-genomic signaling pathways appear critical to maximizing muscle hypertrophy (36). MAPK signaling may phosphorylate the AR increasing its transcriptional capacity.

Testosterone may be anti-catabolic by either decreasing glucocorticoid receptor (GR) expression, interfering with cortisol binding, or the AR-T complex may compete with cortisol-GR complex for *Cis*-element binding sites on DNA (and vice versa). DNA binding domains and ligand binding domains between the AR and GR are 79 and 50% homologous. Glucocorticoids increase expression of atrophy-related genes (i.e., atrogin-1, MuRF1, and forkhead box 01) and androgens reduce atrogene expression, reduce GC-related IGF-I expression inhibition, and down-regulate GR expression in skeletal muscle and muscle satellite cells (39).

Androgens mediate some anabolic functions through myogenesis via multiple pathways. Satellite cells and myoblasts possess ARs and androgen binding increases satellite cell activation, proliferation, mobilization, and differentiation, and incorporation into skeletal muscle (40). Androgens increase myogenesis via increased Notch signaling of satellite cells possibly due to reduced myostatin and increased Akt activation (41) and through increased expression of IGF-I in satellite cells and muscle fibers (28). Androgen binding to ARs on mesenchymal pluripotent cells increases their commitment to myogenesis rather than adipogenesis (42). Androgens may increase follistatin expression and decrease or increase myostatin and down-regulate or up-regulate its gene expression, down-regulate Acvr2b receptor mRNA and Smad 2/3 phosphorylation, decrease myostatin signaling molecules, increase myogenic marker up-regulation, e.g., MyoD, myogenin, myotube formation, and myonuclei number and central positioning (39, 42, 43).

Genomic androgen/AR binding may enhance muscle performance via stimulating the Wnt-β-catenin pathway. Wnt binds to frizzled/lipoprotein receptor protein 6 receptors and activates disheveled and inhibits glycogen synthase kinase-3 (GSK-3) reducing β-catenin dephosphorylation and increases its activity. The FT-AR complex inhibits GSK-3 and increases β-catenin where it translocates to the nucleus, binds to DNA response elements (T-cell factor/lymphoid enhancer factor 1 –TCF/LEF), increases transcription, and activation of muscle satellite cells. As β-catenin lacks a nuclear localization sequence and needs cytosolic proteins with a sequence to assist in translocation, androgen/AR complex may chaperone β-catenin to the nucleus where it binds to specific DNA elements. The presence of T increases AR-β-catenin interaction and transcriptional capacity. Positive correlations were shown

between AR protein content and Wnt5 expression and muscle mass and Wnt5 expression in rats (44). Testosterone treatment increased Wnt5 protein expression and muscle size in a dose-dependent manner (44). Spillane et al. (45) reported significant up-regulation of VL muscle β-catenin following upper and lower body RT at 3 and 24 h PE and increased AR-DNA binding capacity and suggested the increased binding capacity was linked to greater β-catenin pathway signaling.

THE IMPORTANCE OF ANDROGEN SIGNALING FOR MUSCLE STRENGTH AND HYPERTROPHY

Human and animal studies (using a variety of research models) demonstrated the importance of androgens for maintaining and increasing skeletal muscle strength and mass. Muscle strength and mass are reduced significantly (by up to 20%) in male AR knockout mice (6). In satellite cell-specific AR knockout mice, type II to I fiber conversions and reduced muscle strength have been shown (2014). Other muscle-specific AR knockout mice models have shown reduced lean tissue mass and fast-to-slow fiber type conversion without concomitant changes in muscle strength (46). Inoue et al. (47) showed that administration of an AR antagonist in rats (oxendolone) during 2 weeks of electrical stimulation of the gastrocnemius muscle attenuated 70% of stimulation-induced hypertrophy compared to the control condition. The same research group showed that electrical stimulation of rat gastrocnemius increased AR number by 25% within 3 days and the AR up-regulation preceded muscle hypertrophy. Deschenes et al. (48) showed RT in rats increased AR binding capacity in fast-twitch muscles (i.e., extensor digitorum longus) of rats but not slow-twitch (i.e., soleus). In humans, hypogonadism, aging, glucocorticoid use, obesity, and androgen deprivation therapy (ADT) are negative regulators of androgen actions. A study from Kvorning et al. (49) showed that 8 weeks of RT with or without the GnRH analog goserelin (that reduced TT to $\leq$2 nmol/L) significantly attenuated increases in isometric strength and leg lean tissue mass. The authors concluded that suppression of T below 10% of normal levels strongly attenuates the increase in lean tissue mass and muscle strength seen during RT (49).

ANDROGEN RECEPTOR PHYSIOLOGY

The signaling effects of androgens are mediated through the AR which belongs to a family of steroid receptors. The AR gene is located on the q arm of chromosome X at position 11–12 and contains 8 exons that code for approximately 2,757 base pair open reading frames (50, 51). The first exon codes for the N-terminus transcription activation domain; exons 2–3 code for the central DNA binding domain; exons 4–8 code for the C terminus ligand-binding domain (50). The AR consists of ∼920 amino acids (∼110 kD or more when phosphorylated; and consists of 12 α-helices and 2 β-sheets) and is found in nearly all tissues in the human body and other truncated versions with biological activity have been identified (52). The presence of ARs correlates highly with the functions of androgens. AR activity may be altered by phosphorylation at several serine (and threonine and tyrosine) residues particularly in the transcription region (e.g., Ser81, 94, 213, 515, 651), and through methylation, ubiquitination, sumoylation, and acetylation at various sites (>23 sites). For example, phosphorylation of serine residue 651 is needed for full transcriptional activity (53). Phosphorylation may occur during ligand binding and through other signaling pathways indicating that the AR is cross-regulated by other ligand-receptor interactions (54). Thus, phosphorylation may augment androgen/AR transcriptional action (in the presence or absence of androgens) and demonstrate the high intracellular regulatory potential of the AR (55). The AR is activated through ligand-independent mechanisms including IGF-I induced MAPK-ERK1/2, p38, and JNK phosphorylation in C_2C_{12} muscle cells (56). The AR may modulate its phosphorylation state to sensitize itself to anabolic signals in the presence of lower androgens. A recent study from Nicoll et al. (57) showed that men have higher baseline AR protein content than women; however, women had greater AR phosphorylation at rest at ser515 and ser81 residues indicating that the AR activity could be augmented independent of ligand levels.

ANDROGEN RECEPTOR BINDING, STABILIZATION, AND TRANSCRIPTION

Ligand binding occurs at the C terminus of the AR. Upon androgen binding to the ligand binding domain (LBD), dissociation from the heat-shock proteins occurs, hyperphosphorylation, dimerization, and conformational changes occur converting the AR to a transcription factor that interacts with *androgen response elements or AREs* of DNA (58). The AR DNA binding domain contains zinc finger motifs that recognize both consensus and selective AREs. Androgen binding activates and stabilizes the AR and induces N→C terminus interaction which is selectively induced by high-affinity T and DHT, and lower-affinity anabolic steroids (e.g., oxandrolone, fluoxymesterone) (59). Greater stabilization is seen with DHT more so than T as T dissociates from the AR 3 times faster than DHT (60). Testosterone is the primary androgen interacting with ARs in skeletal muscle. Androgens have different potencies, in part, due to affinity and binding properties of the AR.

The androgen/AR complex serves as a transcription factor leading to increased protein synthesis. The N-terminal domain is responsible for transcription activation. Androgen binding to the AR completes the groove that serves as a recruiting surface for co-activators (attract co-regulator motifs, e.g., LxxLL, FxxLF) that form a bridge between the DNA-bound AR and the transcriptional machinery. Co-regulator proteins mostly interact with the N-terminus domain (with some binding at the LBD). More than 250 co-regulators exist many of which are co-activators (61). Co-activators augment transcriptional activity and enhance signaling, e.g., SRC-1, SRC-3, TIF-2, ARA24, ARA160, BAF57, BAF60A, ARA54, ARA70 whereas co-repressors (e.g., SMRT, SIRT1, Ankrd1) reduce transcriptional activity. Many co-activators involve chromatin remodeling,

histone acetyltransferase, methyltansferse, and demethylase, DNA stabilization, and pre-initiation complex (PIC) recruitment whereas some corepressors tighten nucleosomes limiting accessibility (61). Micro RNAs have been shown to mediate AR function via co-repressor expression inhibition (62). The AR LBD coactivator binding groove is a target of drugs to manipulate AR activity especially in the development of anti-prostate cancer drugs (63). However, little is known regarding RT and potential up-regulation of co-activators which may serve as a great area of interest for future research.

Several models have been proposed to explain mechanism(s) involved in gene transcription including chromatin remodeling, direct binding of AR to proteins in the PIC such as transcription factors TFIIB (i.e., transcription factor IIB) and TFIIF (i.e., transcription factor IIF), and AR interactions with complexing proteins and/or co-regulators to enhance assembly of the PIC (64, 65). It appears that a multiple-step model that incorporates combinations of these models may be most accurate. Upon DNA binding and co-activator recruitment, the co-activators covalently modify targeted histone N termini to loosen the nucleosomal structure (via modifying the net charge) to facilitate transcription in the repressed chromatin (61). Transcriptional activation by AR ultimately requires the recruitment of RNA polymerase II to the promoter of target genes. The co-regulators, as well as the ligand-bound activation of AR, enhance assembly of and stabilize the *PIC,* which is the assembly of general transcription factors (64). Polymerase II recruitment is mediated through the assembly of the PIC, the first step of which is binding of TATA binding protein (TBP) near the transcriptional start site. TBP is part of a multi-protein binding complex with TFIID that induce bending of DNA, which brings the sequence of the TATA element closer to interact with general transcription factors and co-regulators. TFIIB binds directly to TBP and functions to recruit the TFIIF-polymerase II complex. TFIIF domains also serve in transcription initiation and elongation. ATPase, the kinase TFIIE, and helicase TFIIH are then recruited to polymerase II to facilitate DNA strand separation before transcription initiation.

ANDROGEN RECEPTOR POLYMORPHISMS AND PERFORMANCE

The first exon contains several regions of repetitive DNA sequences one of which is the CAG (polyglutamine) triplet repeat that begins at codon 58 and extends for >21 repeats. This length varies between 8 and 35 repeats (18–24 is most common). Another is a polyglycine (GGN) repeat in the transactivation region. Genetic polymorphisms yielding a variety of repeats are associated with a variety of conditions including male infertility, hypogonadism, prostate, and testicular cancer, bone disease, neurodegenerative, and cardiovascular disease (66). These could contribute, in part, to responder or non-responder status when examining training adaptations. Long CAG repeats may interfere with androgen actions whereas short repeats (CAG and GGN) are associated with increased AR protein expression and androgen action. However, contradictory results were shown where CAG repeat number was positively related, inversely related, or not related to lean body mass (LBM), T, or FT concentrations, and muscle strength in young and older men (67–70). Nielsen et al. (71) showed inverse relationships between CAG repeat number and thigh and trunk muscle size to where every reduction in repeats of 10 equaled an increase of muscle size by 4%. Thus, performance phenotypes based on AR candidate gene polymorphisms remain unclear.

ANDROGEN RECEPTOR UP-REGULATION AND ADAPTATIONS TO RESISTANCE TRAINING

AR protein content is a critical variable in RT-induced androgen-mediated skeletal muscle protein accretion in healthy men (31). The concentration of ARs in skeletal muscle depends on the muscle fiber type, sex, training status, and androgen concentrations. Several studies investigated AR responses to RT (**Table 1**). Most studies show baseline AR protein content does not change although one study found significant down-regulation (85) and one study reported up-regulation in older men (30). The most expected pattern of change is acute up-regulation of AR mRNA and protein content within 1–2 days of RT followed by a return to baseline unless another workout is performed. Initially, AR protein content may not change or be down-regulated within the first 2 h PE in the fasted state (73). Post-workout protein/CHO feeding may ameliorate this response (77). Notable up-regulation of AR mRNA and protein is seen ∼28 h PE (89) while is most pronounced 48 h PE (74, 75). The response is similar in young and old men (80) and may lessen over time with training experience (81). The AR mRNA and protein up-regulation correlated to TT and FT concentrations in the blood (19, 79). AR protein content explains a large amount of variance in muscle hypertrophy seen during RT (84), and its role may be potentiated with interaction of other hormones such as growth hormone and IGF-I.

GROWTH HORMONE: A NEW COMPLEXITY WITH EXERCISE STRESS

The concept that a "hormone" caused growth was first proposed in 1911 (90). Since that time, and as noted on PubMed, >126,000 publications have reported on some feature of growth hormone (GH). Of that large number, comparatively few (∼2,800) address its role in human exercise. In turn, only a small subset of these exercise studies considered the issue and importance of GH assay choice employed and the large difference it can make in interpreting experimental data. The purpose of this review is to (a) briefly review early history of GH bioassays, (b) summarize the data base that addresses the relevance of assay choice in performing exercise stress studies in humans, and (c) suggest how emerging data concerning GH processing in the pituitary gland may offer new direction(s) for the study of this anabolic hormone in health and aging.

TABLE 1 | Summary of androgen receptor changes following resistance training.

References	Subjects	Muscle	Protocol	Biopsy time	Results
Kadi et al. (72)	UT men, PL on AAS, PL—no AAS	VL, TR	Cross-sectional comparison	BL	PL > % AR-positive myonuclei in TR than UT – P(AAS) > % AR-positive myonuclei than drug-free PL – ↔ in VL between groups
Ratamess et al. (73)	RT men—fasted	VL	SQ: 1 or 6 sets of 10 reps, 80-85% 1RM–2-min RI	1 h PE	1 set = no change AR protein 6 sets = sig. ↓ AR protein – BL AR content correlated with 1RM squat strength
Bamman et al. (74)	UT men and women	VL	SQ: 8 × 8 ECC reps (∼110% 1 RM) or CON reps (∼85% 1 RM)	48 h PE	AR mRNA ↑ by 102% (CON) and 63% (ECC)
Willoughby and Taylor (75)	UT men	VL	SQ, LP, KE–3 sets of 8–10 reps each –75 to 80% 1RM, 3 min RI – 3 sessions separated by 48 h	48 h PE	AR mRNA ↑ 35, 68, 43% after each workout AR protein ↑ 40, 100, 202% after each workout – AR mRNA/protein correlated with PE TT and FT
Vingren et al. (76)	RT men and women—fasted	VL	SQ: 6 × 10 reps –80% 1RM, 2-min RI	10 and 70 min PE	AR protein ↓ at 10 min in women; ↓ at 70 min in men and women – AR protein men > women
Kraemer et al. (77)	RT men	VL	SQ, BP, BOR, SP: 4 × 10 reps each 80% 1RM, 2-min RI – Water + L-carnitine or feeding + L-carnitine post RE	1 h PE	Feeding ↑ AR protein
Spiering et al. (25)	UT men—fasted	VL	5 × 5RM KE following rest (CON) or after upper body RE eliciting TT ↑ by 16% (HT)	10 and 180 min PE	AR protein at 180 min tended to ↓ in CON, ↔ in HT from REST; AR protein following HT > CON
West et al. (78)	MT men and women—fed PE	VL	LP–5 × 10 reps; leg ext/curl superset 3 × 12 reps, 1 min RI	1, 5, 26, 28 h PE	↔ AR mRNA at 1, 5 h AR mRNA ↑ 28 h > 26 h
Poole et al. (19)	UT young and older (60–75 years) men	VL	9 sets of lower-body RE, 10 reps each set, 80% of 1RM, 2-3 min RI—completed 3 workouts	24, 48 h PE	↔ AR mRNA 48 or 24 h post RE over 3 days AR mRNA young men > old – PE TT at 30 min correlated to AR mRNA
Hulmi et al. (79)	RT older (57–72 years) men	VL	Whey or placebo: LP–5 × 10RM, 2-min RI	1 and 48 h PE	AR mRNA trend for ↑ in whey group; when groups combined sig. ↑ in AR mRNA 1 and 48 h Trend for ↑ AR protein in placebo 1 h – Change in AR mRNA 1 h correlated to PE TT response
Ahtiainen et al. (80)	UT young and older (60–65 years) men	VL	Acute RE before & after 21 weeks of RT: protocol—LP – 5 × 10RM, 2 min RI	1 and 48 h PE	↔ Acute AR protein and mRNA response over 21 weeks – AR response correlated to 1RM strength, LBM, and CSA – ↔ BL AR mRNA/protein between old & young men or after RT
Ahtiainen et al. (81)	RT young men	VL	LP – 5 × 10RM, SQ – 4 × 10RM, 2 min RI	1 and 48 h PE	↔ AR mRNA and protein
Ahtiainen et al. (82)	UT young and older (70–75 years) men	VL	Acute RE before and after 12 months of lower-body RT: protocol—LP – 5 × 10RM, 2-min RI	IP (0) and 2 h PE	↔ AR content 0 and 2 h Chronic: ↔ BL VL AR content – No difference between BL VL AR content in young and old men
Kvorning et al. (27)	Young men, limited RT experience	VL	8 weeks of RT: GnRH analog (goserelin, 3.6 mg 3 times to ↓ TT) or placebo; acute RE pre and post RT	BL, 4, 24 h PE	Blocked TT and RT had no effect on AR mRNA acute or chronic at BL
Nilsen et al. (83)	Men with prostate cancer on ADT	VL	16 weeks of RT	BL	↔ BL AR protein content
Sato et al. (30)	UT young and older (mean = 67 years) men	VL	12 weeks of RT: 3 days/week, KE and LC–3 × 10 reps, 70% of 1RM, 3-min RI	BL	AR protein in young men > old men – BL AR protein ↑ in old men (no post biopsies taken for young men)
Morton et al. (31)	Young RT men	VL	12 weeks of RT: high reps (20–25 reps with 30–50% of 1RM) or low reps (8–12 reps with 75–90% of 1RM)	BL	↔BL AR protein content over 12 weeks – AR protein content in responders > non-responders

(Continued)

TABLE 1 | Continued

References	Subjects	Muscle	Protocol	Biopsy Time	Results
			– Divided subjects into responders vs. non-responders		– AR protein content correlated with LBM, type I, and type II muscle CSA
Mitchell et al. (84)	UT young men	VL	16 weeks of RT: 4x/wk—upper/lower body split: 3 × 6–12 reps, 1–2 min RI	BL	↔ BL AR protein content –Δ AR protein correlated to fiber CSA – AR protein & p70S6K phosphorylation accounted for 46% of variance in size
Mobley et al. (85)	UT young men	VL	12 weeks of RT: 3 days/week, 5 exercises —undulating periodization, 4–10 reps	BL	↓ BL AR protein content similar in low, moderate, and high responders
Haun et al. (86)	Young previously RT men	VL	6 weeks of RT: 3 days/week, 10 reps per set, 60% of 1RM, 10–32 sets per exercise per week	BL	↔ BL AR protein content in high and low responders
Roberts et al. (87)	UT young and older (mean = 68 years) men	VL	Acute RE: SQ, LP, KE –3 × 10 reps, 80% of 1RM, 3 min RI	24 h PE	BL AR mRNA in older men > young – ↔ AR mRNA 24 hrs PE in either group – FT negatively correlated with AR mRNA
Brook et al. (88)	UT young and older (~69 yrs) men	VL	6 weeks of RT: unilateral KE, 6 × 8 reps 75% of 1RM	BL, 90 min PE	↔ BL AR mRNA ↔ PE AR mRNA

PL, powerlifters; AAS, anabolic-androgenic steroids; BL, baseline; IP, immediate post exercise; RT, resistance trained; RE, resistance exercise; 1RM, one repetition-maximum; RI, rest intervals; VL, vastus lateralis; TR, trapezius; PE, post exercise; UT, untrained; MT, moderately trained; ECC, eccentric; CON, concentric; SQ, squat; LP, leg press; KE, knee extension; LC, leg curl; BP, bench press; BOR, bent-over row; SP, shoulder press; TT, total testosterone; FT, free testosterone; LBM, lean body mass; CSA, cross-sectional area; ↑ increase; ↓ decrease; ↔ no change.

EARLY HISTORY OF GH BIOASSAY

The isolation of GH from pituitary extracts of many mammalian species, using biochemical techniques available at that time [~1950's–1970's], was described in a review by Papkoff and Li (91). During this early period, the three most often used growth bioassays were (a) the weight gain assay in the plateaued female rat; (b) the weight gain assay in the immature hypophysectomized rat; and (c) the tibia test; an assay originally proposed by Greenspan et al. (92) that measured bone growth at the tibial plate of the hypophysectomized rat following a 4 day injection of GH test sample. In addition, investigators also used other types of biological assays to measure circulating GH hormone that had other endpoints (e.g., lipolysis, carbohydrate metabolism). In fact, results from such differing assay approaches led C.H. Li to propose that a better name for the hormone might be "metabolic hormone" (93). To the best of our knowledge, it was also during this time period (1965) that the first study documenting that human exercise was a potent stimulus for the release of GH from the pituitary appeared (94).

A 15 year period (~1970–1985) marks the time when a majority of clinical and basic investigators appear to have transitioned from measuring circulating GH by biological assay to immunoassay. During this transition period, a critically important experimental series by Ellis et al. (95) was designed to compare results generated between rat growth assays and GH immunoassays. Their data unequivocally showed that bioassays and immunological assay results did not correlate. Plasma GH concentrations measured by this *in vivo* bioassay were estimated to be much greater (~300x) than those measured by immunoassay. Further, in 1978 this group reported that a pituitary growth factor, which escaped detection by immunoassay, nevertheless had strong GH activity in the established rat tibia bioassay (95). Their biochemical studies indicated that this factor was relatively large (~80 kDa). Moreover, the relative concentrations of bioactive GH in the rat pituitary and/or circulation (including human plasma) changed differentially in response to a variety of physiological stimuli (e.g., cold stress, fasting, insulin injection). This study was largely ignored. In retrospect, the authors of this review believe that this pioneering study should have had a more significant impact on future GH research efforts than it did. The ramifications of this concept for the multi-dimensionality of the many GH isoforms are further delineated in a recent review (96).

GH ISOFORMS

A comprehensive review of GH variants, their isolation, availability, and physiological activities is beyond the scope of this review. However, the following points help establish the thesis of this review, viz. that other potent hGH bioactive forms are present in the pituitary and plasma. However, many remain to be fully characterized, both physiologically and structurally. It is clear: GH is not a single substance.

After gene cloning, the first recombinant human GH (rhGH) was produced biosynthetically in 1979 by Genentech (San Francisco, California). Work on this product showed that the 191 amino acid isoform (22 kDa) was identical to a native molecule present in the pituitary gland and plasma (97). This form was active in the tibial bioassay as well as other bioassays having the growth endpoint. Two factors; viz. (a) availability of the recombinant product and (b) closure of the National Pituitary Agency (in 1985) for production of hGH extracted from human

pituitary glands, led to overwhelming use of antibody- based technology (e.g., polyclonal, monoclonal antibodies) and less frequently used cell- based bioassays for GH measurements.

In the ∼30 years following the Ellis report, pioneering biochemical experiments from many laboratories (Lewis, Sinha, Kostyo, and Baumann to name but a few) led to the now familiar realization, summarized by Baumann (98) that …"human growth hormone is a heterogeneous protein, consisting of several isoforms" and that.. "sources of this heterogeneity reside at the level of the genome, mRNA splicing, post-translation modification, and metabolism." According to Baumann (98), and especially relevant to this review, we point out that ∼50% of hGH isoforms in human blood 15–30 min after a secretory pulse are classified as the 22 kDa monomeric form (half bound to GH binding protein). Oligomeric and aggregated forms are believed to make up a significant portion of the remaining isoforms. Baumann concluded, in 2009 (98), what is true today; viz. that the biological significance of such isoform heterogeneity remains largely unknown. Earlier attempts to purify GH variants (between 1975 and 2000) were directed at understanding their physiological effects; however definitive conclusions relating to their bioactivity remained largely unknown. A review by U. J. Lewis (99) entitled "GH: What is it and what does it do?" makes the point another way. The abstract is provocative and relevant for this review …. "The evidence is now irrefutable that growth hormone (GH), long thought to be a single substance, is actually a mixture of several different forms. These multiple forms must be a consideration in any physiologic study if an accurate evaluation of the actions of GH is to be made" (99).

Fragmentation of the native 22 kDa hormone into two peptides [hGH 1–43] and [hGH 44–191] may affect physiology; the shorter fragment has insulin potentiating activity while the larger has anti-insulin activities, thereby implying that the native molecule acts as a prohormone (99). Similarly, exposure of GH to serine proteases will enhance activity of the hormone at the tibial plate (100). If GH has so many metabolic activities, is their mechanism of action via a common receptor? Lewis addresses this point in a 1996 report: "currently it is believed that all of these actions are mediated through the cloned GH receptor, but this is not proven" (101). To the best of our knowledge that is true to this day.

INTEREST IN HUMAN EXERCISE, BIOACTIVE, AND IMMUNO-REACTIVE GH, RE-AWAKENS

Some 20 years after the original report by Ellis et al. (95), pioneering research by Reggie Edgerton, Gary McCall, and Richard Grindeland (at UCLA/NASA Ames) offered evidence for the existence of neural afferent inputs from skeletal muscle that modulated secretion of hGH measured by tibial bioassay. Three trials done between 1995 and 2001 are described in a 2001 review by McCall et al. (102–105). Their designs included: complete bed rest (17 days); astronaut exposure during and after microgravity; and vibration-induced activation of muscle afferents. The exercise component in these trials was either repeated bouts of ankle dorsiflexion or muscle unloading. The interesting findings were that plasma concentrations of bioactive GH changed dramatically, but concentrations of immunoreactive GH were not affected by treatment. These findings clearly challenged the concept that a single molecular form of the hormone is responsible for the growth response (103–105). How activation of a small muscle group, and the neural paths taken, lead to this GH response remains largely unexplored.

How the more standard resistance exercise protocols affected plasma GH, when measured by bioassay and an array of immunoassays, were reported by the Kraemer group between 2001 and 2014. The 2001 study, Hymer et al. (106) was an acute pre-post exercise trial [six sets of 10 at 75% of the 1 repetition maximum (1RM)] involving 35 young (23 year) females tested during the follicular phase of the menstrual cycle. As expected, plasma concentrations of GH, measured by polyclonal, monoclonal radioimmunoassay, and immuno-functional assay [the latter based upon epitope binding of the GH isoform (107), increased after the exercise bout. However, plasma concentrations of GH measured by tibial assay were not different than control samples (**Table 2**)]. Fractionation of these plasma samples by size exclusion chromatography showed that treatment-induced increases in immunoactive GH was associated with molecular forms in mass ranges expected for dimeric (30–60 kDa) and monomeric (<30 kDa) GH.

From the results of this initial 2001 study (106), which involved untrained women, it was clear that the pituitary failed to respond to the exercise stress by secreting additional biologically active GH. To address the question of possible importance of exercise training, Kraemer et al. (111) undertook an extensive 6 month training program using different combinations of resistance training (i.e., either total body or upper body) using a progressive linear periodized training program supplemented by standard endurance training. As expected, each of the training groups experienced significant gains in the strength of the involved musculature over the training period, thus lending internal validity to the training study. Plasma samples were obtained both pre- to post- resistance exercise and pre- and post-training. With training, and as expected, iGH concentrations increased even further and highest assay signals were recorded using monoclonal antibody. bGH concentrations in both unfractionated and fractionated plasma samples were variable with four different training groups (two total body training groups presented in **Table 2**), In this same trial, GH assays of form(s) contained in three molecular weight classes, prepared by size exclusion chromatography, yielded equally interesting results. Thus, smaller (30 kDa) molecular mass variants generated the largest immunoreactive responses; however, larger (>60 kDa) molecular mass variants contained form(s) that were equally as potent as the small (30 kDa) and medium (30–60 kDa) class fractions in terms of generating a bone growth response. We believe this interesting result reflects the importance of either di-sulfide linked GH aggregates, and/or GH bound to GH-binding protein, for generation of somatogenic activity.

But most important exercise-induced changes in GH bioactivity were experienced after 6 months of training (6 × 10 squat at 80% of 1 RM with 2 min rest between sets).

a. The total body strength training group demonstrated in the unfractionated total a significant elevation in resting bGH,

TABLE 2 | Estimated mean comparisons bioassay, total (BGH) BGH with immunoassay (IGH) concentrations obtained at the same time point from various studies before and after resistance exercise (highest value), and analyses.

IGH ($\mu g \bullet L^{-1}$)		BGH ($\mu g \bullet L^{-1}$)		IGH ($\mu g \bullet L^{-1}$) and BGH ($\mu g \bullet L^{-1}$) fractions								
Rest	**Post-ex**	**Rest**	**Post-ex**	**≤30 kD rest**	**≤30 kD Post -ex**	**30-60 kD rest**	**30-60 kD Post-ex**	**≥60 kD rest**	**≥60 kD Post-ex**	**Gender**	**Age ± SD (yrs)**	**References**
1.1	1.2	3,800	10,000*							Male	43.8 ± 63.8	McCall et al. (103)
Nichols				Nichols(IGH)						Female	23 ± 6.4	Hymer et al. (106)
2.5	9.5*			2.5	7.4*	2.0	7.5*	0.5	1.5			
NIDDK				NIDDK(IGH)								
1.0	2.5*			2.5	10.5*	1	4.0*	0.5	1.0			
				BGH								
		2,200	2,000	1,200	1,000	1,480	1,395	1,400	1,490			
4.1	9.5*	1,650	2,400							Female	23.0 ± 1.2	Kraemer et al. (108)
Pre-training										Female	23 ± 3	Kraemer et al. (77)
NIDDK				IGH								*Total-Strength Group*
2.0	3.1*			2.0	8.2*	1.8	4.8*	0.2	0.8			
Nichols												
2.5	11.3*			3.1	8.0*	3.0	9.1*	1.0	2.0*			
				BGH								
		2,450	3,150	1,500	650*	990	750	1,400	4,150			
Post-training												
NIDDK				IGH								
3.2	7.0*			4.8	12.0*#	2.5	8.8*#	0.8	1.5			
Nichols												
4.8	14.2*			2.0	6.1*#	2.5	10.0*	0.8	1.0			
				BGH								
		3,850#	3,450	1,250#	1,500#	1,250	1,250#	1,150	2,450*#			
Pre-training										Female	26.3 ± 4.0	Kraemer et al. (77)
NIDDK				IGH								*Total-Hypertrophy Group*
1.8	2.5*			2.6	8.0*	1.2	3.8*	0.1	0.8			
Nichols												
2.7	8.0*			2.7	8.2*	1.6	7.2*	0.3	2.0*			
				BGH								
		2,950	1,900*	1,550	1,010	1,650	1,100	1,950	1,550			
Post-training												
NIDDK				IGH								
1.8	4.5*			2.4	7.0*#	2.0	4.8*	0.2	0.8			
Nichols												
1.9	13.1*#			1.1	5.0*#	1.3	8.6*#	0.1	1.2*			
				BGH								
		2,900	2,500#	1,090	1,190	1,950#	750*	1,600	2,010#			
				%	%	%				Female	61.6 ± 1.3	Gordon et al. (110)
Old				IGH	IGH	IGH						*Resistance Ex*
2.5	4.8*		980	30	55	15						
				BGH	BGH	BGH						
				15	45	40						
Young				IGH	IGH	IGH						
3.5	17.5*		1,725	40	40	20						
				BGH	BGH	BGH						
				30	40	30						
1.0	10.0*	6,400	11,500							Male	20.1 ± 2.1	Thomas et al. (109)
0.4	7.0*	3,800	6,200							Male	21.0 ± 2.1	Thomas et al. (109)
4.5	16.5*		1,740							Female	23.7 ± 1.0	Gordon et al. (110)
0.6		2360.9								Male	80.5 ± 1.6	Kraemer et al. (1)
2.0		4966.1								Female	80.7 ± 1.4	Kraemer et al. (1)

*Significant increase from corresponding resting value.
#Significant difference from pre-training.

and with training in the >60 kD fraction showing uniquely an increase with acute exercise and this acute response was significantly higher post-training. Additionally, other fractions also demonstrated higher post training values. Thus, the bGH appeared for the first time to be responsive to exercise stress and also demonstrated adaptations to training in these young women. While not shown in **Table 2** the upper body only strength training group also showed similar changes with significant increases in the unfractionated resting values as well as a significant exercise-induced response following training, again showing the influence of training on bGH.

b. In the total hypertrophy group, it was observed that pre-training acute exercise resulted in a significant decrease in the UF samples and this was observed again post-training yet the post-exercise values were significantly higher. Again, while not shown in the table, the upper body group showed no acute exercise changes in the UF samples pre-training but with training, resting values were significantly higher and a significant exercise-induced elevation was observed.

Taken together, these results indicated for the first time that acute and chronic exercise training using conventional large muscle group resistance training protocols will increase (acutely and chronically) plasma concentrations of GH bioactivity in young women. McCall had shown previously that exercise of small muscle groups would also increase plasma concentrations of bGH (103, 104).

Data from other studies also reveal the dichotomy between bioactive and immunoreactive GH. Comparison of bGH plasma levels from 24 vs. 62 year old female volunteers, after acute aerobic cycle exercise, were not different. However, after an additional acute resistance exercise bout, plasma concentrations of bGH from the younger group were significantly higher than those in the older group. These higher concentrations were associated with molecular forms of apparent mass 30–55 kDa (i.e., dimer range) (110).

Comparison of bGH plasma concentrations from lean [BMI = 23] vs. obese [BMI = 36] men revealed that although resistance exercise had no significant effect, their concentration in the leaner group was significantly higher. Similar to other studies, concentrations of GH measured by immunoassay were not different between the two groups (109).

A trial done with free-living 81 year old individuals, failed to uncover differences in plasma GH concentrations (measured by either bioassay or immunoassay) that could be correlated with either fitness or physical performance. Curiously, one half of the group ($n = 21$) had plasma concentrations of bioactive GH that were essentially zero, while the other half ($n = 20$) had concentrations that were readily detectable and in the range of studies listed previously (112).

HOW EXPERIMENTS WITH RATS OFFER CLUES RELEVANT TO HUMAN EXERCISE AND BIOACTIVE GH

Somatotroph Heterogeneity

Cell separation studies indicate that two populations of GH cells (somatotrophs) are present, in roughly equal numbers (∼40%), in the rat pituitary gland. One population (light somatotrophs, also designated the type I cell) has densities <1.071 g/cm^3, while the other (heavy somatotrophs, also designated the type II cell) has densities in ranges >1.071–1.085 g/cm^3. The higher density of the type II cell is attributable to large numbers of 300 nm diameter, GH containing, cytoplasmic secretory granules. Results from a recent experiment (113), designed to determine if the GH released from light vs. heavy somatotrophs is differentially active by bioassay, offer definitive evidence to support the hypothesis that differential responses between bioassay vs. immunoassay results after human exercise (described previously), has a structural (cellular) basis residing within the pituitary gland itself. Results of this experiment showed that: (1) culture media from type II cells contained 5x as much bGH (tibial assay) as that from type I cells; (2) net production of bGH from type II cells was 6x more than that from type I cells ($p < 0.001$), but production of iGH was not different between type I vs. II cells; (3) implantation of type II cells into rat brain ventricles of hypophysectomized recipients significantly increased body weights, tibial widths and gastrocnemius muscle; however, implantation of type I cells had little to no significant effect on these same markers; and (4) type II cells prepared from animals that had been previously fasted or insulin injected showed markedly reduced bGH secretion. Recent studies using RNAseq assays also demonstrate somatotroph heterogeneity in mice, e.g., a subpopulation enriched in sterol/cholesterol synthesis genes (114). Additionally, another study using RNAseq assays also showed a subpopulation of somatotrophs demonstrating sex dependent differences in anterior pituitary cells in female rats (115), Thus, others have also found somatotroph heterogeneity using other molecular techniques harking back historically to some of the first observations of this phenomenon (116, 117).

The GH Secretory Granule

These membrane bound cytoplasmic organelles contain ∼75% of the total bioactive hormone measured in the pituitary homogenate (118). The hormone in the granule is bound cooperatively with two Zn(II) ions per GH dimer (119). Each granule is estimated to contain 5,000–10,000 molecules and its dense core consists of large, crystal-like aggregates which are thought to solubilize on exocytosis (120–122). Some GH granules contain cytochrome C, cytochrome oxidase and ATP; molecules that may mediate GH release (121).

On electrophoresis in non-reducing SDS gels, rat pituitary extracts contain a wide range of di-sulfide linked GH variants (14–88 kDa MW) (123). Electro-elution of protein from different regions of such gels, followed by their chemical reduction, apparently uncovers epitopes hidden in the aggregate, thereby increasing iGH activity up to 6X. Oligomeric forms >44 kDa are found exclusively in extracts prepared from dense, highly granulated, purified type II- bGH producing- somatotrophs (pentamers). Extracts from the less dense, less granulated, type I somatotrophs contain a single dominant 22 kDa peak and a minor 44 kDa species (dimer). Chemical reduction of culture media from type II, but not type I, somatotrophs increases immunoreactivity (5X vs. 1.3X, respectively). This important result confirmed maintenance of granule heterogeneity within the somatotroph in cell culture. Since GH released from the type

II somatotroph, relative to type I cells, is most active in both *in vitro* (cell culture) and *in vivo* (hollow fiber implant) bGH tests, the results of Farrington and Hymer (123) and Grindeland et al. (113), support the contention that bGH activity is associated with disulfide linked aggregates (oligomers) residing in granules of the type II somatotroph, as well as bGH activity in culture media secreted from the type II somatotroph.

Growth Hormone Is Stored as an Amyloid

A major advance in understanding packaging mechanisms of GH molecules within a secretory granule came from the reports of Maji and co-workers showing that the hormone is stored as an amyloid (124, 125). Amyloids are defined by their highly organized cross B-sheet regions in protein aggregates and should be considered as yet another level of protein structure. The cross-B sheet represents a single structural epitope in which individual strands of each sheet run in perpendicular to the fibril axis while B-sheets are parallel to the fibril axis. These highly organized, elongated amyloid fibers are composed of thousands of copies of stacked B sheets composed of peptide/protein. These stacked fibers can trigger further refolding of the natively folded protein. In many proteins the amyloid state is thermodynamically stable at high concentration, but not energetically favorable at lower protein concentration (126). These fibrillary structures are often hallmarks of severe disorders; e.g., Alzheimer disease and diabetes mellitus.

Amino acid residues 72–82 of the 191 amino acid, 22 kDa rHGH monomer have a high aggregation propensity and 4 fibrillation segments, each of ∼6–10 residues. These are B aggregation "hot spots." Only Zn(II) ion, as the specific helper, allows fibrillation; yet even in this configuration, most of the molecule is able to maintain its globular fold (125)! The amyloid configuration not only may ensure efficient release of 22 kDa GH from the amyloid depot, but also protect the GH from enzymatic degradation, high temperature, and large pH ranges. It is now well-accepted that many proteins can assume the amyloid configuration.

Mechanisms underlying amyloid fibril formation, and their relationships/ interactions (sometimes reversible) leading to the formation of either disordered, amorphous aggregates or oligomers (via on/off pathways), will lead to varied configurations of amyloid. These conformations are complex, dynamic and thought critical for understanding protein configuration in health and disease (126). Many proteins form amyloid-like fibrils *in vitro*. Obviously not all proteins are "bad." It must be recognized that common structural principles of amyloids convey their double nature as "good" or "bad" (127).

In the resting state, GH synthesis and processing of functional molecular aggregates (FA) ["good aggregated GH"] follow the regulated path to the cell surface and become primed for stimulated secretion into the blood. As the demand for GH increases with exercise stress, this process may result in errors in the biosynthetic pathway. Mechanisms to repair mis-folded, non-functional GH aggregates (NFA), are shown in **Figure 2**. As summarized by Frottin et al. (128) it has also become apparent that the nucleolus plays and important function in maintaining the homeostatic (proteostasis) quality control of aggregated proteins in the cell to prevent the formation of toxic aggregates or what might also be called non-functional aggregated proteins arising from aberrant cellular processing. Some NFA forms could be released into the circulation, however the concentration of circulating FA and NFA forms remains largely unknown. These

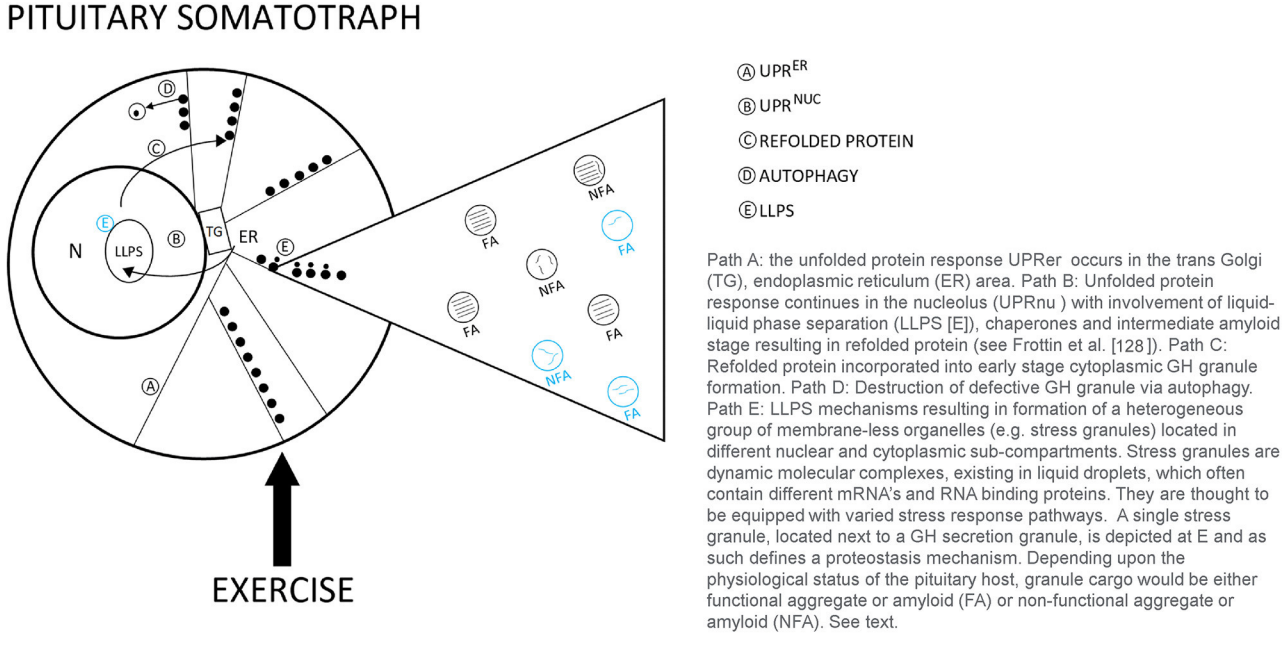

FIGURE 2 | Theoretical model of proteostasis mechanisms likely to be active in the pituitary somatotroph during various types of human exercise stress.

repair mechanisms may be enhanced with exercise training. This model suggests an intriguing line for future research in the quest to understand roles of aggregated GH in stress biology (129–131). The potential for lower values of BGH in the blood might be observed if all of the processing systems for mis-folded non-functional GH aggregates are fully engaged, potentially a training adaptation.

Acute and Chronic Exercise Complexity Remains

While the GH responses to exercise has been characterized for decades understanding the many selective roles in metabolism and other physiological mechanisms related to acute homeostasis and repair and remodeling of tissues remain needed (94, 132–136). It becomes apparent that understanding the role(s) of GH in responding to exercise stress and adapting to exercise training is still in its embryonic stage. This becomes evident when one realizes that GH is not a single entity. The multitude of roles attributed to GH require that a more complex set of mediating mechanisms may be needed to accomplish them. As noted in this section the diversity of GH isoforms from their presence in the anterior pituitary to other biocompartments (e.g., brain, circulation, liver) also suggest that target cells may be responding to different GHs. The mere differences in receptor binding between bio and immune assays and their differential signaling raise questions as to their acute and chronic roles in exercise stress and adaptations. Additionally, growth hormone binding proteins from the liver and their potential to create dimers when binding to 22 kD forms in the blood also raise questions as to how they function in signaling (137–139), yet while increases with acute resistance exercise are observed differences between trained and untrained men have not been observed (140).

Types of exercise may well have an influence as well (63). It may be due to total amount of work or the inability to activate the same motor unit array that contributes to such modality differences. One unifying thought is the influence of pH and H+ ions on IGH (141). This is reflected in its close associations of blood lactate, that when lactate is elevated beyond the anaerobic threshold or is dramatically elevated with a resistance training workout, IGH is highly responsive (134, 142–145). This was demonstrated with resistance exercise in men and women in two studies by Kraemer's research groups (144, 145) where the short rest workouts using 1 min between sets and exercises demonstrated the highest blood lactate responses and IGH responses. Whether this is due to a reduction in the type 2 somatotrophs production less aggregate or a stimulation of predominantly type 1 somatotrophs is unknown. Other factors such as body fat of subjects to fasted or intakes of protein/carbohydrate before and/or after the workout also appear to influence IGH. Since the BGH studies have always been done in the fasted state, nothing is known as to its response patterns. Additionally, with the stability of the BGH in the blood how pulsatility of IGH interfaces with the entire signaling milieu remains to be elucidated.

Finally, how the various splice variants and aggregates of GH are integrated within the larger web of hormonal and molecular signaling remains to be seen as various studies continue to unravel the complex nature of homeostatic regulation with acute exercise and chronic exercise adaptations.

CURRENT PERSPECTIVES ON IGF-I AND PHYSIOLOGICAL ADAPTATIONS AND COMPLEXITY RELATED TO THIS SUPERFAMILY TO TRAINING

Insulin-like growth factors (IGFs) are small polypeptide hormones (70 and 67 amino acids for IGF-I and IGF-II, respectively), structurally related to insulin, and synthesized from a larger precursor peptide that is post-translationally processed into its active form. Of the two, IGF-I has been most extensively studied and is secreted as it is produced by the liver in response to GH stimulation. Only 2% of IGF-I circulates in its free form; most circulates as a binary (20–25%) or ternary complex (∼75%) (146–149). In its binary form, IGF-I circulates with one of seven binding proteins whereas in its ternary form, IGF-I circulates with IGFBP-3 and its acid labile subunit (ALS).

IGF-I (7 kDa) is responsible for metabolic, mitogenic and anabolic cellular responses (150). It is produced locally (i.e., autocrine and paracrine mechanisms) in tissues and cells. IGF-I acts as both a cell cycle initiation and progression factor. Its effects include satellite cell activation, proliferation, survival, and differentiation, increasing myotube size and number of nuclei per myotube, stimulating amino acid uptake and protein synthesis and muscle hypertrophy, neuronal myelinization, axonal sprouting and repairing damage, reducing chronic inflammatory response, increasing free fatty acid utilization, and enhancing insulin sensitivity upon receptor binding and subsequent intracellular signaling and glucose metabolism (1, 151). Expression and secretion of IGF-I increases by myofibers with mechanical loading (152). Secretion by myofibers stimulates autocrine and paracrine myofiber anabolic processes where adjacent satellite cells enter the cell cycle, proliferate, differentiate, fuse with myofibers, and provide myonuclei to maintain or reestablish the myonucleus to myofiber size ratios of the enlarged myofibers (152). Because of these critical anabolic functions, IGF genes have been considered a potential target for gene therapies, gene doping in athletes (153) and staving off advancing muscle weakness (154).

While liver-derived IGF-I is under direct regulation of GH, local mechanical-stretch mechanisms can activate IGF-I synthesis in tissues. The potency of circulating IGF-I remains unclear and needs to be viewed in context with its binding proteins that provide fine tuning of the IGF actions and regulate bioavailability (150). Several studies have shown systemic elevations in IGF-I produced no elevations in protein synthesis or hypertrophy during resistance exercise training whereas up-regulation in the muscle isoform was linked to significant muscle hypertrophy (151).

ACUTE RESPONSES AND CHRONIC ADAPTATIONS OF IGFs TO RESISTANCE TRAINING

There remains much to discover about the roles of systemic vs. locally produced IGF-I in mediating the outcomes of resistance exercise (155). Yet, it appears that local IGF-I is consistently upregulated with both acute and chronic exercises; whereas in certain situations, circulating IGF-I may actually decrease, increase, or not change (21, 155). Studies showing no change in circulating IGF-I can vary due to the temporal frame of measurement following stimulation with GH (21). While the acute responses of IGF-I have been evaluated in the serum/plasma of many different studies of resistance exercise, its contribution to hypertrophy has been difficult to determine due to the milieu of anabolic signaling to skeletal muscle. Kraemer et al. were the first to demonstrate this highly variation to resistance exercise stress of IGF-I (119). However, there is little doubt, IGF-I is a primary player in anabolic signaling targeted to many tissues, including skeletal muscle. It could be that IGF-I acts as a signal that either amplifies or regulates skeletal muscle tissue repair and remodeling (1). Looking at the IGFBPs has provide a more fruitful area of study as they have shown a more reliable pattern of responses to acute resistance exercise protocols. Of importance is the response of IGFBPs which have generated more consistent responses with resistance exercise acutely elevating IGFBP-3 (21). Looking on longer term changes in IGF-I, Nindl et al. (148) monitored overnight IGF-I following heavy resistance exercise and showed IGF-I concentrations remained unaffected. However, IGFBP-2 increased and ALS decreased indicating that binding protein partitioning, rather than changes in systemic IGF-I, appeared to be an important finding. Exercise duration and total work also may impact IGFBP-1 changes but it was not see that the modality had as much impact on the response patterns. With the novel technique of microdialysis to measure IGF-I in the interstitial fluid, Nindl et al. (149) showed total and free IGF-I and IGFBP-3 were elevated. However, IGF-I in interstitial fluid was unaltered following high-power resistance type exercise. It was also observed that the IGF-I receptor phosphorylation was not increased but IGF mRNA content and Akt phosphorylation were increased (149) This supported the speculation that skeletal muscle adaptation is not be directly dependent on systemic IGF-I, but rather be involved with the interactions and signaling across different biocompartments.

Long term resistance exercise training studies examining resting circulating IGF-I concentrations have been demonstrated to be highly variable with reductions, no change, and elevations with no change or reductions in IGFBP-1 and IGFBP-3 (21). It has been demonstrated that in participants who are classified as extreme responders to a long term (16 wk) training program showed no significant changes in IGF-I, IGFBP-1, or IGFBP-3 but a trend showed that IGFBP-3 was lower in the non-responders (156). Resistance-trained men have been shown to have higher resting IGF-I values than untrained men (140) Nevertheless, single measurements of IGF-I need to be carefully interpreted as the roles and contributions remain speculative due to the multiple targets and mechanisms they are involved with in the signaling processes. Of more consequence may be the training responses of locally-produced IGF-I isoforms. Resistance exercise training of sufficient intensity and volume increases IGF-I and MGF mRNA for up to 48 h post RE (21, 157). Furthermore, IGF-I and MGF mRNA have increased 2 h post exercise (but not 6 h) after a single bout of moderate (65% of 1RM; 18–20 repetitions) and moderately-high (85% of 1RM; 8–10 repetitions) intensity resistance exercise training (158). Further studies have shown MGF acts independently and is expressed earlier than other IGF-I isoforms in response to resistance exercise training, and therefore may have greater anabolic potency (159). The recruitment of motor units and their associate muscle fibers creating mechanical damage appears to be an essential stimuli for local production of IGF-I.

IGF-I RECEPTOR AND INTRACELLULAR SIGNALING

Downstream actions of IGF-I are mediated through binding to the IGF-I receptor (IGF-IR), a ligand-activated receptor tyrosine kinase on the cell surface of target tissues. The IGF-IR gene is mapped to chromosome 15q25-26. Activation of receptor tyrosine kinase activity results from ligand binding to the α subunit of the receptor leading to a conformational change in the β subunit (160). This leads to the activation of downstream signaling pathways of IGFs including PI 3-kinase pathway and Ras-mitogen-activated protein kinase (MAP kinase) pathway, for cell proliferation, cell differentiation and cell survival (160). Two types of IGF receptors have been identified. The type I receptor binds IGF-I with greater affinity than IGF-II and also interacts weakly with insulin. The type II receptor binds with greater affinity to IGF-II than IGF-I and does not bind to insulin (161). Resistance exercise influences IGF-IR phosphorylation where high-volume results in greater phosphorylation compared to high-intensity protocols 1 h post exercise (162). Resistance exercise protocols of moderate to high intensity also have been shown to increase IGF-IR mRNA 2 h following acute exercise (158). Mechanical stress also stimulates IGF-R signaling cascades via focal adhesion kinase (FAK), an attachment complex protein necessary for mechanical IGF-I-mediated hypertrophy in skeletal muscle cells (163). To the contrary, anabolic resistance and sarcopenia may be attributed to dysregulation in the IGF stimulated, Akt /Protein Kinase B and mechanistic target of rapamycin (mTOR) signaling pathways in response to resistance exercise and protein intake (164).

INTEGRATED COMMUNICATION FOR ANABOLIC/CATABOLIC SIGNALING: GLUCOCORTICOIDS

Cortisol Regulation

In addition to the anabolic hormones, glucocorticoids, mainly cortisol have a profound influence on human skeletal muscle (165). During stable physiological conditions, circulating cortisol exhibits a circadian rhythm peaking in the morning, slowly decreasing throughout the day, and reaching lowest levels around

midnight (166) (**Figure 3**). Cortisol levels are regulated both at the systemic and tissue level to maintain glucocorticoid homeostasis. Endogenous levels of cortisol are systemically controlled by the hypothalamic-pituitary-adrenal (HPA) axis and locally by the action of 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes. In the periphery, the cellular response to glucocorticoids differs by cell type (167–169), cell cycle stage (167), and exposure to stress (170).

In skeletal muscle, cortisol plays a fundamental role in regulating energy homeostasis and metabolism (171). During exercise, cortisol increases the availability of metabolic substrates, protects from immune cell activity, and maintains vascular integrity (172). The acute cortisol response to exercise is highest when the overall stress (volume and/or intensity of total work) of the training period is high (145, 173). Cortisol is also involved in adaptations to exercise by preparing the body for the next bout of exercise (71, 174), as increases in cortisol are prolonged before returning to basal levels following a bout of exercise. Adaptation of the HPA axis following exercise training is largely manifested by altered sensitivity to cortisol (172). Following acute exercise, there is an increased tissue sensitivity to glucocorticoids that serves to counteract muscle inflammation, cytokine synthesis, and muscle damage (172). Subsequent decreased sensitivity of monocytes to glucocorticoids 24 h following exercise may act to protect the body from prolonged, exercise-induced cortisol secretion (172). Inactivation of cortisol into cortisone acts as another mechanism to protect tissues and cells from the deleterious effects of exercise-related cortisol secretion (175). Inactivation of cortisol to cortisone appears to be an adaptation to exercise, given that athletes display a higher inactivation of cortisol into cortisone (175). However, overtraining appears to impair the inactivation of active cortisol to cortisone in athletes (175), and may impair anabolic processes as high levels of cortisol decrease skeletal IGF-I synthesis by reducing IGF-I transcript levels (176).

TISSUE SPECIFIC REGULATION BY 11β-HSD (11β-HYDROXYSTEROID DEHYDROGENASE)

11β-HSD (11β-hydroxysteroid dehydrogenase) acts as a tissue specific regulator of glucocorticoid action by catalyzing the interconversion of active cortisol and corticosterone with inactive cortisone and 11-dehydrocorticosterone (177). This interconversion regulates glucocorticoid access to intracellular glucocorticoid receptors (178) and glucocorticoid action (179). The cellular hormonal environment can influence 11β-HSD activity, where exposure to insulin, insulin-like growth factor I, and glucocorticoids can alter enzyme activity (179). Raised expression of 11β-HSD1 (Type 1) in skeletal muscle is believed to play role in mechanisms that contribute to the development of metabolic syndrome (180) insulin resistance (181), and hypertension (182).

GLUCOCORTICOID RECEPTORS

Glucocorticoids convey their signal mainly through intracellular glucocorticoid receptors, which in the absence of a ligand are generally localized to the cytosol (183). In the cytoplasm, the glucocorticoid receptor is found in a complex with chaperone proteins that maintain a conformation with high affinity binding

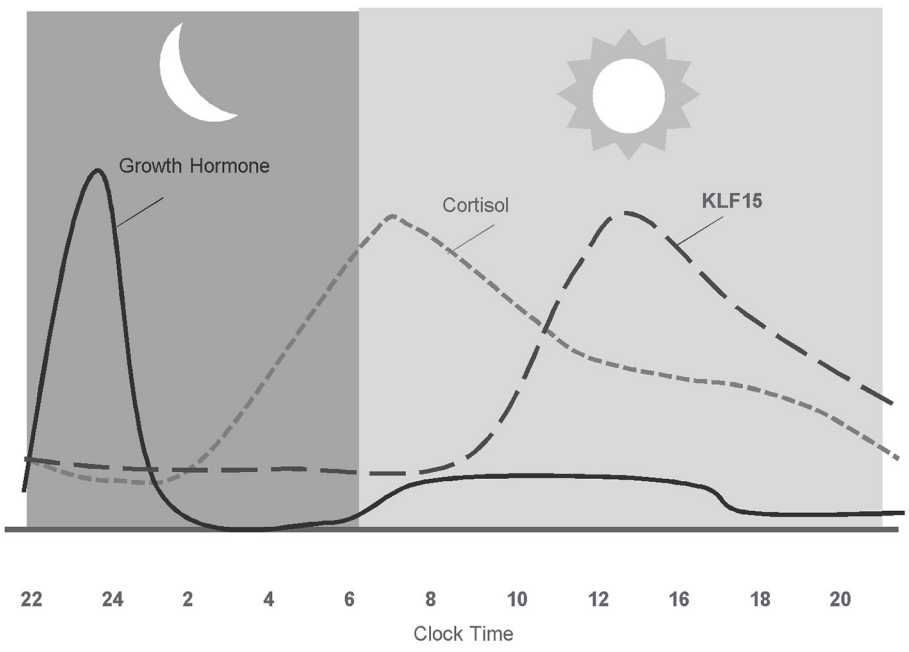

FIGURE 3 | Diurnal pattern of anabolic/catabolic regulators may facilitate anabolic benefit of intermittent exposure.

potential (89). Once a glucocorticoid binds to the receptor, it moves to the nucleus where it interacts with specific DNA sequences known as glucocorticoid response elements (183, 184). Glucocorticoid response elements regulate the transcription of primary target genes by either directly binding to DNA (185), tethering onto other DNA-binding transcription factors (185), or through direct protein-protein interactions with other transcription factors and/or coregulators (186). Glucocorticoid receptor-binding to DNA is highly context specific and relies on the interplay of the receptor with other proteins (187, 188).

Selective targeting of glucocorticoid receptors is mediated by the combined action of cell-specific priming proteins, chromatin remodelers (189), and local sequence features (190). As much as 95% of glucocorticoid receptor binding sites are within preexisting sites of accessible chromatin (190), with some detected in remodeled chromatin (189, 190). Binding is dictated by proteins that maintain chromatin in an open state (188). Activator protein 1 (AP1) is one such protein that is involved in glucocorticoid receptor chromatin interactions and subsequent transcription and recruitment to co-occupied regulatory element (188). Most (62%) GR-binding sites are occupied by the transcription factor C/EBPβ (enhancer-binding protein beta) (189), which regulate multiple genes in the ubiquitin-proteasome pathway (191).

During myogenesis, glucocorticoid receptors are localized in different parts of cells: in the cytoplasm of myoblasts, in the nucleus of myotubes, and in the extracellular matrix, satellite cells, and near mitochondria in mature skeletal muscle fibers in mice (192). Yet, location may differ by fiber type, as most muscle fiber types express glucocorticoid receptors in the cytosol, but only slow fibers express glucocorticoid receptors on the membrane (193). Membrane glucocorticoid receptors are localized in the extracellular matrix and signal rapidly (within 5 min) through the MAPK pathway in mammalian skeletal muscle fibers (192).

Glucocorticoid Receptor Isoforms

The human glucocorticoid receptor is encoded by the NR3C1 gene, located on chromosome 5 (5q31–32) (194), and consists of nine exons (195). There are two major isoforms of glucocorticoid receptor due to alternative splicing of a single gene: GRα and GRβ (196). These isoforms differ at their carboxyl termini (195) (**Figure 4**). GRβ has a truncated glucocorticoid ligand-binding domain, which prevents glucocorticoid binding and causes GRβ to act as a dominant negative inhibitor of GRα (195, 196). In healthy humans, the default splicing pathway is the one leading to GRα (197), with minimal activation of the alternative splicing event leading to GRβ (197). While there are two main isoforms of the glucocorticoid receptor, more than 1,500 variants have been identified and cataloged (198). Such variants include both naturally occurring and stress-induced GR isoforms, where further studies are needed to decipher their roles in stress responses (198). In healthy human cells and tissues, GRα mRNA concentrations are highest in the brain, followed by skeletal muscle, macrophages, lungs, kidneys, liver, heart, eosinophils, peripheral blood mononuclear cells, nasal mucosa, neutrophils, and colon (197). GRβ mRNA expression which is lower than GRα mRNA expression, with the highest concentrations found in eosinophils, followed by peripheral blood mononuclear cells, liver, skeletal muscle, kidney, macrophages, lung, neutrophils, brain, nasal mucosa, and heart (197).

The relative expression of the two alternatively spliced glucocorticoid isoforms and the ratio of GR-α to GR-β expression modulates cellular sensitivity to glucocorticoids (199). Expression of GRβ selectively increases in cells exposed to inflammatory signals; this increased expression leads to glucocorticoid resistance (196, 200) and may reduce the therapeutic potential of glucocorticoids (201). In myoblasts, glucocorticoid exposure results in a dose-dependent decline in GRα expression and a dose-dependent increase in GRβ expression (179). In myotubes, overexpression of GRβ is associated with a blunted catabolic response to glucocorticoids via lower "atrogene" signals (201). Mechanistically, the selective increase in GRβ appears to involve the splicing factor SRp30c (serine/arginine-rich protein p30c) (202, 203). On the other hand, agents that increase GRα expression sensitize cells to glucocorticoids (204). Exercise affects receptor expression (205) and relative expression of receptor isoforms; athletes show less GRα mRNA expression in peripheral blood mononuclear cells than do untrained controls, indicating reduced sensitivity (206). Yet, GR-β does not appear involved in exercise adaptations in peripheral blood mononuclear cells of athletes (206).

GRα Isoform Signal

In skeletal muscle, glucocorticoid hormone action is determined principally by binding to the GRα isoform (179) which can increase or decrease glucocorticoid receptor gene products that contribute to physiologic responses (207) (**Figure 5**). The binding of glucocorticoids to the ligand-binding domain of GRα causes translocation to the nucleus and binding to glucocorticoid

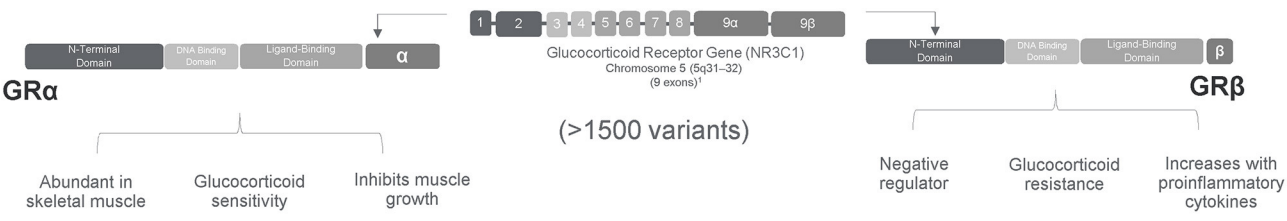

FIGURE 4 | Alternative splicing of a single gene results in two major isoforms of glucocorticoid receptor with more than 1,500 variants.

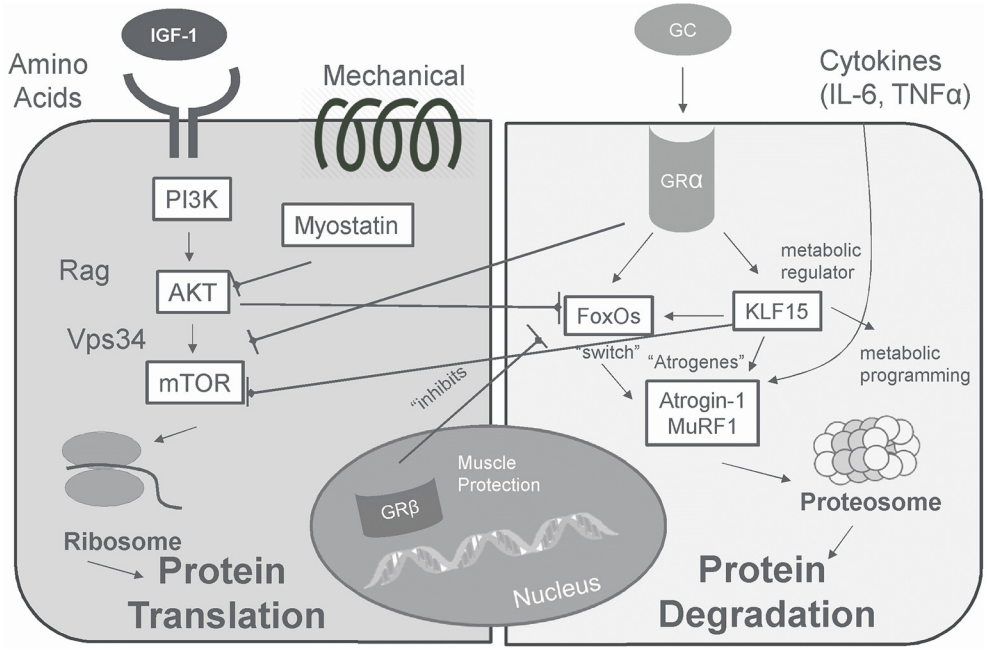

FIGURE 5 | In skeletal muscle glucocorticoids produce a catabolic effect that is opposite that of insulin/IGF-I via GRα.

response elements (GREs) in the promoter region of genes (201). Specifically, GRα binds to GREs in the promoter of forkhead box O (FOXO) transcription factors and enhances expression (208). This results in a FOXO-dependent increase in muscle atrophy F-box/Atrogen-1 (MAFbx) and muscle ring finger 1 (MuRF1), E3 ubiquitin ligases necessary for glucocorticoid -induced muscle myopathy; suppression of MAFbx and MuRF1 inhibits glucocorticoid -induced protein degradation (208). In addition, glucocorticoids may also exert actions through tethering (GR binding to other transcription regulators) and squelching (GR binding to and taking away transcription regulator from DNA), which often lead to transcription repression (185).

Proteolysis Signal

The catabolic actions of cortisol resulting in muscle proteolysis occur largely via the ubiquitin–proteasome and lysosomal systems (186, 209–211). Via these proteolytic systems, expression of genes involved in atrophy ("atrogenes") are increased, which target proteins for degradation by the proteasome machinery (210). Atrogenes include transcription factor FOXO, a major switch for the stimulation of several atrogenes, and two ubiquitin ligases atrogin-1 and MuRF-1, involved in the targeting of protein to be degraded by the proteasome machinery, and LC3 (186, 201, 209, 210). Glucocorticoids also may blunt skeletal muscle protein synthesis by inhibiting IGF-I signaling, a muscle anabolic growth factor, and increasing myostatin signaling, a muscle catabolic growth factor, contributing to muscle atrophy (207, 209, 210).

GR Receptor Expression in Skeletal Muscle

In skeletal muscle, glucocorticoid receptor expression is more abundant in fast than slow twitch fibers (211, 212). Consequently, slow twitch muscle fibers appear to be resistant to the catabolic action of glucocorticoids (213) whereas, fast twitch muscle fibers are more sensitive to the catabolic action of glucocorticoids (214). Glucocorticoid-induced muscle catabolism results from degradation of contractile proteins which begins in the myosin filaments and then spreads to the thin filaments and the z-line (213). In fast fibers, glucocorticoid exposure in the absence of exercise increases the activity of non-lysosomal proteases (214). Yet, in response to exercise, both fast and slow fibers experience increases in myofibrillar protease activity followed by anti-catabolic actions (214). While GR expression does not appear to change following resistance exercise (76), receptor activation occurs at a rate that is independent of both fiber type and delivery of steroid to working muscles during exercise (215).

GRβ Isoform Signal (Negative Regulator)

GRβ functions as a negative regulator of glucocorticoid actions in local tissues (168), where overexpression of GRβ is associated with glucocorticoid resistance. Like other nuclear receptors, the GRβ functions as a naturally occurring dominant negative isoform that blocks the activity of GRα when the two are co-expressed in the same cell (195, 216). The negative action is largely caused by the formation of inactive, or weakly active, heterodimers between GRα and GRβ (216, 217). Unlike the GRα, GRβ has a truncated ligand-binding domain that prevents glucocorticoid binding and causes glucocorticoid resistance (195, 201). The dominant negative activity of GRβ resides within its unique carboxyl-terminal 15 amino acids (217). In addition, unlike GRα, GRβ is located primarily in the nucleus of cells independent of hormone administration (195). In the absence

of GRα, GRβ is transcriptionally inactive on a glucocorticoid-responsive enhancer (195). When both GRα and GRβ isoforms are expressed in the same cell, GRβ inhibits the hormone-induced GRα -mediated stimulation of gene expression (195). Compared to GRα, GRβ does not undergo ligand-induced down regulation and has an increased half-life (195). Elevated levels of GRβ in immune cells correlate with reduced sensitivity to glucocorticoids (168). Expression of GRβ in cells is increased by proinflammatory cytokines [interleukins IL-1, -2, -4, -7, -8 and -18; tumor necrosis factor -alpha (TNFα); and interferons α and γ] (168, 200).

GRβ is responsible for the development of tissue-specific resistance to glucocorticoids in various disorders associated with dysregulation of immune function (168). Increased GRβ expression has been linked to glucocorticoid resistance in asthma, leukemia, cancer, and inflammation (201). GRβ expression in human neutrophils may also provide a mechanism by which cells escape glucocorticoid-induced cell death (218). Cell survival is further enhanced by upregulation of GRβ by proinflammatory cytokines such as IL-8 in the presence of glucocorticoids during inflammation (218). Anti-GRβ molecules have become a target of cancer therapies as GRβ has been shown to be highly expressed in cells from solid and liquid tumor, and blocking them may repress cell migration (219). On the other hand, GRβ may serve as a pharmacological target for skeletal muscle growth and protection from glucocorticoid-induced catabolic signaling (201). Increased expression of GRβ promotes glucocorticoid resistance in skeletal muscle, thus stabilizing muscle mass during exposure to high doses of glucocorticoids (201).

Muscle protection via GRβ is associated with increased levels of muscle regulatory factors, enhanced proliferation in myoblasts, and increased myotube fusion (201). Myotubes overexpressing GRβ have lower forkhead box O3 (FOXO3a) mRNA levels and a blunted muscle atrophy F-box/atrogen-1 (MAFbx) and muscle ring finger 1 (MuRF1) response to glucocorticoids (201). GRβ also enhances insulin-stimulated growth through suppressed phosphatase and tensin homolog (PTEN) gene expression and increased phosphorylation of Akt (220). Moreover, overexpression of GRβ may preserve skeletal muscle mass in the presence of glucocorticoids by increased MyoD (1.8-fold) and myogenin (2.5-fold) gene expression, two muscle regulatory factors necessary for skeletal muscle development and regeneration (201). In addition, overexpression of GRβ enhances myotube formation and reduces glucocorticoid responsiveness in mouse muscle cells (201). Another protective mechanism by which GRβ contributes to preserved muscle mass may be through repression of the tumor necrosis (TNF) α and interleukin (IL)-6 genes (221), and inhibited GRα -mediated repression of an NF-kappaB-responsive promoter (217). Yet, glucocorticoid exposure alone does not appear to impact GRβ protein levels in mouse muscle cells (201) and human cells (222).

To the contrary, insulin exposure increases GRβ protein expression (201). Thus, insulin resistance in response to glucocorticoid therapy may contribute to muscle atrophy via reduced protein synthesis and increased protein degradation by genomic and non-genomic interference with several kinases in the insulin-signaling pathway (201). Although further work is needed to determine the impact of physical exercise training on GRβ, studies in human myoblast and myotube cultures (without neural innervation, mechanical loading, and *in vivo* conditions) revealed that treatment with glucocorticoids alone may not be sufficient to elicit changes in GRα or GRβ mRNA or protein expression (222).

Glucocorticoid Sensitivity

Sensitivity to glucocorticoids varies among individuals, among tissues from the same individual, and even within the same cell depending on the phase of the cell cycle (223). Hereditary studies show that differences in the glucocorticoid receptor gene make 6.6% of the normal population relatively hypersensitive to glucocorticoids, and 2.3% relatively resistant (169). Yet, glucocorticoid resistance may also be acquired and localized to the sites of inflammation (169) with pathological conditions (224). Glucocorticoid sensitivity is largely determined by a number of factors including the intracellular density and distribution of glucocorticoid receptors (183), 11βHSD1-mediated intracellular synthesis of active cortisol from inactive cortisone (179), tissue-specific presence of coregulatory proteins, the phosphorylation status of GR, the sequence of the GR-binding site and flanking DNA on target genes (184, 225), post-translational modifications of GR, the availability of specific co-activators and co-repressors, epigenetic regulators, the chromatin landscape (187, 190), and cross-talk with MyoD family inhibitor domain-containing proteins (226).

With exercise training, the body adapts to regulate glucocorticoid sensitivity in some cell types (172). Increased tissue sensitivity to glucocorticoids following (6–24 h) acute exercise may serve to counteract muscle inflammatory reaction and cytokine synthesis and then decrease exercise-induced muscle damage or inflammatory response (172). Subsequent decreased sensitivity of monocytes to glucocorticoids 24 h following exercise may act to protect the body from prolonged, exercise-induced cortisol secretion (172). Intracellular adaptation of glucocorticoid regulators to exercise is tissue specific, resulting in decreases in glucocorticoid action in skeletal muscle and increases in glucocorticoid action in the liver and visceral fat (227). While exercise attenuates glucocorticoid induced muscle atrophy (228), glucocorticoid exposure (via prednisolone exposure) reduces exercise performance, increases blood glucose concentrations and white blood cell counts and alters Leydig cell function (229).

KLF15—A TARGET OF GLUCOCORTICOID RECEPTOR IN SKELETAL MUSCLE

A peripheral clock system is present in a human adrenocortical cells where periodic oscillations of clock genes are influenced by glucocorticoids, mainly through GRα (230). In human leukocytes, glucocorticoid receptor expression parallels that of plasma cortisol with values peaking in the morning at

04:00–08:00 h and being lowest at 23:00–24:00 h (231). The diurnal variations in the glucocorticoid receptor may serve to coordinate the reactivity of the target cells to cortisol (231). Corresponding to the peripheral clock system are responses to glucocorticoid exposure where, although chronic and sustained exposure to glucocorticoids promotes catabolic consequences for skeletal muscle, intermittent exposure appears to have a more favorable impact (232, 233). In fact, intermittent administration of glucocorticoids appears to promote sarcolemmal repair and muscle recovery from injury (232) and muscle performance (233). In contrast, sustained glucocorticoid exposure induces muscle atrophy. Differences in muscle responses to intermittent compared to sustained exposure to glucocorticoids are likely mediated by transcription factor KLF15, which also increases with weekly exposure, but is suppressed with daily exposure (232).

Transcription factor Kruppel-like factor 15 (KLF15) is a direct target of the glucocorticoid receptor in skeletal muscle (212). Within skeletal muscle it regulates lipid utilization (234), coordinates the transcriptional circuitry responsible for metabolism (234), mediates the metabolic ergogenic effects of glucocorticoids via metabolic programming (233), and affects exercise capacity (212, 234). In addition to its metabolic role, KLF15 regulates myofiber typing (235), mTOR activity (233), and myofiber size (212). KLF15 displays a diurnal pattern of expression, and regulates branched-chain amino acid (BCAA) metabolism and utilization in a circadian fashion (236). Glucocorticoid exposure (237), acute endurance exercise (234), and hyperglycemia lead to increased KLF15 expression. As a direct target gene of the glucocorticoid receptor with a diurnal response pattern, KLF15 signaling may explain the complex role of glucocorticoids in metabolism and protein balance and mechanistically favor the intermittent value of glucocorticoids via exercise or pharmaceuticals.

CONCLUSIONS

Hormones are largely responsible for the integrated communication network responsible for modulating cellular signaling for protein synthesis (165). All aspects from production, release, transportation, and tissue uptake to intracellular signaling affect the cell signaling and communication that govern basic activities of cells and coordinate all cellular actions. Among the "anabolic giants," testosterone is the primary anabolic hormone in men. It's anabolic influence largely dictated through genomic and non-genomic signaling, satellite cell activation, interaction with other anabolic signaling pathways, upregulation or downregulation of the androgen receptor, and potential roles in co-activators and transcriptional activity. Growth hormones exhibit differential influences depending on the "type" of the hormone being assayed and the magnitude of the physiological stress. The actions of IGF-I are regulated by a family of binding proteins (IGFBPs 1–6), which can either stimulate or inhibit biological action depending on binding. Circadian patterning and newly discovered variants of glucocorticoid isoforms largely dictate glucocorticoid sensitivity and catabolic, muscle sparing, or pathological influence. The downstream integrated anabolic and catabolic mechanisms of these hormones not only affect the ability of skeletal muscle to generate force, they also have implications in pharmaceutical treatments (238), aging (176), metabolic syndrome (180), insulin resistance (181), and hypertension (182). Thus, advances in our understanding of hormones that impact anabolic: catabolic processes have relevance for athletes and the general population, alike.

AUTHOR CONTRIBUTIONS

WK, NR, WH, BN, and MF contributed to the conception of the work, drafting the article, critical revision of the article, and final approval of the version to be published.

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Digit Ratio (2D:4D) and Physical Performance in Female Olympic Athletes

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Background: The second to fourth digit ratio (2D:4D ratio) is suggested to be a negative correlate of prenatal testosterone. Little is known about the role of the 2D:4D ratio in relation to serum and urinary androgens for physical performance in female athletes. We aimed to compare the 2D:4D ratio in female Olympic athletes with sedentary controls, and to investigate the 2D:4D ratio in relation to serum and urinary androgens and physical performance in the athletes.

Methods: This cross-sectional study included 104 Swedish female Olympic athletes participating in power, endurance and technical sports and 117 sedentary controls. The 2D:4D ratio was calculated using direct digit measurements. Serum androgens and urinary androgen metabolites were analyzed by liquid chromatography-tandem mass spectrometry. The athletes performed standardized physical performance tests and body composition was established by dual-energy X-ray absorptiometry.

Results: The 2D:4D ratio was significantly lower in the athletes compared with controls although serum testosterone levels were comparable between groups and within normal reference values. The 2D:4D ratio correlated negatively with urinary levels of testosterone glucuronide and 5α- and 5βAdiol-17G, whereas there were no correlations to serum androgen levels. Furthermore, the 2D:4D ratio correlated negatively with strength tests and positively with 3,000-meter running in the athletes.

Conclusion: Female Olympic athletes had a lower 2D:4D ratio, possibly reflecting a higher prenatal androgen exposure, than sedentary controls. Furthermore, the 2D:4D ratio was related to urinary levels of androgen metabolites and physical performance in the athletes but not to serum androgen levels. It is suggested that the 2D:4D ratio could reflect androgen metabolism and may be of importance for sporting success in female athletes.

Keywords: 2D:4D ratio, testosterone, physical performance, androgen metabolites, female athletes

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INTRODUCTION

Prenatal testosterone and estrogen levels are suggested to influence the formation of the second to fourth digit ratio (2D:4D ratio), with high environmental levels of androgens during fetal life being associated with a low 2D:4D ratio (1–3). However, this concept has recently been debated (4–7). The digit ratio, proposed as a sexually dimorphic trait (2, 8, 9), is believed to be set during the first trimester of fetal development (2, 10) and does not change substantially with age (9). Furthermore, the 2D:4D ratio has been associated with Differences of Sex Development (DSD) (11, 12).

The digit ratio has also been suggested to be related to physical performance (13, 14). Previous studies have shown that male athletes have a lower 2D:4D ratio than non-athletes (15–19). However, only a few previous studies have investigated the digit ratio in female athletes in comparison to controls (15, 19–21). An association between the 2D:4D ratio and physical performance have been demonstrated in female athletes for alpine skiing (18), endurance running (22) fencing (23) and rowing (24). Similarly, positive correlations between the 2D:4D ratio and physical fitness (25), and sporting ability (26) have been demonstrated in women taking part in leisure sports. These reports are either based on rather small study groups and/or not including populations of Olympic athletes.

Androgens are considered beneficial for athletic performance by exerting positive effects on muscle tissue, erythropoiesis, immune system, and behavioral patterns, and may also contribute to a decreased risk of injuries and increased health status in athletes (27). In women, the active androgens testosterone and dihydrotestosterone (DHT) are synthesized in the ovaries and the adrenal glands, and by conversion in peripheral tissue of precursor androgens produced in the adrenal cortex, such as androstenedione (A4), dehydroepiandrosterone (DHEA), its sulfate (DHEAS) and 5-androstene-3β, 17β-diol (5-DIOL) (28). Androgens are finally mainly metabolized by uridine diphospho (UDP)-glucuronosyl transferases (UGTs) and to some extent by sulfotransferases (SULTs) and excreted in urine (29).

We have recently published data showing that female Olympic athletes have higher levels of serum androgen precursors compared to controls and that serum androgens are positively associated with physical performance in the athletes (30). These results are of relevance for the ongoing discussion regarding hyperandrogenism in female athletes (27). Adult serum androgens have also been studied in relation to the 2D:4D ratio in non-athletic populations of men and women, demonstrating inconclusive results (31, 32). No previous studies have examined the 2D:4D ratio in relation to the androgen profile in both serum and urine, as well as physical performance in female top athletes.

The aim of the present study was to investigate the 2D:4D ratio in female Olympic athletes and untrained controls, and to study the 2D:4D ratio in relation to the androgen profile in serum and urine and physical performance in the athletes.

MATERIALS AND METHODS

Study Population

The present study included a representative population of Swedish female Olympic athletes ($n = 104$) participating in the summer or winter Olympic games, and 117 controls, for whom digit measurements were obtained (30). The controls were age- and body mass index (BMI)—matched, having a maximum of 2 h endurance and/or strength training per week and no prior participation in elite level competition. All participants were > 18 years of age. Olympic athletes were recruited in connection with pre-Olympic training camps and controls recruited via advertisement (recruitment was conducted from November 2011 to April 2015). The subjects were investigated at the Women's Health Research Unit, Karolinska University Hospital or in connection with pre-Olympic training camps. All participants filled out a general health questionnaire including training hours per week, and hormonal contraceptive use and for the athletes' information concerning sport discipline, age at training debut and age at elite level debut was obtained. Data on menstrual function was collected via questionnaire and confirmed by measurement of serum hormones for participants not using hormonal contraceptives. Blood- and urine samples were collected in a fasted, rested state, between 07.00 and 10.00 am and stored at −20°C until further analysis.

The project was approved by the Regional Ethics Committee (EPN 2011/1426-32). Informed written consent was obtained from all participants.

2D:4D Ratio

Digit measurement expressed in millimeters (mm) was performed for digit two (2D) and digit four (4D) (**Figure 1**) using a Vernier digital caliper 0–150 mm (USA, Cocraft) with a precision of 0.01 mm. Digit length was directly measured from the mid-point of the proximal crease of the proximal phalanx to the distal tip of the distal phalanx for 2D and 4D (9, 15) on both left ($n = 103$ athletes, $n = 116$ controls) and right hand ($n = 104$ athletes, $n = 117$ controls). The 2D:4D ratio was calculated by dividing 2D length by 4D length. In addition, right minus left 2D:4D ratio (Dr-l), suggested as an additional negative marker for prenatal testosterone, was calculated (2). The digits were independently measured by two raters. For rater A, the intraobserver agreement was 0.90 for right hand and 0.90 for left hand. For rater B, the corresponding intraobserver agreement was 0.93 and 0.96, respectively. The inter-rater correlation was 0.87 for the right hand and 0.85 for the left hand (33).

Body Composition

For 65 athletes and 100 controls, body composition [bone mineral density (BMD) (g/cm^2), fat mass (%), lean mass (kg)] was established by dual-energy X-ray absorptiometry (DXA), Lunar Prodigy Advance (GE Healthcare, Madison, WI) at the Karolinska University Hospital, Solna as previously described (30).

Physical Performance

Athletes were offered standardized physical performance tests managed by the SOC at Bosön, Stockholm, Sweden. They

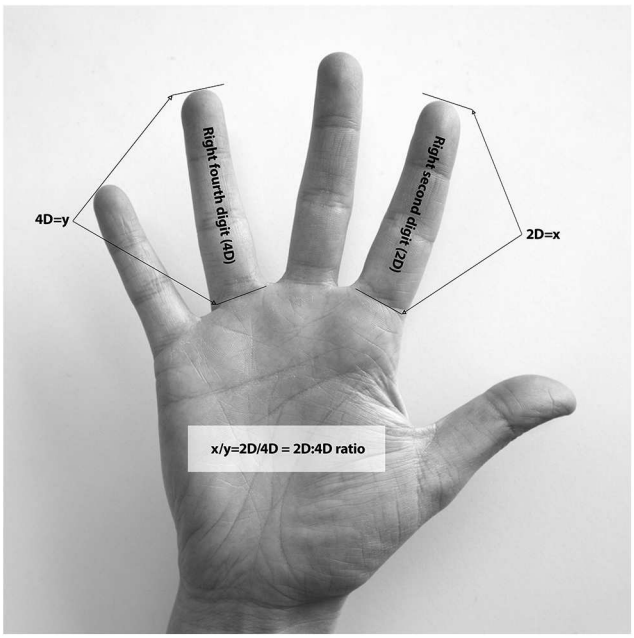

FIGURE 1 | Measurement and calculation of the second to fourth digit (2D:4D) ratio.

participated in physical performance tests measuring explosive power [countermovement jump (CMJ) ($n = 57$), squat jump (SJ) ($n = 58$)], strength (bench press ($n = 45$), chins ($n = 49$)) and 3,000 meters running ($n = 20$).

Serum Steroid Profile

Serum levels of androgens [testosterone, DHEA, DHEAS, DHT, A4 and 17- alpha-hydroxyprogesterone (17-OHP)] and estradiol (E2) were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Endoceutics laboratory, Quebec, Canada, as previously described (34). Free androgen index was calculated (testosterone nmol/L divided by sex hormone-binding globulin (SHBG) nmol/L * 100). Follicle-stimulating hormone (FSH), luteinizing hormone (LH), anti-müllerian hormone (AMH) and SHBG were determined by electrochemiluminiscence immunoassay at the Department of Clinical Chemistry, Karolinska University Hospital as previously described (30).

Urinary Steroid Profile

For 93 athletes, urinary samples were obtained and urinary levels of conjugated (glucuronide and sulfated) androgens [testosterone (T-G, T-S), epitestosterone (EpiT-G, EpiT-S), androsterone (ADT-G, ADT-S), etiocholanolone (Etio-G, Etio-S), DHEA-G, DHEA-S, 5α-androstane-diol (5αAdiol-3G and 5αAdiol-17G) and 5β-androstane-diol (5βAdiol- 3G and 5βAdiol-17G)] were determined by LC-MS/MS as previously described (35) at the accredited doping laboratory, Department of Laboratory Medicine, Karolinska Hospital Huddinge. Specific gravity (SG) was measured for all urine samples by a Digital Urine SG Refractometer (ATAGO UG1, Tokyo, Japan). Using a correction formula, Ccorrected = Cmeasured * ((1.020–1)/(SG−1)), each sample was corrected to a specific gravity of 1.020, adjusting for urine dilution. Limit of detection (LOD) was estimated to be below 0.4 μg/mL for all analytes. For two athletes a urine sample was not obtained and for nine athletes the amount of urine was insufficient for analysis. Testosterone:epitestosterone (T:E) ratio was calculated by dividing testosterone glucuronide (T-G) by epitestosterone glucuronide (EpiT-G).

Statistical Analyses

Statistical analyses were performed using Statistica™ 13 software (Statsoft® Inc., Tulsa, OK, USA). Continuous data was presented as mean ± SD or as median and interquartile range (25th−75th percentile) depending on distribution. Comparison of the 2D:4D ratio and body composition between groups was performed using the student's *t*-test. The proportion of women using hormonal contraceptives and having menstrual dysfunction was calculated by Chi-Square test and type of sport by Fisher's exact test. Effect size for continuous variables was calculated using Cohen's ***d*** and for categorical variables with Phi = $\sqrt{}$(Chi-2/n). Correlations were evaluated by Spearman's rank-order correlation or Pearson's correlation. *P*-values <0.05 were considered significant.

RESULTS

General Characteristics of Female Athletes and Controls

Female athletes and controls were similar regarding age and BMI (**Table 1**). As expected, training hours/week was significantly higher among athletes compared to controls (**Table 1**). Age at training debut was 9.34 ± 4.74 years and age at elite debut was 17.56 ± 3.32 years for the athletes. As previously published, hormonal contraceptive use was similar between groups, but menstrual dysfunction was significantly more common among female athletes compared to controls (30). Furthermore, the Olympic athletes demonstrated a more anabolic body composition, including higher total BMD, lower body fat percent and higher amount of lean mass compared to the controls (30) (**Table 1**). In addition, as previously published, the athletes had significantly lower levels of estrone and higher serum levels of the androgen precursors DHEA and 5-DIOL than controls (30). However, both groups had serum steroid levels within the normal range (30).

2D:4D Ratio in Relation to Serum and Urinary Androgen Levels in Athletes and Controls

The 2D:4D ratio right hand was significantly lower in the female Olympic athletes than the controls ($p < 0.05$) (**Table 1**, **Figure 2**). Furthermore, the athletes demonstrated a significantly lower Dr-l compared to the controls (**Table 1**). No significant correlations were found between serum androgen levels and the 2D:4D ratio in the controls or the

TABLE 1 | General characteristics and body composition in Olympic athletes and controls.

Parameter	Controls	Athletes	Effect size[a]
n	117	104	
Age #	26.2 ± 5.5	25.9 ± 5.6	0.044
BMI #	22.0 ± 2.6	22.0 ± 2.0	0.008
HC use (*n* (%) #	46 (39)	40 (38)	0.009
MD (*n* (%) #	3 (4)	15 (23)**	0.28
Training (hour/week)	0.93 ± 0.85	17.93 ± 5.67***	4.32
Digit ratio			
2D:4D ratio right[b]	0.98 ± 0.04	0.97 ± 0.03*	0.29
2D:4D ratio left[c]	0.97 ± 0.03	0.97 ± 0.03	0.052
Dr-l	0.02 ± 0.03	0.01 ± 0.03**	0.383
Body composition			
N	100	65	
Total BMD (g/cm^2) #	1.15 ± 0.07	1.25 ± 0.08***	1.315
Body fat (%) #	31.7 ± 6.6	18.4 ± 5.9***	2.108
Lean mass total (kg) #	40.4 ± 4.1	49.9 ±5.9***	1.964

Values presented as mean ± SD or percentage. 2D:4D ratio right, Second to Fourth digit ratio right hand; 2D:4D ratio left, Second to Fourth digit ratio left hand; BMD, Bone Mineral Density; BMI, Body Mass Index; Dr-l, right – left 2D:4D ratio; HC, Hormonal Contraceptives; MD, Menstrual dysfunction.

 # Data previously published (30), [a] Cohen's d (continuous variables) or Phi (categorical variables), [b] data available for calculation for 104 athletes and 117 controls, [c] data available for calculation for 103 athletes and 116 controls.

**p < 0.05, **p < 0.01, ***p < 0.001.*

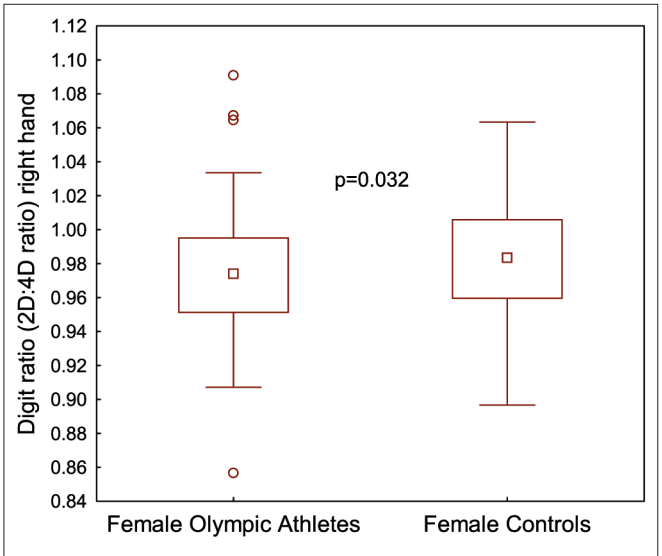

FIGURE 2 | Digit ratio (2D:4D ratio) right hand expressed in millimeters (mm) for female Olympic athletes ($n = 104$) and female controls ($n = 117$).

female Olympic athletes. However, in the athlete group, there were significant negative correlations between the 2D:4D ratio right hand and the urinary steroid metabolites T-G, 5αAdiol-17G and 5βAdiol-17G, see **Figures 3A–C**. However, there were no corresponding correlations for Dr-l.

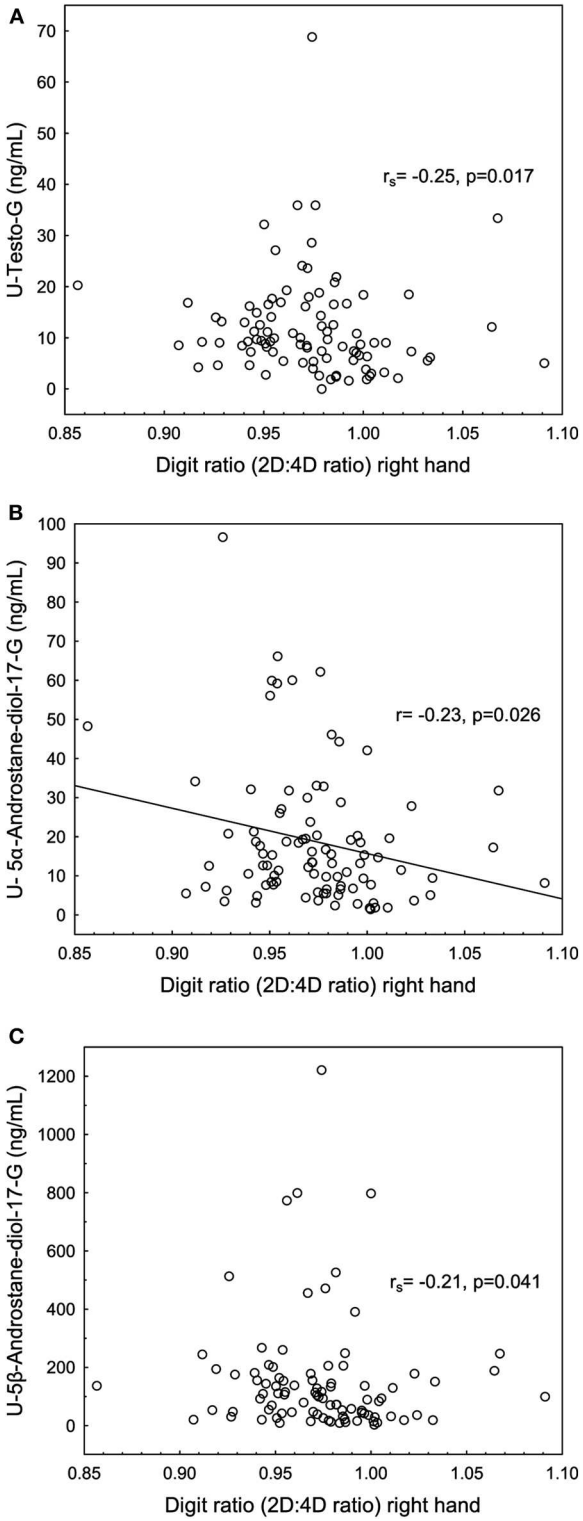

FIGURE 3 | Significant negative correlations between the second to fourth digit ratio (2D:4D ratio) right hand expressed in millimeters (mm) and **(A)** urine testosterone glucuronide (U-Testo-G) (ng/mL), **(B)** U−5α-Androstane-diol-17G (5aAdiol- 17G) and **(C)** U-5β- Androstane-diol-17G (5βAdiol-17G) in female Olympic athletes.

2D:4D Ratio, Urinary Androgens and Physical Performance in Athletes

Significant negative correlations were found between the 2D:4D ratio right hand and bench press and chins, see **Figures 4A,B** and a significant positive correlation between the 2D:4D ratio right hand and 3,000 meters running performance see **Figure 4C**. No significant correlations were found between Dr-l and physical performance.

In turn, 5αAdiol-3G and 5βAdiol-17G correlated positively to chins ($r_s = 0.33$, $p < 0.05$ and $r_s = 0.37$, $p < 0.05$, respectively) and Etio-G correlated negatively to running time 3,000 m ($r_s = -0.51$, $p < 0.05$).

DISCUSSION

To our knowledge, this is the first study investigating the 2D:4D ratio in relation to both the serum and urinary androgen profile and physical performance in female top athletes. We found a lower 2D:4D ratio right hand, suggesting a higher prenatal androgen exposure, in female Olympic athletes than untrained controls. In addition, we found negative correlations between the 2D:4D ratio and the urinary steroid profile, and both these variables correlated significantly with physical performance including strength tests and middle-distance running in the female Olympic athletes.

Few previous investigations (15, 19–21) have reported data on the 2D:4D ratio in female athletes compared with controls. These studies have been performed in female athletes at national level (20), varsity athletes (non- elite athletes) (15), college tennis players (21) and youth handball players (19) demonstrating a significantly lower digit ratio for the athletes compared to controls. However, our study is the first including a large number of female Olympic athletes and untrained controls. In agreement with most previous studies, we found significant differences in the 2D:4D ratio only for the right hand (15, 19, 21). It has been demonstrated that the right hand shows a greater sex difference than the left hand leading to the suggestion that the right hand is more representative of prenatal androgen influence (8).

The relation between prenatal androgen exposure and the 2D:4D ratio is supported by an association between a low 2D:4D and high levels of fetal testosterone relative to estradiol in amniotic fluid in humans (1). Furthermore, male human fetuses have higher androgen levels in amniotic fluid (36) and significantly lower 2D:4D ratio than female fetuses (10). However, a relationship between the 2D:4D ratio and adult sex hormone levels have not been confirmed (31, 32). In agreement, we found no correlation between serum androgen levels and the 2D:4D ratio. More recent studies suggest that the digit ratio is not correlated to resting serum androgen levels, but could be associated with androgen levels in response to physical training (2, 37). However, in our study data was collected in a resting state and not in close connection to training or competition.

On the other hand, we found for the first time, significant negative correlations between the 2D:4D ratio and several urinary

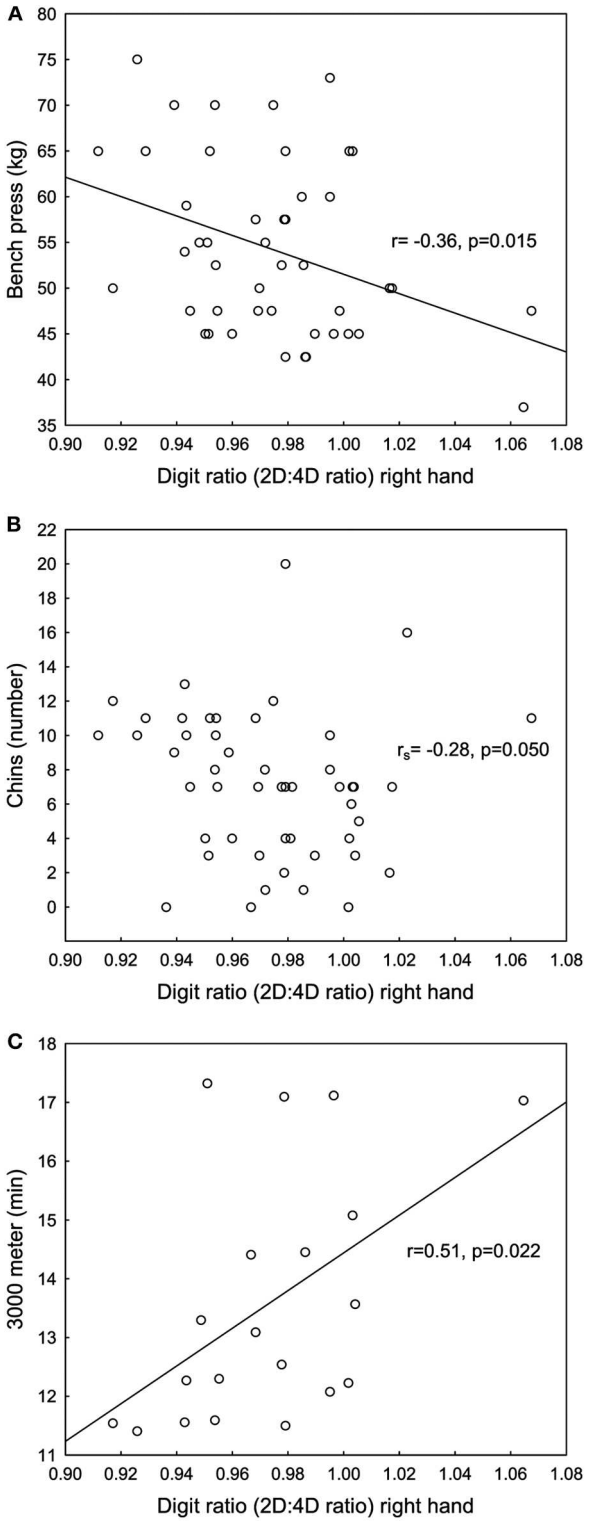

FIGURE 4 | Significant negative correlations between the second to fourth digit ratio (2D:4D ratio) right hand and **(A)** bench press and **(B)** chins and a significant positive correlation between the 2D:4D ratio right hand and **(C)** 3,000 meters running measured in minutes for female Olympic athletes.

androgen metabolites (T-G, 5αAdiol-17G, 5βAdiol-17G). Our findings could reflect a possible difference in androgen phase II metabolism depending on 2D:4D ratio among female athletes. As opposed to the circulatory androgens, the urinary androgen concentrations are dependent on the expression and activity of phase II enzymes i.e., UGTs and SULTs. T-G, 5βAdiol-17G and 5αAdiol-17Gs are all conjugated at the 17β- OH-position preferable by UGT2B17, whereas T-G and 5αAdiol-17G may also be inactivated by UGT2B15, UGT2A1, and UGT1A4 (38, 39). The expression and activity of UGT2B17 and UGT2B15 are known to be higher in men than in women (40, 41). It is possible that polymorphisms in UGTs and other androgen metabolizing enzymes, as well as other factors that determine the expression and activity of UGTs, may be associated with the androgen load of the fetus in the first trimester. Several fetal UGTs are expressed already in the first trimester (42). Further studies are warranted to establish any putative link between phase II metabolism and the 2D:4D ratio.

In support of a role of prenatal androgen exposure for physical performance, we found significant correlations between the 2D:4D ratio and athletic performance tests in the Olympic athletes. Previous studies on 2D:4D ratio and physical performance have focused on male athletes (16, 17, 22), whereas there is more limited research in female athletes. The few previous studies in female athletes have demonstrated significant correlations between a lower 2D:4D ratio and faster rowing times (24), faster skiing times (18), better endurance running performance (22) and better national fencing rank (23). In our study of top-level female athletes, the 2D:4D ratio was significantly related to both strength tests and 3,000 m running, a test mainly representative of aerobic capacity.

There are several possible underlying mechanisms for the association between the 2D:4D ratio and athletic performance. We and others have previously demonstrated associations between adult serum androgen levels and muscle mass and strength in female athletes (30, 43, 44). However, we found no correlations between serum androgen levels and the 2D:4D ratio. In contrast, there were correlations between the 2D:4D ratio and urinary steroid metabolites, which in turn correlated to physical performance tests. As urinary androgen levels are dependent on androgen metabolizing enzymes, these urinary metabolites are more representative for the androgen metabolism than circulating androgens. Furthermore, the androgen metabolism is dependent on genetic variations. We hypothesize that genetic variation of the androgen metabolism and thereby androgen activity, could influence both the development of the 2D:4D ratio during fetal life and the predisposition for physical performance.

In conclusion, we found a lower 2D:4D ratio related to urinary androgen levels and physical performance in female Olympic athletes. The association between the digit ratio and urinary androgen levels, but not serum androgen levels, may indicate that the 2D:4D ratio reflects variations in androgen metabolism rather than absolute circulating androgen levels. A low digit ratio was associated with increased aerobic and strength performance in the athletes. Although the link between the 2D:4D ratio and physical performance, is still not fully clarified, our results suggest that prenatal androgen exposure, in addition to the adult androgen levels, may be of importance for athletic capacity in female Olympic athletes.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Regional Ethics Committee (EPN 2011/1426-32). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EE, BB, and AH were involved in the concept/design of the study, acquisition of data, and data analysis. J-OT and ME performed the quantification of urinary androgens. EE, BB, LE, J-OT, ME, and AH were involved in the manuscript preparation, critical revision of the article and approval of the article. All authors listed met the conditions required for full authorship.

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Conflict of Interest: BB is the medical director for the Swedish Olympic Committee (SOC) and AH is medical adviser to the SOC, the International Association of Athletic Federation (IAAF) and the International Olympic Committee (IOC). The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Microdialysis-Assessed Exercised Muscle Reveals Localized and Differential IGFBP Responses to Unilateral Stretch Shortening Cycle Exercise

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Microdialysis allows for a preview into local muscle metabolism and can provide physiological insight that blood measurements cannot.

Purpose: To examine the potential differential IGF-I system regulation in interstitial fluid during unilateral stretch shortening cycle exercise.

Methods: 10 men (26 ± 7 year) performed unilateral jumping [stretch shortening cycle (SSC) exercise at 50% of optimal jump height] until volitional fatigue on a sled apparatus. Biological sampling took place using a catheter inserted into an antecubital vein (serum), and 100 kDa microdialysis probes inserted into the thigh muscle of each exercise/control leg (dialysate). Serum was drawn before (Pre; −3 h) and after SSC [Post I (+0 h), II (+3 h), or III (+20 h)]; dialysate was sampled for 2 h before (Pre), during/immediately after (Ex), and 3 h into recovery (Rec) following SSC. IGF-I system parameters (free/total IGF-I and IGFBPs 1–6) were measured with immunoassays. Interstitial free IGF-I was estimated from dialysate IGF-I and relative recovery (ethanol) correction. Data were analyzed with repeated measures ANOVA.

Results: Serum total IGF-I remained elevated +3 h (Post II: 182.8 ± 37.6 vs. Pre: 168.3 ± 35.0 ng/mL, $p < 0.01$), but returned to baseline by +20 h (Post III vs. Pre, $p = 0.31$). No changes in serum free IGF-I were noted. Serum BP-1 and −3 increased over baseline, but not until + 20 h after SSC (Post III vs. Pre: 7.6 ± 4.9 vs. 3.7 ± 2.3 and 1,048.6 ± 269.2 vs. 891.4 ± 171.2 ng/mL, respectively). We observed a decreased serum BP-6 +3 h after SSC ($p < 0.01$), followed by a return to baseline at +20 h ($p = 0.64$ vs. Pre). There were no exercise-induced changes in serum BP-2, −4, or −5. Unlike serum, there were no changes in dialysate or interstitial free IGF-I in either leg ($p > 0.05$). Dialysate BP-1 remained increased in both exercise and control legs through 3 h into recovery (Rec vs. Pre, $p < 0.01$). Dialysate BP-3 also demonstrated a prolonged elevation over Pre SSC concentrations, but in the exercise leg only (Ex and Rec vs. Pre, $p < 0.04$).

We observed a prolonged decrease in dialysate BP-5 (Ex and Rec vs. Pre, $p < 0.03$) and an increase in BP-4 IP in the exercise leg only. There were no changes relative to Pre SSC in dialysate BP-2 or −6.

Conclusions: Unilateral exercise drives differential regulation of the IGF-I system at both local and systemic levels. More specifically, this is the first study to demonstrate that localized exercise increases IGFBP-3, IGFBP-4 and decreases in IGFBP-5 in muscle interstitial fluid.

Keywords: microdialysis, IGF-I, stretch shortening cycle exercise, interstitial fluid, binding proteins, muscle

INTRODUCTION

The insulin-like growth factor-I (IGF-I) system serves as an important metabolic modulator for physiological processes related to growth, development, muscle repair/regeneration and altered energy and activity paradigms (1–8). The IGF-I system circulates and is present across a number of different biocompartments (i.e., blood, interstitial fluid, and muscle) (9). The IGF-I system is comprised of IGF-I itself and a family of six different binding proteins (BPs 1–6) (1, 10–12). The biological action IGF-I is influenced by a family of BPs that can serve to either stimulate or inhibit IGF-I action (1, 10–12). A recent special edition in Frontiers in Endocrinology highlights the important role of IGFBPs for increasing functional diversity and utility for discerning context-dependent roles for IGF-I action and signaling (3, 10). While IGFBPs are known to serve as carrier, sequestering, and trafficking reservoirs both potentiating and inhibiting IGF-I action, they have also been demonstrated to possess IGF-independent actions (1, 10–12). We have previously observed that acute resistance exercise had no impact on overnight IGF-I concentrations, but alterations were detected for IGFBPs suggesting that exercise could influence the manner in which IGF-I is partitioned among its family of BPs (13). More recently, our laboratory has further reported the importance of measuring IGFBPs by demonstrating that 8 weeks of exercise training resulted in increased basal IGFBPs 2 and 3, but no change in total IGF-I and have recommended moving beyond solely relying on measures of total IGF-I concentrations for insight into IGF-I physiological action (14).

The modulatory influence of IGFBPs on IGF-I action is well established across various *in vitro* tissues and cell types, but much less studied and understood within the context of exercise responses and adaptations (2, 7, 15). Awede et al. (16) were the first to demonstrate the influence of loading on IGFB-4 and IGFBP-5 gene expression using a murine model, illustrating the potentially critical mechanistic role for IGFBPs mechano-transduction in muscle adaptation. Further, most exercise studies have measured the IGF-I system within the systemic circulation. IGF-I activity in interstitial fluid most proximal to tissues and cells could provide potentially meaningful context to understanding how exercise conveys hormonal and biochemical signals (4, 7, 9, 15, 17–19). Microdialysis is a method to sample interstitial fluid allowing for a preview into local muscle metabolism, and can provide physiological insight that blood measurements cannot (9, 17, 20–23). For example, we have previously demonstrated that post-exercise IGF-I increases in the systemic circulation are not reflected in post-exercise interstitial fluid (ISF) (9). To date, we are only aware of two studies measuring IGFBPs in ISF following exercise (21, 24). Berg et al. (21) reported no change in IGFBP-1 and IGFBP-3 proteolysis in exercised muscle via microdialysis and Olesen et al. (24) reported that IGFBP-4 was increased in peritendinous tissue after running exercise, suggesting a key role in human collagen synthesis.

As both IGF-I and IGFBPs are produced systemically and locally, it has proven difficult to discern the relative impact of exercise-mediated whole-body metabolic-stress (i.e., systemic) vs. muscle/load specific (i.e., local) on subsequent IGF-I system responses (2, 7, 15). Also of particular note, stretch-shortening cycle (SSC) exercise is a unique model to study the interaction between neural, mechanical, structural and biochemical events (25, 26). An advantage of SSC over isolated concentric and eccentric contractions is that SSC exercise more closely mimics the loading of the neuromuscular system observed in normal human locomotion (26). To more fully examine the IGF-I system (both IGF and IGFBPs) across biocompartments in exercised muscle, we sought to determine whether IGFBPs were differentially influenced by SSC exercise using microdialysis to sample ISF from both an exercised limb vs. a control limb with simultaneous blood sampling.

METHODS

Subjects

Ten healthy men (age: 26 ± 7 year; height: 180 ± 8 cm, body mass: 77.4 ± 10.7 kg) volunteered after being briefed on all study methods, risks, and discomforts, and after providing their verbal and written consent. Participants' height was measured to the nearest 0.1 cm using a stadiometer and body mass was measured using a standard electronic scale to the nearest 0.1 kg. The study protocol was performed according to the Declaration of Helsinki, and was approved by the Commission on Ethics of the University of Jyväskylä prior to implementation.

Experimental Design Overview

Subjects were asked to refrain from caffeine, alcohol, and exercise for 24 h prior to the experimental visit. On the morning of the visit, overnight-fasted subjects had a venous catheter inserted into their antecubital vein for systemic blood draws, and microdialysis probes inserted into their thigh (vastus lateralis; VL) muscle to sample local skeletal muscle ISF (dialysate). Using

sterile procedures, microdialysis probes were inserted into the VL of each leg, which has been described elsewhere in detail (9). Briefly, a small amount (∼2 cc per insertion site) of 1% lidocaine was injected just under the skin above the distal VL on both legs. Next, two pre-sterilized non-linear 100 kDa molecular weight cut-off probes (30 mm membrane; MDialysis Inc., N. Chelmsford, MA) were placed in the VL muscle of each leg using an 18-gauge needle and manufacturer provided removable sheath introducer on each leg. The introducer was removed, leaving only the probe membrane embedded in the muscle. Once all probes were inserted and secured to the leg, the inlet tubing was connected to a portable CMA adjustable flow rate pump (CMA-107; set to 2 μL/min) containing a syringe filled with sterile perfusate solution [0.9 % sodium chloride, 30 g/L Dextran 40 (Pharmacosmos, Holbaek, Denmark), and 10 mM ethanol (EtOH)]. Dextran was added to prevent fluid loss across the large pore size membrane (ultrafiltration), and EtOH was added to qualitatively estimate changes in microvascular blood flow and to calculate interstitial concentrations. After a 5 min flush sequence, perfused probes were left in tissue for at least 45–60 min prior to any sampling. Due to the extended time period of the experimental visit, subjects were provided a small food bar (180 kcal) before and after the exercise protocol.

Acute Exercise Protocol

In order to study the localized effects in an exercise vs. control muscle, subjects performed a unilateral jumping exercise utilizing the stretch shortening cycle (SSC) following a standardized 0 min warm-up on a bicycle ergometer 60–70% of maximum HR. Utilizing a specially designed sled apparatus described previously (26), subjects performed unilateral jumps with the dominant leg, keeping the contralateral leg isolated/passive during exercise. Thus, we were able to have the one leg serve as an internal control when comparing local muscle IGF-I responses to exercise. Subjects were secured on the sled apparatus using straps to prevent unnecessary movement during the jumping motions. The unilateral jumps began from a knee angle set to 107° from flexion (measured via a goniometer) with the sled set at 23° from the horizontal plane. The jumping protocol was set at 50% of the individual optimal jump (optimal parameters were determined in a separate assessment) and continued until volitional fatigue. For the separate assessment, optimal drop heights were determined individually for each subject by having them dropped on the sledge-jump from different heights to determine their rising height (25, 26).

The number of fatiguing jumps performed ranged from 60 to 1,280.

Biological Sampling

At pre-determined time points before (Pre; −3 h before exercise) and after (Immediately Post/Post I: 0 h; Post II: +3 h; Post III: +20 h) the SSC exercise, subjects had blood drawn from the venous catheter. Serum was separated after allowing the blood sample to clot at room temp for 30 min and after centrifugation at 3,000 g for 15 min. Skeletal muscle dialysate samples were collected 2-h prior to (Pre), immediately following exercise (Ex); including time during the SSC exercise), and 3 h post-exercise recovery (Rec). Sample vials were weighed prior to and following each collection in order to monitor flow rate. Dialysate was run immediately for EtOH, and the remaining aliquot was stored at −20°C until specific IGF-I system analysis occurred. **Figure 1** depicts the sampling timelines for blood and interstitial fluid.

Assays

Serum and dialysate samples were analyzed for several IGF-I system components, and all samples for a particular analyte were run in the same assay batch to minimize intra-assay variance. Serum Total IGF-I was analyzed on the Immulite 1000 (Siemens Healthcare Diagnostics, Malvern, PA; LKGF1 kit, reported sensitivity of 20 ng/mL). Serum and dialysate Free IGF-I was analyzed with an ELISA from Beckman Coulter (Brea, CA; DSL-10-9400 kit, reported sensitivity of 0.015 ng/mL), and quantified on a Dynex MRX Revelation absorbance reader (Dynex Technologies, Chantilly, VA). Serum and dialysate IGFBP-1 through BP-6 were analyzed on a multiplexed bead-based fluorescent assay from Millipore (Billerica, MA; HIGFBP-53K multiplex kit, reported sensitivity of 0.013, 0.325, 0.145, 0.573, 1.15, and 0.078 ng/mL, respectively), and quantified on the Luminex 200 Instrument. Intra-assay CVs for the respective assays were as follows: Total IGF-I = 4.4%; Free IGF-I = 8.7%; BP-1 through BP-6 ranged 6.2–12.5%.

Perfusate and dialysate samples were run for EtOH concentrations using a clinical analyzer. A subsequent calculation of the outflow: inflow ratio (O:I) from [dialysate EtOH]/[perfusate EtOH], allowed a qualitative estimate of local microvascular blood flow (where O:I is inversely proportional to local blood flow), and subsequent estimation of interstitial analyte concentrations from measured dialysate analyte concentrations since EtOH is not metabolized by the local tissue. Thus, changes in EtOH concentration reflect changes in blood flow.

Interstitial Estimates

As the interstitial concentration of an analyte will depend on changes in production and clearance rates, the changes in microvascular blood flow to a tissue (e.g., changes in EtOH O:I ratio) can be used to estimate/calculate interstitial concentrations from assayed dialysate concentrations. Using the external standard approach and separate *in vitro* experiments to determine the relative recovery rates of analyte to EtOH under various conditions, we calculated the *in vivo* interstitial concentration of multiple IGF-I system components (free IGF-I, IGFBPs 1-6) using the following equation: [*in vivo* interstitial analyte] = [(*in vitro* EtOH relative recovery/*in vitro* analyte relative recovery) × (*in vivo* dialysate analyte)]/[1–(*in vivo* EtOH O:I)]. This interstitial estimation is similar to interstitial corrections previously reported (23, 27).

Statistical Analyses

All data are presented as mean ± standard deviation, unless otherwise noted. Acute changes in serum IGF-I system components were assessed with repeated measures ANOVA (RMANOVA), using within-subjects time factor (Pre, Post I, II, III). Acute changes in dialysate/interstitial IGF-I system

FIGURE 1 | Sampling schematic for blood via venipuncture and interstitial fluid via microdialysis. Samples were obtained at the same timepoints for pre, immediate post, and +3 h post timepoints. An additional blood sample was obtained at +20 h post.

components were assessed with a RMANOVA, using within-subjects time factor (Pre, Ex, Rec) and were analyzed in each leg (exercise, control) separately. If the RMANOVA model detected a significant F-ratio ($p < 0.05$), *post-hoc* comparisons were tested with an LSD test. All analyses were conducted using SPSS Statistics v. 21 (IBM, Artmonk, NY).

RESULTS

Serum Total and Free IGF-I

As a result of unilateral SSC exercise, there was an acute increase in serum total IGF-I at the Post I and Post II time points ($p < 0.01$ for both vs. Pre), and by Post III, serum total IGF-I had returned to baseline values ($p = 0.31$ vs. Pre). In contrast, there were no acute changes in serum free IGF-I with the SSC exercise ($p = 0.55$) (Refer to **Figure 1**).

Serum IGF-I Binding Proteins 1-6

BP-1 demonstrated a delayed increase in serum concentrations but not until the +20 h time point (different from all preceding time points, $p < 0.01$). There were no changes observed with BP-2 ($p = 0.52$). As with BP-1, there was an increase in serum BP-3, but not until the +20 h time point, which was the only significant difference from baseline ($p = 0.05$ vs. Pre). We observed a main time effect for serum BP-4 ($p < 0.01$); however, *post-hoc* testing revealed that the apparent increase at the immediate post time point did not reach statistical significance ($p = 0.07$ vs. Pre). There were significant reductions in the circulating BP-4 pool during the recovery time period (Post II and Post III vs. Post I, $p < 0.05$). There was no acute change observed for serum BP-5 ($p = 0.06$). In a pattern different from all other serum BP responses, we observed a significant decrease in serum BP-6 at the + 3 h post time point ($p < 0.02$ vs. all other time points) (Refer to **Figure 2**).

Dialysate Free IGF-I

As with serum free IGF-I, there were no acute changes with dialysate free IGF-I following SSC exercise, in either the exercise ($p = 0.62$) or control ($p = 0.70$) leg musculature (Refer to **Figure 3**).

Dialysate IGF-I Binding Proteins 1-6

Figure 5 displays the results for interstitial fluid IGFBPS. For dialysate BP-1, a significant main time effect was observed in both control and exercise leg musculature ($p < 0.01$ for both). Within the exercise leg, there was an increase throughout exercise and into recovery (Ex vs. Pre, $P < 0.01$; Rec vs. Ex, $p = 0.01$), whereas in the control leg, there was only a significant increase observed during the +3 h post recovery period (Rec vs. Ex, $p = 0.01$). Similar to serum BP-2, there were no acute changes in dialysate BP-2 with exercise (exercise leg, $p = 0.14$; control leg, $p = 0.94$). Dialysate BP-3 demonstrated an acute and sustained elevated concentration relative to baseline in the exercise leg (Ex and Rec vs. Pre, $p < 0.05$), whereas the control leg did not have a significant response ($p = 0.06$). For dialysate BP-4, there was a main time effect for the exercise leg only ($p = 0.05$), while the control leg did not change ($p = 0.17$). *Post-hoc* testing revealed that within the exercise leg, only the immediately post exercise time point was trending toward BP-4 being significantly increased (Ex vs. Pre, $p = 0.06$). Interestingly, there was a decrease in dialysate BP-5 in the exercise leg only, which remained below baseline values during the post recovery period (Ex and Rec vs. Pre, $p < 0.02$). Dialysate BP-5 did not change in the control leg ($p = 0.29$). Dialysate BP-6 did not change in either the exercise or control leg ($p = 0.28$

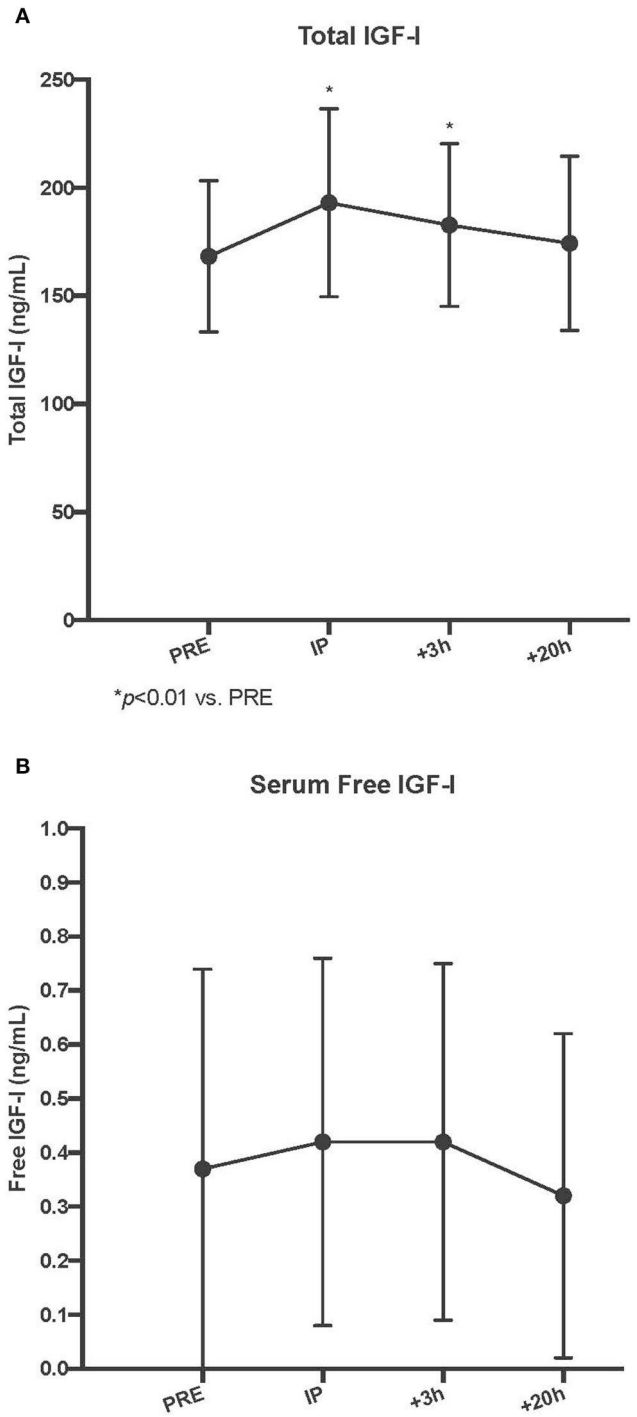

FIGURE 2 | Circulating serum total **(A)** and free insulin-like growth factor-I (IGF) **(B)** concentrations pre, immediately post (IP), 3 h post +3 h, and 20 h post (+20 h) to unilateral stretch shortening exercise. *denotes statistical difference from pre-exercise ($p < 0.05$).

and 0.55, respectively) as a result of unilateral SSC exercise (Refer to **Figure 4**). **Table 1** lists the comparison of IGF-I system component response patterns across the blood and muscle ISF for exercises and control leg during unilateral stretch shortening cycle exercise.

EtOH O:I

We observed that microvascular blood flow was increased (decreased EtOH O:I ratio) during the exercise time period (O:I = 0.15 ± 0.07), which was different from both resting (O:I = 0.19 ± 0.06) and recovery (O:I = 0.20 ± 0.07) time points ($p < 0.01$ for both), but was not different between exercise (O:I = 0.18 ± 0.06) and control (O:I = 0.18 ± 0.06) leg musculature (main effect: $p = 0.67$).

Interstitial Free IGF-I

Our estimated interstitial concentrations indicated that we recovered ∼15% IGF-I through our microdialysis probe across both exercise and control leg musculature *in vivo*. Although the amount recovered was not different between exercise and control legs ($p = 0.56$), the recovery was slightly higher with exercise (15.82 ± 1.23%) over resting (15.16 ± 1.17%) and recovery (15.04 ± 1.27%) time points ($p < 0.01$ for both). Further, although the corrected interstitial IGF-I concentration was higher than both serum and dialysate concentrations as expected, the corrected interstitial free IGF-I muscle protein concentration demonstrated a similar pattern as the dialysate IGF-I concentration, such that there were no acute changes in either the exercise or control legs (main time effect, $p = 0.77$ and 0.66, respectively).

DISCUSSION

The experiment in the current study was designed to extend our knowledge of exercise influences on the IGF-I system by using microdialysis during unilateral stretch-shortening cycle leg exercise to measure IGF-I and its associated family of IGFBPs in muscle interstitial fluid (ISF). By sampling muscle ISF in a control vs. exercise leg, we were able to selectively delineate the effects of systemic vs. local effects on the IGF-I system in the extracellular space (i.e., interstitial fluid) surrounding contracted vs. non-contracted muscle. Novel and salient findings are that when compared to the control leg, the ISF of the exercised leg revealed localized and differential IGFBP responses. Specifically, exercised vs. control muscle ISF demonstrated increases in IGFBP-3 and IGFBP-4, and decreases in IGFBP-5 concentrations. We interpret these data and other emerging IGFBP data (1, 3, 12, 14, 28–30) to support our working hypothesis that a major influence of exercise on the IGF-I system across various biocompartments is via IGFBPs and perhaps even more so than alterations in total IGF-I circulating concentrations.

Exercise resulted in increased IGFBP-3 and IGFBP-4 and decreased IGFBP-5 muscle ISF concentrations. Given some of the known potentiating and inhibitory functions for IGFBPs, these findings could be generally considered favorable for IGF-I biological activity and subsequent muscle adaptation (1, 10, 31, 32). While IGFBP-3's main role in to modulate IGF-I bioavailability in the blood, it is interesting to note that IGFBP-4 and −5 are the most abundant IGFBPs in muscle (33). Comparing our results to those of the literature are difficult

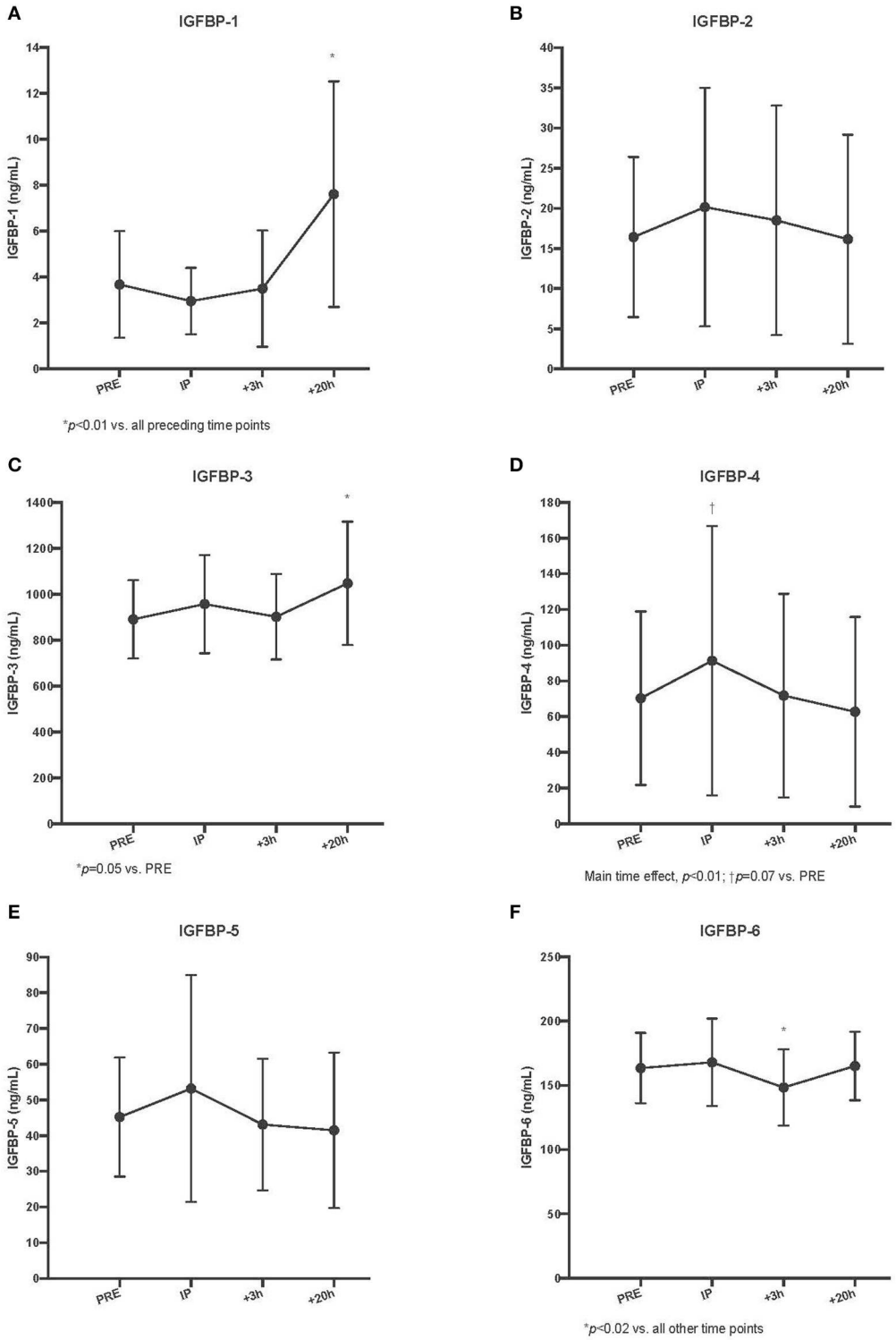

FIGURE 3 | Circulating insulin-like growth factor-I binding protein (IGFBP) concentrations for IGFBP-1 **(A)**, IGFBP-2 **(B)**, IGFBP-3 **(C)**, IGFBP-4 **(D)**, IGFBP-5 **(E)**, and IGFBP-6 **(F)** pre, immediately post (IP), 3 h post (+3 h), and 20 h post (+20 h) to unilateral stretch shortening exercise. *denotes statistical difference from pre-exercise ($p < 0.05$).

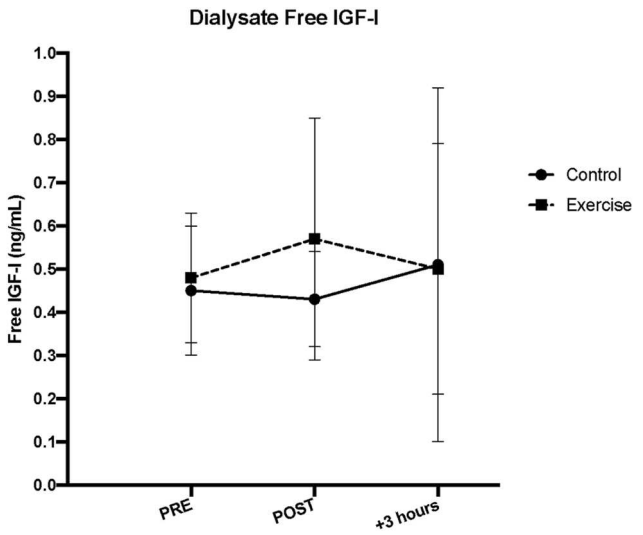

FIGURE 4 | Interstitial fluid free insulin-like growth factor-I (IGF-I) concentrations measured via microdialysis dialysate in exercise vs. control leg pre, immediately post (IP), and 3 h post (+3 h) unilateral stretch shortening exercise.

as we are aware of only two previous studies that measured IGFBPs in exercising muscle via microdialysis and reported no change in IGFBP-1 and IGFBP-3 proteolysis (21) and no change in IGFBP-3 and 4 (34). However, Olesen et al. (24) have measured IGFBPs via microdialysis in peritendinous connective tissue after running and reported increases in local IGFBP-4 concentrations. The current study is the first to concomitantly measure the entire IGFBP family (i.e., BPs 1-6) in both blood and muscle ISF, and thereby provides some important insight for exercise effects across these two important biocompartments. It is possible that the different temporal IGFBP concentration response patterns observed across these biocompartments may represent different physiological cascades impacting whole body metabolism and physiology vs. local muscle metabolic and recovery adaptations (35).

Under certain conditions, IGFBPs 4 and 5 have been shown to have both stimulatory and inhibitory actions (1, 16, 36, 37). Awede et al. (16) were the first to demonstrate regulation of IGFBP-4 and 5 gene expression by loading in mouse skeletal muscle. With overload, Awede et al. (16) demonstrated a 200% increase in IGFBP-4 mRNA levels and a 60% decrease in IGFBP-5 mRNA and a subsequent 200% increase with in IGFBP-5 mRNA with unloading, providing some of the earliest evidence implicating IGFBPs in adaptation to skeletal muscle loading. While Ewton et al. (31) have reported a dual role or IGFBP-5 in L6A1 myoblasts dependent on the culture medium, the underlying mechanisms for an inhibitory role reside during the early proliferative response of L6A1 cells to IGF-I by inhibition of: tyrosine phosphorylation of the cytoplasmic signaling molecules, IRS-1 and Shc, the activation of the MAP kinases, ERK1 and ERK2, the elevation in steady-state levels of the mRNA of the nuclear transcription factor c-fos, the early inhibition of elevation of myogenin mRNA, and increase in cell number (31). Other evidence also suggests an inhibitory role for IGFBP-5 such as comprised survival, growth, muscle development and fertility in mice (38), IGFBP-5 interaction with thrombospondin-1 to induce negative regulatory effects on IGF-I action (39), IGFBP-5 blocking of muscle differentiating IGF-I actions (40), and IGFBP-5 induced cell senescence (41). Thus, we interpret the decrease in ISF IGFBP-5 immediately post-exercise and +3 h into recovery in the exercise leg only to be a positive response with regard to muscle adaptation and conclude that this is also specific and localized within the ISF IGF-I system that has not been previously reported. The additional observation of ISF IGFBPs 3 and 4 changing before corresponding discernable changes in the circulation also substantiate the value of sampling and measuring the IGF-I system outside of the blood biocompartment to provide greater physiological insight and context (4, 7, 9, 18, 20, 21, 24, 34).

The exercise response for IGFBP-4 ($p = 0.06$ for the immediate post time point) was in the opposite direction (i.e., increased), less pronounced and not sustained when compared to IGFBP-5 response (decrease). While Miller et al. (34) did not report an IGFBP-4 increase after one-legged kicking exercise, Oleson et al. (24) reported an elevation in IGFBP-4 following exercise in peritendinous connective tissue ISF and noted that this increase preceded an elevation of procollagen type I carboxy-terminal propeptide (PICP). IGFBP-4 was also the only IGFBP to manifest an apparent increase at similar time points (immediate post exercise in both blood and ISF). While it is difficult to assign a definitive role for IGFBP-4 in muscle hypertrophy, a recent review by Clemmons suggests that given the available evidence in human studies, increased IGFBP-4 increases the supply of available IGF-I and if sufficient proteolytic activity is present this results in enhanced free IGF-I bioavailability and an anabolic response (1). In muscle ISF, IGFBP-3 was increased at the immediate post time point, similar to IGFBP-4. In the blood, IGFBP-3 was increased only at the +20 h post time point. Even though IGFBP-3 increases are considered as positive and stimulatory for circulating IGF-action and signaling, it is likely that similar conditions must exist for IGFBP-3 to be anabolic in muscle microenvironment (1, 10, 15).

While ISF IGFBPs 3, 4, and 5 demonstrated specific and localized exercise effects (changes were only observed in the exercised leg), IGFBP-1 increased in both the exercise and control leg likely reflecting an overall metabolic systemic effect (1, 12, 28, 32). In a study examining the effects of exercise mode and duration on 24-h IGF-I system recovery responses, IGFBP-1 was the only IGFBP that was sensitive to the exercise duration (IGFBP-1 was increased with longer duration exercise) (28). It was concluded that IGFBP-1 was a sensitive circulating biomarker reflecting the physiological strain by exercise. IGFBP-1 is typically considered inhibitory to IGF-I action and inversely proportional to insulin release (32). A recent report suggests that active recovery following heavy resistance exercise may attenuate circulating IGFBP-1 perhaps assisting in the facilitation of the recovery processes (30). Of note, circulating IGFBP-1 in the blood was increased for the +20 h post time point likely indicating the exercise was sufficiently metabolically taxing and recovery processes were still in effect the day after the exercise. When all blood and ISF IGFBP findings are considered, the

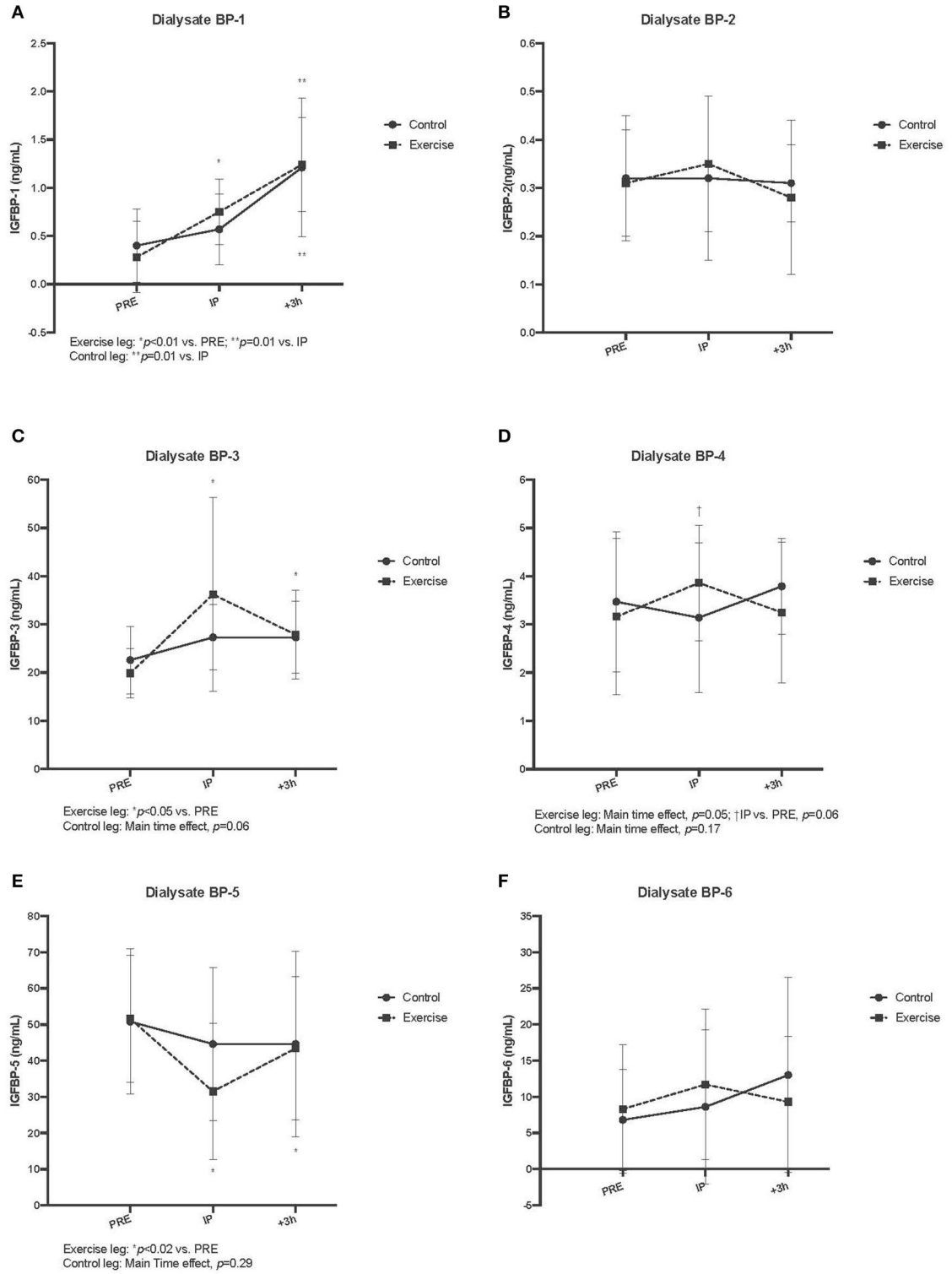

FIGURE 5 | Interstitial fluid free insulin-like growth factor-I binding protein (IGFBP) concentrations measured via microdialysis dialysate in exercise vs. control for IGFBP-1 **(A)**, IGFBP-2 **(B)**, IGFBP-3 **(C)**, IGFBP-4 **(D)**, IGFBP-5 **(E)**, and IGFBP-6 **(F)** pre, immediately post (IP), 3 h post (+3 h), to unilateral stretch shortening exercise. *denotes statistical difference from pre-exercise ($p < 0.05$). Main and interaction effects are also noted in the figures.

TABLE 1 | Comparison of IGF-I system component response patterns during unilateral stretch shortening cycle exercise for blood (measured via veninpuncture) and exercised and control muscle interstitial fluid (ISF) (measured via microdialysis).

IGF-I system component	Blood	Muscle ISF Exercise leg	Muscle ISF Control leg
Total IGF-I	↑ (IP, +3 h post)	NA	NA
Free IGF-I	↔	↔	↔
IGFBP-1	↑ (+20 h post)	↑ (IP, +3 h post)	↑ (+3 h post)
IGFBP-2	↔	↔	↔
IGFBP-3	↑ (+20 h post)	↑ (IP, +3 h post)	↔
IGFBP-4	↑ (IP)	↑ (IP; $p = 0.06$)	↔
IGFBP-5	↔	↓ (IP, + 3 h post)	↔
IGFBP-6	↓ (+3 h post)	↔	↔

↑, increase; ↓, decrease; ↔, no statistical significant change ($p > 0.05$); NA, not measured. This table was adapted from ***Figures 1–4****.*

20+ h post time point was most coincident for IGFBP-1 in that elevations were observed in both blood and ISF. However, the temporal resolution pattern reveals that ISF IGFBP-1 increases post-exercise can be observed ∼17 h before detected in the blood.

While the IGFBP family likely contributes to an amplified level of functional and regulatory diversity that serves to facilitate fine-tuning of IGF bioactivity and signaling (10), it is also recognized that IGFBPs possess IGF-independent actions (36). Of the major actions credited to IGFBPs (10): sequestration of IGF-1 away from the IGF-I receptor, promotion of IGF signaling by proteolytic cleavage and liberation of IGFs from IGF/IGBP complexes, trafficking and concentrating IGF-I toward receptor to provide availability and access, and IGF independent actions via binding to the IGFBP receptor, the independent actions of IGFBPs perhaps represent the most intriguing consideration within the context of our current findings. A number of characteristics of IGFBPs contribute to their amplified flexibility and versatility in influencing exercise-mediated adaptations (10): (1) distinct spatiotemporal expression patterns of IGFBP genes, (2) differences in ligand-binding affinity and selectivity, (3) different roles in the circulation including formation of binary and ternary complexes, (4) different abilities to interact with cell surface proteins, extracellular proteins, and other growth factors, (5) different subcellular localization, and (6) independent actions.

No significant changes were observed in interstitial fluid free IGF-I in either leg during the sampling period. This finding is consistent with previous reports also reporting no significant changes in ISF IGF-I following exercise (9, 20, 24). In our previous report indicating no change in ISF IGF-I after explosive, high-power exercise, we attempted to maximize the likelihood of detecting any potential change by using a larger molecular cutoff microdialysis probe (100 kDA) than previous studies had used (9). However, in our previous study, we removed the microdialysis catheters during exercise to safeguard against possible damage to the catheter integrity during muscle contractions. Removing the microdialysis catheter during exercise required a 45-min equilibration period post-exercise catheter insertion before ISF IGF-I could be sampled, therefore, we could not entirely dismiss the possibility that a bolus of IGF-I release was missed due to a diminished temporal resolution. Subsequent pilot work prior to the current study indicated that microdialysis catheter insertion during exercise was both viable and safe. Hence, any bolus of IGF-I release during exercise into the ISF should have been collected with the current experimental methodology. Circulating total, but not free, IGF-I was elevated at However, we cannot entirely dismiss the possibility that a rapid, small and transient ISF IGF-I change was missed due to microdialysis temporal resolution limitations. The immediate post and +3 h post and these results are consistent with some, but not all previous reports as the literature provides equivocal results for exercise and circulating IGF-I (refs). The lack of any measurable exercise-induced changes in IGF-I concentrations in muscle ISF among the current and other studies could suggest potentiated biological activity may reside in its partitioning among its family of binding proteins (7, 28, 35).

In summary, by measuring IGF-I and the IGFBP family in blood and muscle ISF via microdialysis after exercise, this study represents the most comprehensive characterization to date. Blood and ISF measures of the IGF-I system were fairly discordant implying that IGF-I measurement across biocompartments provides different information with regard to IGF-I action. By employing a unilateral stretch shortening cycle exercise, we were able to demonstrate differential and localized IGFBP responses in muscle ISF (i.e., increased IGFBP-3 and 4 accompanied by decreased IGFBP-5). We conclude that muscle contractions yield a local extracellular milieu whereby specific IGFBPs are altered. The physiological significance could be either through direct independent IGFBP actions or by influencing IGF-I bioactivity by sequestration or trafficking/delivery mechanisms. We also suggest that specific exercise-mediated IGF-I system influences might be better detected in ISF whereas blood measures may be more reflective of generalized whole body metabolic effects.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Central Finnish Hospital District, Jyvaskyla IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BN, JA, ML, KH, and HK contributed to the experimental design. BN, JA, SG, RT, and HK contributed to data collection. All authors contributed to data analysis, writing, and interpretation.

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Eating, Sleep, and Social Patterns as Independent Predictors of Clinical, Metabolic, and Biochemical Behaviors Among Elite Male Athletes: The EROS-PREDICTORS Study

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Objectives: Physiological hormonal adaptions in athletes and pathological changes that occur in overtraining syndrome among athletes are unclear. The Endocrine and Metabolic Responses on Overtraining Syndrome (EROS) study evaluated 117 markers and unveiled novel hormonal and metabolic beneficial adaptive processes in athletes. The objective of the present study was to uncover which modifiable factors predict the behaviors of clinical and biochemical parameters and to understand their mechanisms and outcomes using the parameters evaluated in the EROS study.

Methods: We used multivariate linear regression with 39 participants to analyze five independent variables—the modifiable parameters (caloric, carbohydrate, and protein intake, and sleep quality and duration of concurrent cognitive activity) on 37 dependent variables—that were elected among the parameters evaluated in the EROS study.

Results: Carbohydrate intake predicted quick hormonal responses to stress and improved explosive responses during exercise. Protein intake predicted improved body composition and metabolism and caloric intake, regardless of the proportion of macronutrients, predicted muscle recovery, and alertness in the morning. Sleep quality predicted improved mood and excessive concurrent cognitive effort in athletes under intense training predicted impaired metabolism and libido.

Conclusions: The results support the premise that eating, sleep, and social patterns modulate metabolic and hormonal function, clinical behaviors, and performance status of male athletes, and should be monitored continuously and actively to avoid dysfunctions.

Keywords: hormonal conditioning, endocrinology of physical activity, sports endocrinology, hormones and sports, Endocrine and Metabolic Responses on Overtraining Syndrome (EROS) study, overtraining syndrome

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INTRODUCTION

Physical activity has multiple benefits, including decreased risk for multiple diseases, increased life expectancy, and improved quality of life (1–3). To achieve these benefits, a balance among major lifestyle habits, including training, resting, and eating patterns, is critical. Classically, healthy habits include sufficient caloric, protein, and carbohydrate intake, adequate sleep quality and

duration, and avoidance of concurrent excessive psychological or cognitive stress, especially during moderate-to-intense training (4). However, our understanding of whether and how these habits may predict and modulate behaviors of hormonal, metabolic, clinical, and other biochemical parameters is poor. Conversely, it has been extensively reported that excessive training may disrupt physiological processes, induce multiple dysfunctions, and eventually lead to overtraining syndrome (OTS). It is also uncertain whether and how eating, social, and sleep patterns disrupt adaptive physiological changes in athletes, leading to the pathophysiology of OTS (4, 5).

Unlike the cardiovascular and musculoskeletal systems, extensively described in athletes, the peculiarities and not fully elucidated hormonal and metabolic adaptations to sports challenged the research on the endocrinology of physical activity and sport. The hormonal adaptations to physical activity were poorly understood, and consequently, research of biochemical markers on OTS has been compromised since levels expected for athletes were unknown.

Therefore, we conducted the Endocrine and Metabolic Responses to Overtraining Syndrome (EROS) studies (6–9), in which we evaluated 117 parameters, including exercise-independent hormonal responses to stimulation tests, basal hormones, muscular, immunologic, classic inflammatory, lipid, and hematologic parameters, body composition and metabolic rates, psychological, sleeping, and detailed eating patterns, in both athletes affected by OTS and healthy athletes, comparing to healthy athletes and healthy sedentary, respectively, in a three-arm study. The EROS study was designed to address some of the challenges and limitations of the assessment methods of the studies on athletes and unveil novel insights from overcoming the methodological limitations, including: (1) The employment of two control-groups, of healthy athletes and also of healthy sedentary, which allowed the analysis of the results from a more comprehensive perspective, since the simultaneous evaluation of the influence of the physical activity under healthy state and how this influence is altered under OTS is possible due to the concurrent comparisons with sedentary controls. In addition, findings on healthy athletes, when compared to non-active participants, were also relevant, particularly for the present study; (2) In the case of the OTS group, recruitment of athletes suspected of OTS in real life, aiming to evaluate actual and natural-occurring OTS, strictly diagnosed with diagnostic flowchart proposed by the latest guideline on OTS, including the exclusion of confounding diagnoses and the *sine-quo-non* presence of the key criteria of a minimum of 10% reduction in sports-specific performance; (3) Exclusive employment of extensively validated and standardized tests, and endorsed by specialized societies, in order to have reliable results and conclusions; (4) Performance of exercise-independent stimulation tests, aiming to avoid sub-optimized responses due to differences in performance, which also allow comparisons with non-physically active controls; (5) Concurrent evaluation of multiple and broad different aspects for the identification of which sorts of dysfunctions are present in OTS, in order to allow further analyses of how these dysfunctions correlate and interact in both development of OTS and in normal physiology, detection of independent triggers of OTS, and possible determinants of behaviors between the parameters evaluated in all athletes.

In the EROS study, we analyzed three groups: healthy athletes, OTS-affected athletes, and non-athletes. The main objective of this EROS study was to understand the behaviors associated with multiple parameters in male elite athletes, and how these parameters are modified by the presence of OTS by comparing the OTS-affected and healthy athletes with the sex-, age-, and body mass index (BMI)-matched non-athletes.

Each parameter was compared among the three groups, for which both overall and pairwise comparisons were conducted, aiming to understand the behavior of each evaluated marker in healthy athletes by comparing them with the non-athletes, and OTS athletes, thereby comparing affected with healthy athletes.

The changes in the methodology of the EROS study allowed the identification of novel findings and the clarification of previously inconsistent results. The most remarkable findings unveiled by the primary arms of the study, which included the EROS-HPA axis (6), the EROS-STRESS (7), the EROS-PROFILE (8), the EROS-BASAL (9), as well as the novel insights in OTS (10), the findings in high intensity functional training (EROS-HIFT) (11), and the demonstration of enhancement of hormonal responses to stimulations (12), include:

1. Through a 7-day thorough and precise diet record, athletes affected by OTS had a prior diet of ∼2 times less carbohydrates, two times less protein, and two times less overall caloric intake shown as g/kg/day, g/kg/day, and kcal/kg/day, respectively, when compared to healthy athletes, and three times less carbohydrate than sedentary controls;
2. Healthy athletes had better sleep quality (but not longer) and had shorter working or studying duration (h/day);
3. At an insulin tolerance test (ITT), performed to evaluate hormonal responses to a stressful stimulation (hypoglycemia), healthy athletes disclosed optimized and prolonged GH and cortisol responses compared to non-physically active controls, and was the only group to disclose a significant response of prolactin to stimulations, which was lost under OTS;
4. Direct stimulation of the adrenal glands using a synthetic ACTH did not yield any difference between healthy and affected athletes, and sedentary;
5. Testosterone levels were higher in healthy athletes than both sedentary and OTS-affected athletes;
6. The testosterone-to-estradiol ratio, an indirect marker of aromatase activity, was ∼2 times lower in OTS-athletes, compared to healthy athletes and to sedentary;
7. All other basal hormones were similar between groups;
8. Basal lactate levels were lower in healthy athletes than non-physically active participants, and also lower than levels in OTS-affected athletes;
9. Creatine kinase (CK) was exacerbated in affected athletes, compared to healthy ones, after similar period since last training with similar training patterns;
10. Neutrophils were higher in healthy athletes than OTS, while lymphocytes were lower compared to sedentary. The neutronphil-to-lymphocyte ratio, a proposed marker of

diseases prognosis, was increased in healthy, but not in affected athletes;
11. Catecholamines and the catecholamine-to-metanephrine ratio were exacerbated in OTS, compared to healthy athletes;
12. Healthy athletes had benefits from training in terms of vigor, fatigue, irritability, humor, tension, and lucidity moods, when compared to non-active participants, which were lost in OTS sedentary;
13. Healthy athletes had higher measured-to-expected basal metabolic rate (BMR) ratio and fat oxidation than sedentary and OTS;
14. Healthy athletes had lower body fat, higher muscle mass, and were better hydrated than OTS-affected athletes and sedentary.

These findings, including a total of 50 novel markers and processes identified in both healthy and OTS-affected athletes, supported the hypothesis of the existence of multiple adaptations of clinical, metabolic, biochemical, and body parameters that athletes, while the majority of the physiological adaptive changes are compromised in OTS, which may explain the hallmark of OTS, the loss of performance.

Associations, interactions, synergisms, stimulations, predictions, and inhibitions were further evaluated in joint *post-hoc* analyses of the primary findings of the EROS study, using different and more complex statistical analyses (e.g., multivariate linear regression, logistic regression, and linear correlation analyses).

In terms of biochemical parameters as correlated with other behaviors performed in the EROS-CORRELATIONS (13), further findings were identified:

1. Testosterone: estradiol T:E ratio predicted measured-to-predicted basal metabolic rate (BMR) ratio;
2. T:E ratio and total testosterone level were inversely predicted by fat mass;
3. Estradiol was not predicted by any clinical or biochemical parameter;
4. GH, cortisol, and prolactin responses to an ITSS were strongly correlated between them;
5. Hormonal responses to the ITT were positively correlated with fat oxidation, predicted-to-measured BMR ratio, muscle mass, and vigor, and inversely correlated with fat mass and fatigue;
6. Salivary cortisol 30 min after awakening and the T:E ratio were inversely correlated with fatigue;
7. Tension was inversely correlated with libido and directly correlated with body fat;
8. Predicted-to-measured BMR ratio was correlated with muscle mass and body water;
9. Fat oxidation was directly correlated with muscle mass and inversely correlated with fat mass;
10. Muscle mass was directly correlated with body water;
11. Extracellular water was directly correlated with body fat and inversely correlated with body water and muscle mass.

In summary, overall hypothalamic-pituitary responses to stimulation were diffuse and indistinguishable between the different axes, late hormonal responses, cortisol after awakening and T:E ratio were correlated with vigor and fatigue, T:E ratio was correlated with body metabolism and composition, testosterone was predicted by fat mass, and estradiol predicted anger. Hydration status was inversely correlated with edema, and inter-correlations were found among fat oxidation, hydration, and body fat.

In regards with the most important modifiable habits, also termed as "modifiable patterns," and which include eating, training, sleeping, professional, and social characteristics, the EROS-DISRUPTORS arm (14) demonstrated among OTS-affected athletes that three dietary patterns, including daily carbohydrate, daily protein, and daily overall calorie intake, were found to be, each one alone, independent triggers of OTS. Conversely sleeping, social, and training patterns depended on the combination with other factors to induce OTS. This arm also demonstrated that once triggered, OTS was inherently able to induce further reductions of cortisol, GH, and adrenocorticotropic hormone (ACTH) responses to stimulations, T:E ratio, neutrophils, neutrophil-to-lymphocyte ratio, vigor levels, hydration status, and muscle mass, while increase of tension levels and visceral fat, independently of other factors.

Despite the novel findings in the healthy and OTS-affected athletes and the learnings from the EROS-CORRELATIONS and EROS-DISRUPTORS arms, we were unable to identify how the modifiable habits can predict or modulate the behavior of basal and stimulated hormonal levels, biochemical, muscular, inflammatory, and immunologic levels, and psychological, and physical metabolism and composition parameters in athletes, when irrespective of OTS.

We hypothesized that a balance between training, resting, and nutrition is crucial for the occurrence of the multiple beneficial adaptations that have been detected in athletes. Hence, in the present study, named as EROS-PREDICTORS, we aimed to identify the influence of each habit patterns evaluated in the EROS study (eating, social, and sleep patterns) on the behaviors of the clinical, metabolic, and hormonal parameters, and when and how these patterns can dysfunctionally modify these behaviors, leading to OTS.

Remarkably, unlike EROS-DISRUPTORS, in which modifiable behaviors were evaluated as potential triggers for OTS, the present manuscript analyzes how modifiable habits shape the clinical and biochemical behaviors, irrespective of the presence of OTS. The sample analyzed in the EROS-DISRUPTORS were those affected by OTS vs. healthy athletes, whereas in this manuscript athletes were analyzed altogether, considering the fact that OTS is a result of a *continuum* process (4, 5) of the physiological adaptations in athletes.

MATERIALS AND METHODS

Subject' and Parameters' Selection

The full participant selection process and primary results of the EROS study were previously presented (6–9). The raw

data can be accessed at https://osf.io/bhpq9/. This study was approved by the ethical committee of the Federal University of São Paulo (approval number: 1093965). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Participants were recruited through sports coaches and social media. Age, sex, weight, and height, and intended to participate in (if suspected for Overtraining Syndrome: OTS; if healthy athlete: ATL; and if non-physically active: NPAC) were questioned prior to a first face-to-face interview.

Exclusion criteria included: extremes of age (<18 y/o and >50 y/o), undertrained athletes (training <300 min/week, <moderate-to-intense intensity, and <6 months consecutively), misdiagnosis of OTS (lack of unexplained decreased performance, presence of confounding dysfunctions that could be the cause of decreased performance), use of drugs or

TABLE 1 | Markers evaluated by the EROS study and included in the present analysis.

Study/Tests (76 parameters)	Markers
EROS-HPA axis—15 parameters	
Basal ACTH and cortisol and their response to an insulin tolerance test (ITT)	(1) Basal ACTH (pg/mL), and (2) cortisol (μg/dL) (3) ACTH, and (4) cortisol during hypoglycemia (5) ACTH, and (6) cortisol 30 min after hypoglycemia (7) ACTH, and (8) cortisol increase during ITT
Cortisol response to a *cosyntropin* stimulation test (CST)	(9) Cortisol at 30 min, and (10) at 60 min after injection
Salivary cortisol rhythm (SCR)	(11) Salivary cortisol (ng/dL) at awakening, and (12) 30 min after (13) at 4 p.m. and (14) at 11 p.m. (15) Cortisol awakening response (CAR)
EROS-STRESS—11 parameters	
GH and Prolactin response to an ITT	(1) Basal (GH) (μg/L), and (2) prolactin (ng/mL) (3) GH, and (4) prolactin during hypoglycemia (5) GH, and (6) prolactin 30 min after hypoglycemia (7) Prolactin increase during ITT
Glucose behavior during an ITT	(8) Basal serum glucose (mg/dL) (9) Serum glucose during hypoglycemia (mg/dL) (10) Adrenergic symptoms during hypoglycemia (0–10) (11) Neuroglycopenic symptoms during hypoglycemia (0–10)
EROS-BASAL—26 parameters	
Hormonal markers	(1) Total testosterone (ng/dL), and (2) Estradiol (pg/mL) (3) IGF-1 (pg/mL), (4) TSH (μUI/mL), and (5) Free T3 (pg/mL) (6) Total catecholamines, and (7) metanephrines (both μg/12 h) (8) Noradrenaline, (9) Epinephrine, and (10) Dopamine (all μg/12 h) (11) Metanephrines, and (12) Normetanephrines (both μg/12 h)
Biochemical markers	(13) Erythrocyte sedimentation rate (ESR, mm/h), and (14) Hematocrit (%) (15) C-reactive protein (CRP, mg/dL), and (16) Lactate (nMol/L) (17) Vitamin B12 (pg/mL), and (18) Ferritin (ng/mL) (19) Neutrophils, (20) Lymphocyte, and (21) Eosinophils (all /mm^3) (22) Creatine kinase (CK, U/L)
Ratios	(23) Testosterone-to-estradiol, and (24) Testosterone-to-cortisol ratios (25) Neutrophil-to-lymphocyte, and (26) Platelet-to-lymphocyte ratios
EROS-PROFILE—24 parameters	
General patterns	(1) Duration of night sleep (h), and (2) Self-reported sleep quality (0–10) (3) Self-reported libido (0–10) (4) Number of hours of activities besides professional training (h/day)
Eating patterns	(5) Calorie intake (kcal/kg/day) (6) Carbohydrate intake (g/kg/day) (7) Protein intake (g/kg/day) (8) Fat intake (g/kg/day)
Psychological patterns	(9) Profile of Mood State (POMS) questionnaire (total score: −32 to +120) (10) Anger (0–48), and (11) Confusion subscales (0–28) (12) Depression (0–60), and (13) Vigor subscales (0–32) (14) Fatigue (0–28), and (15) Tension subscales (0–36)
Body metabolism analysis	(16) Measured-to-predicted basal metabolic rate (BMR, %) (17) Percentage of fat burning compared to total BMR (%)
Body composition	(18) Body fat percentage (%), and (19) Muscle mass weight (kg) (20) Body water percentage (BW, %), and (21) Extracellular water compared to total BW (%) (22) Visceral fat (cm^2), (23) Waist circumference (cm), and (24) chest-to-waist circumference ratio

hormones, and altered biochemical or hormonal levels, that may also justify the reduced performance (6–12).

In the present study, from the 117 parameters evaluated by the EROS study (6–9), we elected those were not qualitative, intrinsically linked to other parameters, unvalidated, or missed in more than 5% of the participants, in a total of 76 parameters, from two groups of athletes (OTS-affected and healthy athletes; 39 participants) of the four arms of the EROS study (**Table 1**) (6–9). From the elected parameters, we excluded those that were not influenced by modifiable patterns, as they were unaltered between the groups of athletes, irrespective of the modifiable patterns.

For the present analysis, from a total of 51 selected participants divided into three groups (OTS = 14; ATL = 25; and NPAC = 12), the two groups of athletes (OTS and ATL groups) were included, in a total of 39 participants. Non-active participants were not included, as we aimed to be identify behavioral predictions in athletes, not sedentary.

For the evaluation of the modifiable habits, we performed a 7-day specific dietary record, which was followed regularly for at least 3 months. Sleeping duration and quality was self-reported, while specific questions regarding social, professional, and cognitive aspects were performed, as specified previously (6–12).

Statistical Analysis

For the five modifiable patterns (caloric-, carbohydrate-, and protein intake, sleep quality, and the duration of concurrent cognitive activity) and 37 parameters that yielded significant differences between healthy and OTS athletes (**Figure 1**), in a total of 42 variables, we used multivariate linear regression with the five modifiable patterns as the independent variables

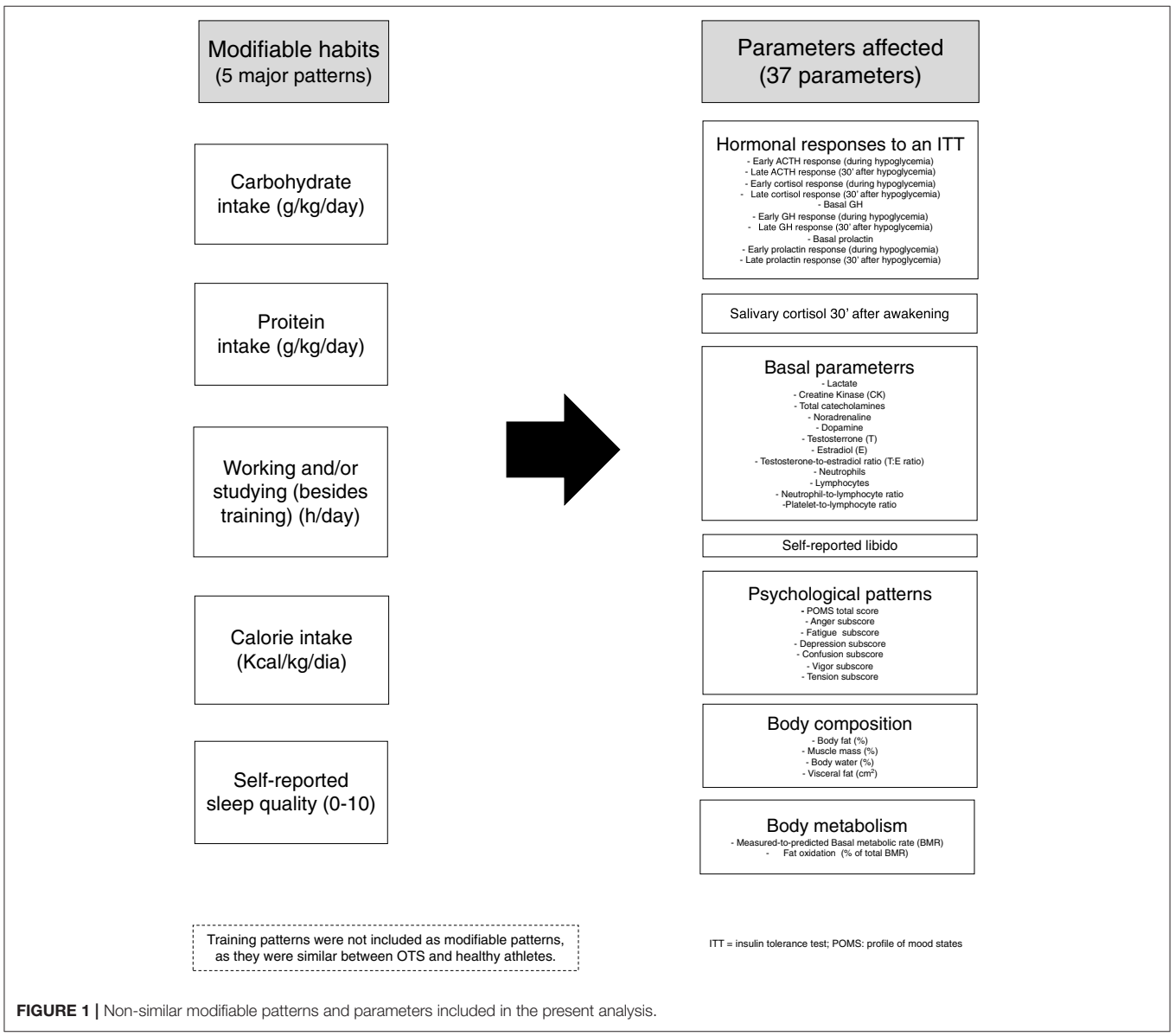

FIGURE 1 | Non-similar modifiable patterns and parameters included in the present analysis.

and the 37 non-similar clinical and biochemical markers as the dependent variables.

Multivariate linear regression analyzes were performed using the backward method of variable selection method (removal criterion $= p > 0.01$) to analyze the significance of the contributions of the 42 variables, including the five modifiable patterns and 37 non-similar parameters (**Figure 1**).

The significance of the contribution of the variable to the model was estimated and compared to the removal criteria ($p > 0.01$). When a potential predictor met the removal criteria, it was removed from the regression model. The model was then re-estimated for the remaining variables, and the process was repeatedly performed until none of the predictors achieved the removal criteria. The standardized residual variables of the last model analyzed were examined for normality and homoscedasticity criteria. The cutoff for the presence of multicollinearity was a tolerance index 0.40^3 for the variables in the last model. A $p < 0.05$ was considered statistically significant for the independent predictors. All statistical analyses were performed using SAS 9.4 (SAS Institute, Inc., Cary, NC).

Given the context of the present study and its main objective, the number of participants in the present study was found to be sufficient for the number of variables and outcomes for both multivariate logistic regression analyses. Compared to previous studies, we consider that we performed a broad and comprehensive analysis, encompassing multiple aspects.

Once this as a complex and multifactorial disorder, we considered that the lack of previous understanding of OTS may have been due to the lack of evaluation of multiple aspects. In addition, it is noteworthy that the level of statistical analysis employed in the present manuscript cannot be found previously in studies on endocrinology of physical activity and sport and on OTS.

In terms of correlations, although $r > 0.4$ ($p < 0.01$) is generally considered to be of moderate association, there is no rule or universally accepted sizes of correlation to be considered as weak, moderate, or strong. Since we studied entirely different biological aspects, and each of these aspects is extensively influenced by a large number of different predictors from different natures, it is unlikely to find a single linear correlation > 0.5 (> -0.5), since each parameter tends to be driven by multiple factors. Hence, in this particular case, according to the literature, a correlation > 0.4 is sufficient to be considered as a strong correlation, or at least moderate-to-strong. The *p*-value for the

TABLE 2 | Modifiable patterns as independent predictors of hormonal responses to stimulations (multivariate linear regression analysis).

Parameter	*p* of the influence of the modifiable variables	Level of influence by the modifiable variables (Adjusted *R*-Square)	Modifiable variables with significant correlations (and *p*-value)	Equation for the estimation of the parameter level in male athletes
Hormonal responses to stimulations				
Early cortisol response to an ITT (during hypoglycemia) (μg/dL)	0.029	23.8%	(1) CHO intake (direct) ($p = 0.025$)	Cortisol (μg/dL) = 8.33 + 0.5 × (CHO intake) + 1.36 × (protein intake)
Late cortisol response (30′ after hypoglycemia) (μg/dL)	0.0005	26.1%	(1) Presence of OTS (inverse) ($p = 0.0005$)	Cortisol (μg/dL) = 17.86 – 3.81 (if OTS)
Early ACTH response to an ITT (during hypoglycemia) (pg/mL)	0.012	17.5%	(1) Calorie intake (direct) ($p = 0.0035$)	ACTH = −67.74 + 2.83 × (calorie intake) + 0.92 × (Total POMS)
Late ACTH response to an ITT (30′ after hypoglycemia) (pg/mL)	0.007	19.9%	(1) Presence of OTS (inverse) ($p = 0.002$)	–
Cortisol response to an ITT (μg/dL)	0.004	22.0%	(1) Presence of OTS (inverse) ($p = 0.0017$)	–
Basal GH (μg/L)	0.033	9.3%	(1) Extra-activities (inverse) ($p = 0.033$)	GH (μg/L) = 0.97 – 0.08 × (extra activities)
Early GH response to an ITT (during hypoglycemia) (μg/L)	0.017	12.0%	(1) CHO intake (direct) ($p = 0.017$)	GH (μg/L) = −0.78 + 1.29 × (CHO intake)
Late GH response (30′ after hypoglycemia) (μg/L)	0.0012	23.0%	(1) Presence of OTS (inverse) ($p = 0.0012$)	–
Early prolactin response to an ITT (during hypoglycemia) (ng/mL)	0.009	15.0%	(1) CHO intake (direct) ($p = 0.009$)	Prolactin (ng/mL) = 8.36 + 2.43 × (CHO intake)
Late prolactin response (30′ after hypoglycemia) (ng/mL)	0.0002	37.8%	(1) Protein intake (direct) ($p = 0.0004$) (2) CHO intake (direct) ($p = 0.038$)	Prolactin (ng/mL) = −28.49 + 1.60 × (CHO intake) + 10.64 × (protein intake) + 2.46 × (extra activities)
Prolactin response to an ITT (ng/mL)	0.0133	17.0%	(1) Protein intake (direct) ($p = 0.0036$)	Prolactin (ng/mL) = −356.25 + 108.6 × (protein intake) + 30.57 × (extra activities)

CHO, Carbohydrate; ITT, Insulin tolerant test; POMS, Profile of mood states; BMR, Basal metabolic rate; T/E, Testosterone-to-estradiol; OTS, Overtraining syndrome; Calorie intake, kcal/kg/day; CHO intake, g(CHO)/kg/day; protein intake, g(protein)/kg/day; extra activities, working and/or studying hours besides training; sleep quality, self-reported sleep quality (0–10).

linear correlations was lower and partial correlations were not considered to avoid incidental misinterpretative correlations.

Parameters that were independently influenced by the presence of OTS were adjusted according to the level of its influence, aiming to homogenize the groups of athletes. These results were published in the EROS-DISRUPTORS arm (14), and included: (1) cortisol 30 min after hypoglycemia, in response to an ITT (26.1% of influence by OTS); (2) cortisol increase during ITT (22.0%); (3) GH 30 min after hypoglycemia, in response to an ITT (23.0%); (4) testosterone-to-estradiol (T:E) ratio (30.7%); (5)

TABLE 3 | Modifiable patterns as independent predictors of basal hormones and biochemical parameters (multivariate linear regression analysis).

Parameter	*p* of the influence of the modifiable variables	Level of influence by the modifiable variables (Adjusted *R*-Square)	Modifiable variables with significant correlations (and *p*-value)	Equation for the estimation of the parameter level in male athletes
Basal hormones				
Estradiol (pg/mL)	0.008	20.3%	(1) Calorie intake (inverse) ($p = 0.002$) (2) CHO intake (direct) ($p = 0.013$)	Estradiol (pg/mL) = 50.28 – 0.68 × (calorie intake) + 2.32 × (CHO intake)
Testosterone-to-oestadiol ratio (T/E)	0.0007	30.7%	(1) Presence of OTS (inverse) ($p = 0.0002$)	T/E = 14.1 – 0.86 × (CHO intake) + 12.9 (in case of OTS)
Total nocturnal urinary catecholamines (mg/12 h)	0.0187	11.7%	(1) Extra activities (direct) ($p = 0.0187$)	Total NUC = 49.5 + 20.6 × (extra activities)
Dopamine (mg/12 h)	0.0136	13.1%	(1) Extra activities (direct) ($p = 0.0136$)	Dopamine = 25.7 + 20.1 × (extra activities)
Basal biochemistry				
Creatine kinase (CK)	0.02	11.3%	(1) Calorie intake (inverse) ($p = 0.02$)	CK = 1488 – 20.5 × (calorie intake)
Lactate	0.0035	22.9%	(1) Calorie intake (inverse) ($p = 0.001$)	Lactate = 1.62 – 0.02 × (calorie intake)
Neutrophils (/mm^3)	0.045	13.8%	(1) Calorie intake (inverse) ($p = 0.044$) (2) Presence of OTS (inverse) ($p = 0.015$)	Neutrophils = 4210 – 60.7 × (calorie intake) + 154.4 × (CHO intake) – 1,724 (if OTS)
Lymphocytes (/mm^3)	0.025	10.8%	(1) Protein intake (inverse) ($p = 0.025$)	Lymphocytes = 2767 – 207 × (protein intake)

CHO, Carbohydrate; T/E, Testosterone-to-estradiol; OTS, Overtraining syndrome; Calorie intake, kcal/kg/day; CHO intake, g(CHO)/kg/day; protein intake, g(protein)/kg/day; extra activities, working and/or studying hours besides training; sleep quality, self-reported sleep quality (0–10).

TABLE 4 | Modifiable patterns as independent predictors of moods and feelings (multivariate linear regression analysis).

Parameter	*p* of the influence of the modifiable variables	Level of influence by the modifiable variables (Adjusted *R*-Square)	Modifiable variables with significant correlations (and *p*-value)	Equation for the estimation of the parameter level in male athletes
Psychology				
POMS confusion subscale	0.0002	33.7%	(1) Sleep quality (inverse) ($p = 0.002$) (2) Calorie intake (inverse) ($p = 0.019$)	POMS confusion subscale = 15.25 – 0.92 × (sleep quality) – 0.1 × (calorie intake)
POMS depression subscale	0.0001	30.8%	(1) Sleep quality (inverse) ($p = 0.0001$)	POMS depression subscale = 17.22 – 1.66 × (sleep quality)
POMS vigor subscale	<0.0001	83.6%	(1) Sleep quality (direct) ($p = 0.0002$) (2) Presence of OTS (inverse) ($p < 0.0001$)	POMS vigor subscale = 3.7 + 1.15 × (sleep quality) – 11.96 (if OTS)
POMS fatigue subscale	<0.0001	85.7%	(1) Sleep quality (direct) ($p = 0.0059$) (2) Presence of OTS (direct) ($p < 0.0001$)	POMS fatigue subscale = 24.5 – 0.9 × (sleep quality) + 15.3 (if OTS)
POMS tension subscale	<0.0001	42.8%	(1) Presence of OTS (direct) ($p < 0.0001$)	–
Adrenergic symptoms (0–10)	0.003	23.7%	(1) Calorie intake (direct) ($p = 0.0008$) (2) CHO intake (inverse) ($p = 0.023$)	Symptoms = −0.09 + 0.16 × (calorie intake) – 0.45 × (CHO intake)
Libido (0–10)	0.018	11.9%	(1) Extra-activities (inverse) ($p = 0.018$)	Libido = 10.3 – 0.4 × (extra activities)

CHO, Carbohydrate; POMS, Profile of mood states; OTS, Overtraining syndrome; Calorie intake, kcal/kg/day; CHO intake, g(CHO)/kg/day; protein intake, g(protein)/kg/day; extra activities, working and/or studying hours besides training; sleep quality, self-reported sleep quality (0–10).

neutrophils (13.8%); (6) neutrophil-to-lymphocyte ratio (13.6%) (7) Profile of Mood States (POMS) vigor subscale (83.6%); (8) POMS fatigue subscale (85.7%); (9) POMS tension subscale (42.8%); (10) muscle mass (33.7%); (11) body water (50.5%), and (12) visceral fat (38.2%). Parameters that were not modified by the presence of OTS did not require adjustments according to the population (if OTS-affected or if healthy athletes), since these markers behaved independently from OTS. In addition orrelations that were unlikely to have any biological plausibility were excluded.

Compared to the EROS-DISRUPTORS arm, since this arm had a larger number of variables (total of 44) and demonstrated sufficient statistical power for the present analysis, in the EROS-PREDICTORS, in which we employed a lower number of variables (42 parameters), statistical power was sufficient (6–14). Indeed, largely consistent differences between athletes, strict linear correlations, and small number of outsiders were aspects that strengthen the statistical power of the present study. The raw statistical analysis is also available at the depository (https://osf.io/bhpq9/).

It is important to highlight that the findings in the present are should be considered as suggestive, instead of conclusive, regardless.

RESULTS

The results of the multivariate linear regression analyses, including *p*-values, level of association of the independent predictors, and the proposed equations for the estimation of each marker for modifiable factors are detailed in **Tables 2**–**5**. A summary of expected (according to biological plausibility for causal relationships and previous scientific data) and actual predictions are shown in **Figure 2**.

The most significant findings among male athletes regarding eating, sleep, and social patterns as independent predictions are as follows. Carbohydrate intake predicted 12–24% of all early hormonal responses to an ITT, and 37.8% of late prolactin responses when analyzed together with protein intake. Sleep quality and caloric intake inversely predicted 33.7% of the confusion subscale of the POMS questionnaire, and sleep quality predicted vigor and fatigue levels. Protein intake, together with total caloric intake, predicted more than half of the body's water content (within the normal range). Protein intake inversely predicted 31% of the body's fat content; conversely, it independently and positively predicted muscle mass and body water. Caloric intake, but not each macronutrient separately, negatively predicted 10% of creatine kinase (CK) levels, promoting muscle recovery after training sessions, after the training patterns were similar among the athletes. Finally, the amount of working and studying predicted more than 10% of the nocturnal catecholamines, and reduced libido by more than 10%. A summary of the predictions of each modifiable pattern on the behaviors of clinical and biochemical markers, and their consequences, are presented in **Figure 3**.

DISCUSSION

The EROS study unveiled adaptations and dysfunctions in acute and chronic hormonal responses to stimulations, other hormones, immunologic, inflammatory, and muscular parameters, and body composition and metabolism in healthy athletes and OTS, respectively, and the correlations between

TABLE 5 | Modifiable patterns as independent predictors of body metabolism and composition (multivariate linear regression analysis).

Parameter	*p* of the influence of the modifiable variables	Level of influence by the modifiable variables (Adjusted *R*-Square)	Modifiable variables with significant correlations (and *p*-value)	Equation for the estimation of the parameter level in male athletes
Body metabolism and composition				
Fat oxidation (% of total BMR)	<0.0001 (together with body water and T/E ratio)	58.8%	(1) Extra activities (inverse) ($p = 0.0001$)	Fat oxidation = −66.96 + 2.30 × (body water) + 0.51 × (T/E ratio) – 4.99 × (extra activities)
Fat mass (%)	0.0001	31.0%	(1) Protein intake (inverse) ($p = 0.0001$)	Fat mass = 20.35 – 3.1 × (protein intake)
Muscle mass (%)	0.0006	33.7%	(1) Protein intake (direct) ($p = 0.0135$) (2) Presence of OTS (inverse) ($p = 0.0282$)	Muscle mass = 47.84 + 1.42 × (protein intake) – 3.47 (if OTS)
Body water (%)	<0.0001	50.5%	(1) Protein intake (direct) ($p = 0.0061$) (2) Calorie intake (inverse) ($p = 0.021$) (3) Presence of OTS (inverse) ($p = 0.001$)	Body water = 60.75 + 1.69 × (protein intake) – 0.12 × (calorie intake) – 5.77 (if OTS)
Visceral fat (cm^2)	0.0002	38.2%	(1) Calorie intake (direct) ($p = 0.0076$) (2) Protein intake (inverse) ($p = 0.023$) (3) Presence of OTS (direct) ($p = 0.0026$)	Visceral fat = 47.4 – 11.9 × (protein intake) + 1.3 × (calorie intake) + 45.1 (if OTS)

CHO, Carbohydrate; BMR, Basal metabolic rate; OTS, Overtraining syndrome; Calorie intake, kcal/kg/day; CHO intake, g(CHO)/kg/day; protein intake, g(protein)/kg/day; extra activities, working and/or studying hours besides training; sleep quality, self-reported sleep quality (0–10).

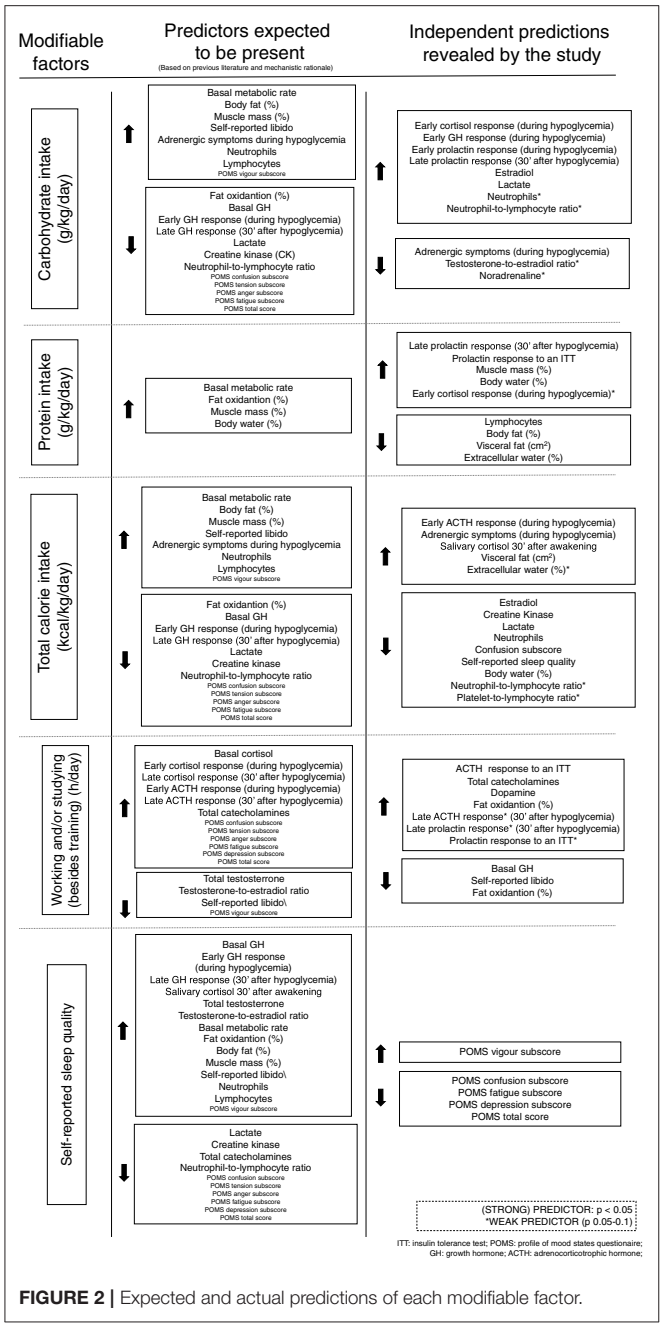

FIGURE 2 | Expected and actual predictions of each modifiable factor.

these parameters and eating, social, and sleep patterns. All these findings were conflicting or unclear prior to the present study (15–19).

In the present arm of the EROS study, our objective was to explore and unravel which modifiable factors modulate the clinical, metabolic, and biochemical markers assessed in the EROS study and their mechanisms of action, by employing innovative design and evaluated parameters, *post-hoc* joint analyses were conducted using more complex statistical tools, unlike the techniques used in previous studies with healthy athletes. This helped identify potential independent predictors (independent variables) of evaluated parameters (dependent variables), particularly, when the biological plausibility of the criteria for causality in the relationships were met. The main findings of the EROS study in male athletes are shown in **Table 6**.

From the identification of eating, social, and sleep patterns as independent predictors of beneficial or harmful outcomes, we aimed to recommend more precise approaches for the continuous improvement of athletes, by the optimization of eating, social, and sleep habits to improve the performance and the overall well-being of athletes.

Other modifiable factors, such as the use of drugs, hormones, smoking, drinking alcohol, and other social behaviors were exclusion criteria, and therefore, were not analyzed. We intuitively assumed that athletes were fully aware of the need to avoid drugs, anabolic steroids (unless clinically needed), smoking, alcohol intake (except during special social events), and sleep deprivation due to excessive hedonic living.

Carbohydrate Intake

Carbohydrate intake had multiple effects on the behavior of hormones and other biochemical parameters. It was an independent predictor of the overall early hormone responses to an ITT, accounting for up to 24% of responses (early hormonal responses to an ITT can predict sports performance that demands sudden and explosive reactions). Our hypothesis is that improved responses require a greater availability of energy, and carbohydrates are notorious prompters and an easy source of energy; therefore, which may justify why carbohydrate intake and its consequent prompt availability has been demonstrated to be an independent predictor of early hormone responses to stimulations. Accordingly, we hypothesized that carbohydrate deprivation may have led to decreased and delayed hormonal responses, which would indirectly impair athletes performance, as identified in the primary findings of the EROS study (6, 7) (**Figure 4**).

In contrast to the suppressive effect of acute carbohydrate intake on GH release (20), chronic carbohydrate intake had a stimulating effect on the GH response, showing a dual effect of carbohydrate intake on GH-release patterns.

Similarly to the dual effects on GH release, carbohydrate intake has also demonstrated an apparent dual effect on aromatase activity (i.e., conversion from testosterone to estradiol) was found. While a very low carbohydrate intake may be related to a pathological increase in aromatase activity (9), which corroborates previous similar findings (21, 22). Notwithstanding, excessive carbohydrate intake may also increase aromatase activity, causing increased estradiol and a decreased testosterone-to-estradiol (T/E) ratio, as observed in our previous findings (9, 15, 16, 18, 19). This finding may justify the not fully elucidated finding of higher estradiol levels in obese males, since higher estradiol levels in these males cannot be not fully explained by the hypertrophy of adipocytes (9). Despite the protective role of overall caloric intake among elite athletes, excessive carbohydrate intake may have a pro-inflammatory role (9, 23), as typical markers of unspecific subclinical metabolic inflammatory states have been correlated with excessive carbohydrate intake, including increased aromatase activity, increased lactate levels

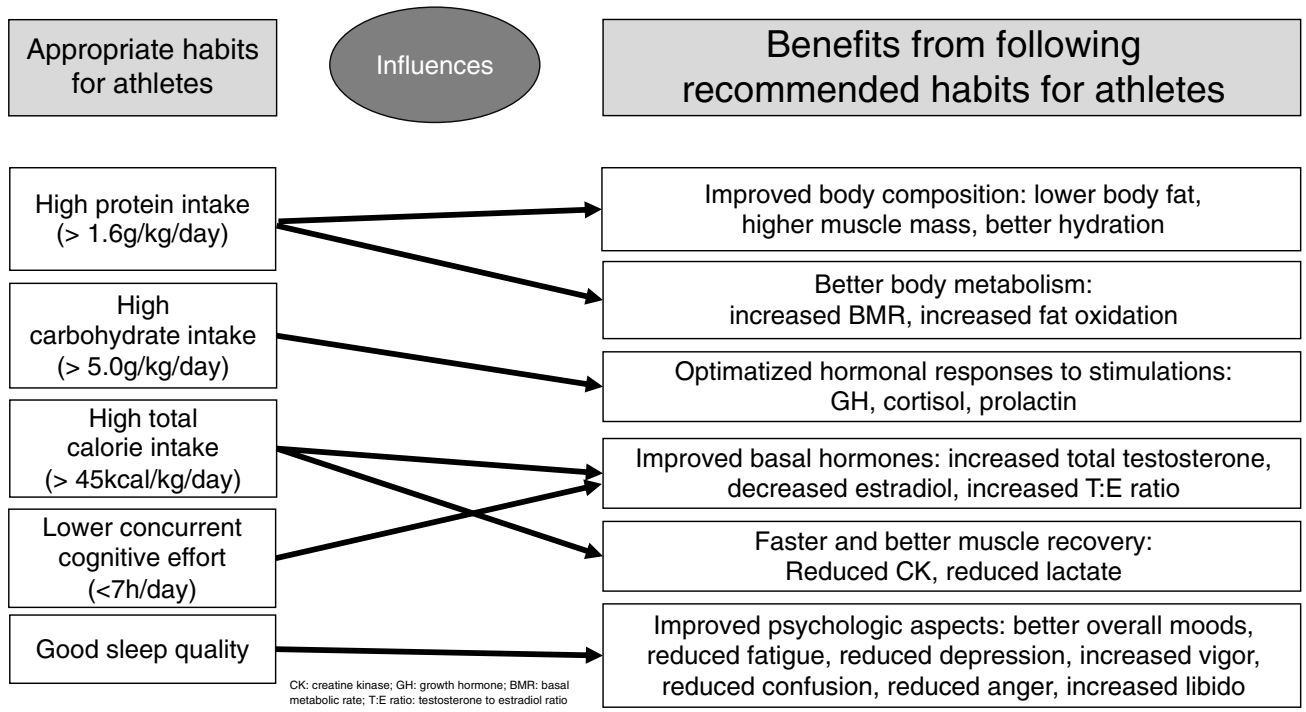

FIGURE 3 | Summary of the influences of modifiable patterns on clinical and biochemical behaviors.

without concurrent increase in CK levels (unrelated to muscle stimulation) (9), and slight non-significant increased neutrophils. Neutrophils are independently associated with inflammatory status, and cardiovascular and neoplastic diseases, in the absence of clinical infections or the use of glucocorticoids (24, 25).

Despite claims that lower carbohydrate intake does not impair performance, even for elite athletes (26), higher carbohydrate intake was shown to have positive effects on hormonal profile. Nonetheless, excessive intake has the potential to induce a pathological increase in aromatase activity. In addition, the EROS studies showed carbohydrate intake below 5.0 g/kg/day predicted harmful effects on hormonal responses and performance (6–8, 21–23).

Protein Intake

Protein intake was found to predict the most important parameters of body metabolism and composition positively, in an independent manner, including increased BMR, fat oxidation, muscle mass, and hydration, while protecting against body and visceral fat, accounting for 30–50% of the variation in body fat. Protein intake significantly and inversely predicted ($p = 0.029$) extracellular water, i.e., it protected against the loss of water from the "third space," thereby preventing edema. All these findings point to the conclusion that protein is a major determinant of body characteristics (6, 15, 16, 18, 19).

The daily whey protein intake by 88% of the athletes may have contributed to the independent benefits found in the present study, since whey consumption has been independently associated with decreased body fat (27), reduced inflammatory parameters (28), and the prevention of fat weight gain (29).

Overall, higher protein intake for athletes had beneficial effects on metabolism and body composition. Previous caution about protein intake related to concerns about kidney and liver safety has been unsubstantiated (27–29), and the present study reinforces that additional protein intake has several benefits without risks of kidney or liver dysfunctions. The EROS study showed that protein intake should be at least 1.6 g/kg/day (7, 15, 16), which is consistent with the latest sports nutrition guideline for athletes (30) and previous researches (27–29) although there is no evidence for a maximum intake limit.

Indeed, we hypothesized that a higher ("unlimited") protein intake among male athletes would have a protective role in the body metabolism and composition, without a plateau or inverse effect at any point, at least up to 4.5 g/kg/day.

Overall Caloric Intake

Overall caloric intake, independent of the macronutrient content, had four major influences: positively predicted salivary cortisol 30 min after awakening, enhanced the speed and quality of muscle recovery, prevented aberrant exacerbations of aromatase activity, and prevented a pathological increase in neutrophils without the presence of an apparent infection.

Higher caloric intake, regardless of its content, may increase elite male athletes alertness in the morning, assumed from the increased salivary cortisol 30 min after awakening, and possibly helps increase the speed of the clearance of markers of muscle recovery (CK and lactate). These findings suggest that unlike

TABLE 6 | Most remarkable findings of the EROS study in healthy athletes.

Study/Tests	Remarkable findings in healthy athletes
	EROS-HPA axis
Basal ACTH and cortisol and their response to an insulin tolerance test (ITT)	(1) Prompter cortisol response (compared to non-athletes and OTS-affected athletes) (2) Optimized cortisol response (compared to non-athletes and OTS-affected athletes)
Salivary cortisol rhythm (SCR)	(3) Higher salivary cortisol 30 min after awakening (compared to non-athletes and OTS-affected athletes)
	EROS-STRESS
GH response to an ITT	(4) Higher basal GH (compared to non-athletes and OTS-affected athletes) (5) Prompter GH response (compared to non-athletes and OTS-affected athletes) (6) Optimized GH response (compared to non-athletes and OTS-affected athletes)
Prolactin response to an ITT	(7) Prompter prolactin response (compared to non-athletes and OTS-affected athletes) (8) Optimized prolactin response (compared to non-athletes and OTS-affected athletes)
	EROS-BASAL
Hormonal markers	(9) Higher total testosterone (ng/dL) (compared to non-athletes and OTS-affected athletes) (10) Higher total catecholamines and noradrenaline (compared to non-athletes)
Biochemical markers	(11) Lower lactate (compared to non-athletes and OTS-affected athletes) (12) Lower neutrophils (compared to non-athletes and OTS-affected athletes) (13) Higher lymphocytes (compared to non-athletes and OTS-affected athletes)
Ratios	(13) Lower neutrophil-to-lymphocyte (compared to non-athletes and OTS-affected athletes)
	EROS-PROFILE
General patterns	(14) Better self-reported sleep quality (compared to non-athletes and OTS-affected athletes)
Psychological patterns	(15) Better overall moods, and anger, confusion, vigor, depression, tension, and fatigue subscales (compared to non-athletes and OTS-affected athletes)
Body metabolism analysis	(16) Higher measured-to-predicted basal metabolic rate (BMR) (compared to non-athletes and OTS-affected athletes) (17) Higher percentage of fat burning compared to total BMR (compared to non-athletes and OTS-affected athletes)
Body composition	(18) Lower body fat percentage (compared to non-athletes and OTS-affected athletes) (19) Higher muscle mass weight (compared to non-athletes and OTS-affected athletes) (20) Higher body water percentage (compared to non-athletes and OTS-affected athletes) (21) Extracellular water compared to total BW (compared to non-athletes) (22) Lower visceral fat (compared to non-athletes and OTS-affected athletes)

the predictions for other outcomes, for muscle recovery higher caloric intake seems to be more important than the amount of each macronutrient.

Despite the positive findings associated with overall caloric intake, this was detected as an independent and direct predictor of visceral but (although not for total fat), and it also was a predictor of lower muscle mass when not accompanied by increase of protein intake. Indeed, carbohydrate abuse is frequently associated with low and insufficient protein intake, leading to sarcopenic obesity (31). Thus, for some aspects of body composition, the source of calories is at least as important as the total caloric intake, once the effect of higher caloric intake when from protein may have opposite effects compared to non-protein higher overall caloric intake.

In conclusion, increase of caloric intake in elite athletes improved the quality of muscle recovery, hormonal environment, and sports performance. The total amount of needed calories was more important than their source. The EROS study found athletes should consume a minimum of 35 kcal/kg/day (6, 9) to achieve this caloric intake. Any macronutrient (i.e., protein, carbohydrate, or fat) can be added to the diet, even if the amount exceeds the athletes daily caloric needs.

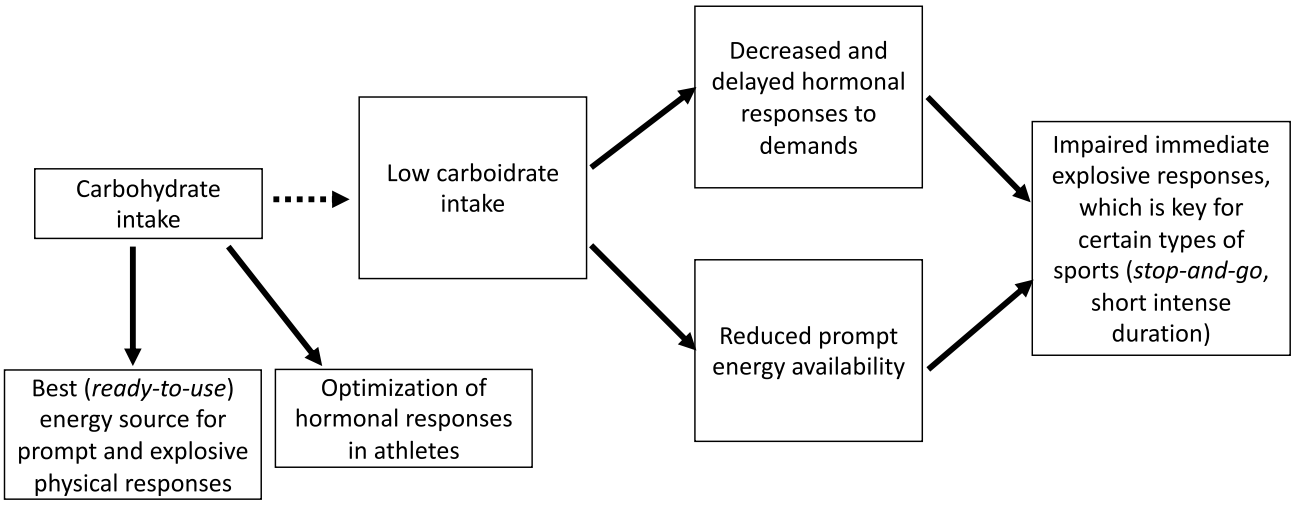

FIGURE 4 | Proposed mechanisms for the impaired performance observed in prolonged low carbohydrate intake.

Other Activities

For all elite athletes, excessive concomitant physical and cognitive efforts may lead to harmful effects, although different from those related to insufficient caloric, protein, and carbohydrate intake. The number of hours of studying and/or working was an independent enhancer of ACTH response to stimulation. However, this did not translate into enhanced cortisol release, as would be expected in response to ACTH. The lack of cortisol response to enhanced ACTH release can be hypothesized to be a sort of hypo-responsiveness of the adrenals to ACTH stimulation. Conversely, direct adrenal stimulation in the same participants did not disclose differences in cortisol responsiveness, irrespective of cognitive demands, and was not predicted by any other factor or marker, which weakens this hypothesis.

Basal GH levels were inversely predicted by excessive mental activity, indicating that more studying or working led to lower GH levels when not in pack, although this was not reflected in the GH response to stimulations.

The duration of working and/or studying among elite athletes directly predicted urinary catecholamines. Since catecholamines have acute positive effects on fat oxidation and metabolic rate, a paradoxical reduction in fat oxidation and BMR were detected with increased cognitive activity.

Although catecholamines acutely increase fat oxidation, chronic exposure may have the opposite effect, in a similar manner that happens in hypercortisolism states. Indeed, a chronic *fight-or-flight* readiness effect, typically observed in chronic psychological stress, can lead to fat weight gain and decreased BMR (32), despite the expectedly observed increase of cortisol and catecholamines. Possibly, a decreased sensitivity of the fat tissue to catecholamines is in accordance with a lack of fat loss to be expected under excessive catecholamines (32, 33). The unexpected lack of fat loss has is observed in patients with pheochromocytoma (catecholamine-producing tumors), who are chronically exposed to higher catecholamine levels, or under chronic stress (33).

Considering the present findings and the results from the previous arms of the EROS study, we speculate that when both physical and cognitive demands are concurrently present fat oxidation and BMR get impaired, which is resulted from an environment exposure to chronic stress (6–14, 32). This is correlated with impaired metabolism associated with insufficient resting and recovery, as cognitive stress precludes appropriate physical recovery. Athletes should avoid excessive cognitive activities during periods when volume and intensity of training increase, for example, during seasons. Contrariwise, periods that demand high cognitive effort should not be accompanied by intensification of training load.

Sleeping

While duration of sleep did not predict any marker or outcome, sleep quality was the most important predictor of psychological outcomes, and the only modifiable factor that modulated overall mood states.

Also, sleep quality was an independent and inverse predictor of total caloric intake; i.e., better sleep quality could be able to reduce overall caloric intake, irrespective of other factors, such as training characteristics. However, greater sleep quality did not lead to additional reduction and consequent insufficient caloric intake.

Deprivations and Overtraining Syndrome

Collectively, the subjective analysis of the findings of the present study shows that concurrent strict lifestyle in the long run may bring more harms than previously thought. Despite the benefits of adequate caloric and carbohydrate intake, food deprivation, and carbohydrate phobia ("carbphobia") are present in some athletes, especially those in sports in which categories are based on body weight and body shape is culturally acclaimed, such as high-intensive functional training (HIFT), e.g., CrossFit®,

which attempts to simultaneously lower body fat and improve performance (15, 16). These behaviors can lead to fatigue and temporary underperformance, consistent with our finding that lower caloric intake reduces alertness in the morning and impairs muscle recovery, while lower carbohydrate intake may lead to a paradoxical decrease in pace and strength; together these findings are termed overreaching (5). If overreaching is not addressed by an increase in caloric and carbohydrate intake and compensatory rest, athletes can progress to a state of prolonged and hard to recover from decrease in performance, chronic fatigue, and mood disturbances, which characterize classic OTS. In one of the EROS studies, a relatively low caloric (not hypocaloric) and low carbohydrate intake were the two major OTS triggers (6).

Excessive work or studying might lead to multiple harmful effects in athletes, including worsening of hormonal levels, libido, sleep quality, and performance (6, 15, 16, 18, 19). Sleep quality impairs performance, libido, and all psychological functions. We recommend, therefore, against concurrent intense levels of physical and cognitive activity during championships, or intensified training. Athletes should decrease the intensity and duration of studying and/or working, and when more intense studying or working is needed, the volume of training should be decreased. During intensification of training, a maximum work or study duration of 7 h is recommended, following the findings of the EROS study (6).

Multiple modifiable patterns were found to modulate clinical and biochemical behaviors, and we learned answers are unlikely to be found if studies evaluate each aspect separately. The level of importance of each modifiable factor varies by the type of sport. For instance, carbohydrate intake plays an important role in explosive, stop-and-go, and short and intense sports, in which prompter and enhanced hormonal responses and prompter energy availability are the two major factors influencing performance. An overall balance between training, eating, and resting is the most important factor for endurance sports, when prolonged optimization of hormonal responses are desired for a longer time-to-fatigue and a maximum maintenance of pace throughout the training session.

Limitations

The findings of the EROS study are only applicable for male athletes that practice both endurance and strength exercises, either together (as in high-intensive functional training or CrossFit) or separately (e.g., when athletes practice both weight lifting and middle distance running), as basal and stimulated hormonal and metabolic levels are highly sex-specific and possibly sport-specific. Whether the findings are applicable to exclusive endurance, strength, or explosive sports, is unknown. However, the clinical applications of the present findings can be extrapolated in the absence of more specific data, for practice purposes, as many of the adaptive changes and behaviors found in this study should occur in other populations of athletes. Hence, further studies with larger samples of athletes are crucial to confirm whether our data are reproducible; longitudinal studies are needed because the present study's design precludes drawing conclusions from the sequence of events in response to interventions in modifiable patterns, including training, eating, and social aspects. Additionally, due to unexpected findings regarding changes in hormones and other biochemical markers, for further researches we suggest additional parameters for further studies, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), sex hormone-binding globulin (SHBG), IGF binding globulin-3 (IGFBP-3), tumor necrosis factor-alpha (TNF-alpha), interleukin-1 beta (IL-1beta), CD3, CD4, CD8, CD8/CD4 ratio, lactate dehydrogenase (LDH), free thyroxin (fT4), intra-tissue cortisone:cortisol ratio, and cortisol binding globulin (CBG). Comparisons between exercise-dependent and -independent stimulations should also be performed. Compared to liquid chromatography mass spectrometry/tandem mass (LC/MS-MS/MS), electrochemiluminescence (CLIA) has sufficient relative precision for in-between (pairwise) group comparisons (34–39).

Final Discussion

The EROS-PREDICTORS arm of the EROS study showed that: (1) carbohydrate intake predicts quick hormonal responses to stress and improves explosive responses during exercise; (2) protein intake improves body composition and metabolism; (3) caloric intake, independent of the its source, predicts muscle recovery; (4) sleep quality improves mood; and (5) excessive concurrent cognitive effort in athletes participating in intense training impairs metabolism and libido. These results support the premise that eating, sleep, and social patterns affect metabolic, hormonal, and clinical behaviors in athletes, and should be addressed to prevent dysfunctions.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author and in a depository (https://osf.io/bhpq9/).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of the Federal University of São Paulo (approval number: 1093965). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FC and CK developed the central idea of the present manuscript. FC performed the tests of the EROS study, compiled the data, analyzed the results, and participated in the discussions. CK supervised and reviewed the results and actively participated in the discussion. All authors have read and approved the manuscript.

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Growth Hormone and Insulin-like Growth Factor-I Molecular Weight Isoform Responses to Resistance Exercise Are Sex-Dependent

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Purpose: To determine if acute resistance exercise-induced increases in growth hormone (GH) and insulin-like growth factor-I (IGF-I) were differentially responsive for one or more molecular weight (MW) isoforms and if these responses were sex-dependent.

Methods: College-aged men (n = 10) and women (n = 10) performed an acute resistance exercise test (ARET; 6 sets, 10 repetition maximum (10-RM) squat, 2-min inter-set rest). Serum aliquots from blood drawn Pre-, Mid-, and Post-ARET (0, +15, and +30-min post) were processed using High Performance Liquid Chromatography (HPLC) fractionation and pooled into 3 MW fractions (Fr.A: >60; Fr.B: 30–60; Fr.C: <30 kDa).

Results: We observed a hierarchy of serum protein collected among GH fractions across all time points independent of sex (Fr.C > Fr.A > Fr.B, $p \leq 0.03$). Sex × time interactions indicated that women experienced earlier and augmented increases in all serum GH MW isoform fraction pools ($p < 0.05$); however, men demonstrated delayed and sustained GH elevations ($p < 0.01$) in all fractions through +30-min of recovery. Similarly, we observed a sex-independent hierarchy among IGF-I MW fraction pools (Fr.A > Fr.B > Fr.C, $p \leq 0.01$). Furthermore, we observed increases in IGF-I Fr. A (ternary complexes) in men only ($p \leq 0.05$), and increases in Fr.C (free/unbound IGF-I) in women only ($p \leq 0.05$) vs. baseline, respectively.

Conclusions: These data indicate that the processing of GH and IGF-I isoforms from the somatotrophs and hepatocytes are differential in their response to strenuous resistance exercise and reflect both temporal and sex-related differences.

Keywords: exercise endocrinology, sex differences, HPLC, fractionation, molecular weight variants

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INTRODUCTION

Resistance exercise is a potent stimulus, perturbing homeostasis and promoting both favorable physiological and metabolic adaptations (1). The associated responses to resistance exercise involve numerous signaling pathways and are speculated to be partially mediated by the release of growth hormone (GH) and insulin-like growth factor-I (IGF-I) (2). GH is a polypeptide hormone

secreted by the anterior pituitary gland, which contributes to multiple biological processes, such as anabolism, protein synthesis, and substrate mobilization (3, 4). An explanation for such pleiotropic nature is that GH exists as a family of more than 100 isoforms (5) differing in biological and immunological activity (6). The metabolic and anabolic responses associated with GH are mediated via the interaction of GH with the GH-receptor both directly by tyrosine kinase activation and indirectly by induction of insulin-like growth factor 1 (IGF-1) (7). Thus, the true effects of the multitudes of GH isoforms released in the different molecular weight fractions remain unclear or at least represent a composite effect based on fractional sizing analysis. IGF-I is a ubiquitous growth factor, also existing as several forms including free and binary/ternary complexes (8) and residing among several biocompartments (9), again with major roles in numerous metabolic and physiological functions (10). The systemic (e.g., hepatic) or local (e.g., skeletal muscle) release of IGF-I initiates its biological effects through several pathways, including PI3K-Akt-mTOR, and MAPK extracellular signal-regulated kinases (ERKs) (9, 11–13). Thus, the changes in these two superfamilies of hormones represent a powerful signaling network in human physiology.

A multitude of different studies (14–21) have provided insight into the influence of resistance- and endurance-based exercise on immunoreactive GH concentrations, yet far fewer studies have investigated the effects of exercise perturbations on GH molecular weight (MW) variants (19, 22). Regarding the latter, Wallace et al. observed that the increase, peak concentration, and disappearance differed among GH isoforms as a result of acute aerobic exercise in men (23). These included the 20- and 22-kDa isoforms, two of the earliest identified variants released from human pituitary cells (24). Hymer et al. were the first to describe acute increases in unfractionated GH and two MW fraction pools (<30, and 30–60 kDa) immediately following resistance exercise in untrained women (25), which was observed across several immunoreactive and immunofunctional assays. Contrasting these data, Kraemer et al. (26), examined the effects of acute resistance exercise intensity on GH isoforms stratified by MW in non-resistance exercise-trained women. Although no between-group differences in unfractionated GH were observed based on total work performed, differences in the smallest MW fraction (<30 kDa) were noted. Thus, distinct GH MW isoforms may respond differently than unfractionated GH, presenting a divergent response pattern by sex.

Previous studies have investigated the role of IGF-I concentrations in potentiating exercise-associated adaptations, as well as recovery from acute and chronic exercise (27–29). Similar to GH, acute exercise is an effective stimulus to alter and examine IGF-I profile responses. However, very little is known regarding the effects of acute exercise on fractionated IGF-I isoforms, as demonstrated with GH. For example, Durzynska et al. (30) characterized the different pre-pro forms of IGF-I peptide, which provided details within skeletal muscle-specific IGF-I MW isoforms. To our knowledge, this has yet to be examined in the systemic circulation.

Considering peptide heterogeneity and the dynamic interactions between GH and IGF-I, and the multiple biological adaptations attributed to these hormones, we proposed to examine their responses simultaneously in the blood to determine if diverse patterns exist reflective of the different temporal cybernetic release patterns in glands and tissues. By examining the blood biocompartment, this would enhance the understanding of the temporal time frames in men and women for the patterned responses of different GH and IGF-I MW isoforms, thus supporting further targeted investigations. Therefore, the purpose of this investigation was to determine if GH and IGF-I MW isoform responses to acute resistance exercise were due to specific MW isoforms in sex-independent or -dependent manners. We hypothesized that acute resistance exercise would drive sex-specific responses differently among GH and IGF-I family constituents (e.g., MW isoforms).

MATERIALS AND METHODS

Subjects

Healthy, college-aged, men ($n = 10$; age: 28 ± 5 y, height: 171.9 ± 5.1 cm, weight: 86.2 ± 12.7 kg, BMI: 29.2 ± 4.5 kg·m^{-2}), and women ($n = 10$; age: 21 ± 2 y, height: 165.4 ± 5.8 cm, weight: 67.1 ± 11.0 kg, BMI: 24.5 ± 3.3 kg·m^{-2}) participated in this study. The volunteers had all experimental methods explained to them, and only participated after giving their free and voluntary written informed consent in accordance with the Declaration of Helsinki. All were screened *via* a health history examination, a physical examination by a physician, and were excluded if they had any conditions and/or taking any medications known to affect hormonal responses. All women were interviewed by the screening physician and deemed to have been eumenorrheic (having cycle lengths between 28 and 32 days for the previous several months leading up to the study) and not taking oral contraceptives. The women from this study were part of a broader longitudinal study (31), during which they were scheduled to complete multiple acute exercise trials during the same relative phase of their menstrual cycle. Attempts were made to have them complete their initial exercise trial as close to the early follicular phase as possible. We did not measure sex hormones to standardize timing, however, and only relied on verbal reports with regard to their menstrual cycle status/timing. Participants were untrained, and performed <3 exercise sessions per week for at least 6 months before the study, and no subjects were currently serving in the active duty military population. Sample sizes were determined using power calculations from previous studies that the authors have conducted in similar populations and protocols, which were primarily based on expected variability in IGF-I (typically less robust exercise-induced responses than GH), where 10 subjects per group was expected to yield 80% statistical power. All methods were reviewed and approved by the Human Use Review Committee of the U.S. Army Research Institute of Environmental Medicine. The investigators adhered to the policies for the protection of human subjects as prescribed in Army Regulation (AR) 70–25, and the research was conducted in adherence with the provisions of 32 CFR Part 219.

Acute Resistance Exercise Test

Following a 10 h overnight fast, and refraining from strenuous exercise for 48 h, subjects were asked to report to the laboratory in the morning to perform an acute resistance exercise test (ARET). All ARET sessions took place between 600 and 1200 h in order to standardize the time of the perturbation. The ARET was chosen due to previous success in subject tolerance and the ability to perturb the hormonal milieu, as described elsewhere (25, 32). Briefly, the ARET was comprised of 6 sets of the individual's 10 repetition maximum (6 × 10-RM) squat, separated by 2-min inter-set recovery periods. The initial 10-RM weight was ∼75% of the subject's 1-RM measured during pre-experimental testing sessions at least 48 h prior. The goal of each subsequent set was a 10-RM load, where the load was adjusted as needed to facilitate completion of 10 full range of motion repetitions with good form.

Blood Sampling and Handling

Prior to the ARET, a venous catheter was inserted in a forearm vein with a saline lock to maintain catheter patency. Subjects had venous blood obtained before (Pre), after 3 sets (Mid), immediately post (Post), as well as 15-min (+15) and 30-min (+30) following the ARET. Blood samples (∼7 mL) collected into SST vacutainers (BD, Franklin Lakes, NJ), clotted at room temperature for 30-min, and then centrifuged at 1,500 × *g* for 20-min. Serum aliquots were frozen and stored at −80°C until High Performance Liquid Chromatography (HPLC) processing and subsequent analyses were performed.

Serum Fractionation

At each time point noted above, serum was processed using HPLC and Sephacryl gel filtration columns [S-100HR sizing column (26 mm ID); GE Healthcare Bio-Sciences, Pittsburgh, PA] employing similar methods described previously (25). Briefly, the gel columns were calibrated with a MW standards kit (Pharmacia, Uppsala, Sweden), and proteins from each processed serum sample were eluted from the column based on size. Resultant collection tubes were subsequently pooled into 3 MW fraction ranges corresponding to >60 kDa (Fr.A), 30–60 kDa (Fr.B), and <30 kDa (Fr.C). Fractionated MW cut-off values were chosen to represent specific isoforms within the circulating GH-IGF-I axis, highlighting the heterogeneous nature of these related hormones (**Figure 1**).

Immunoassays

All samples were run in duplicate; however, within-subject (fractionated and unfractionated) samples were run in the same plate to minimize inter-assay variability, with both intra- and inter-assay CV's <10 % for the GH and IGF-I assays. For GH analysis, unfractionated and fractionated serum samples from each time point were analyzed using a commercially-available bead-based human GH fluorescence assay (Millipore, Billerica, MA) with a reported sensitivity of 0.004 ng/mL. Concentrations were determined using a commercially available immunoassay platform (Luminex200; LuminexCorp, Austin, TX), and fluorescence values were quantified using Masterplex QT, v2.5 (Hitachi, San Bruno, CA). For IGF-I analysis, unfractionated and fractionated samples were analyzed using a commercially-available ELISA (DG-100; R&D Systems, Minneapolis, MN), with a reported sensitivity of 0.026 ng/mL. Absorbance values were quantified on a Dynex MRX Revelation absorbance reader (Dynex Technologies, Chantilly, VA). It should be noted that with the low abundance of circulating free IGF-I (e.g., IGF-I molecules measured in Fr.C) several samples failed to meet the assay sensitivity. In such cases, values were reported asthe lowest concentration detectable in the assay (e.g., assay sensitivity).

Data Analysis

GH and IGF-I data were checked for normality with regard to fractions and time within sex using a Shapiro-Wilk test, and the majority were found to be normally distributed indicating that parametric statistics were appropriate. Subsequently, a 2-way (fraction × time) ANOVA with repeated measures (RM-ANOVA) with all subjects combined was used to determine whether GH or IGF-I responses to exercise were due to MW isoforms independent of sex. To answer the sex-dependent heterogeneity questions, a 3-way (fraction × time × sex) RM-ANOVA model for the interaction term was utilized. Subsequently, within-fraction 2-way (sex × time) RM-ANOVA models were run after identifying the significant 3-way interaction. When the RM-ANOVA detected a significant *F*-ratio, *post-hoc* analysis (least significant difference) was used to determine statistical differences for within- and between-subject factors. All values are expressed as mean ± SD, and α was ≤ 0.05, and statistical analyses were performed on SPSS version 21 (IBM, Armonk, NY).

RESULTS

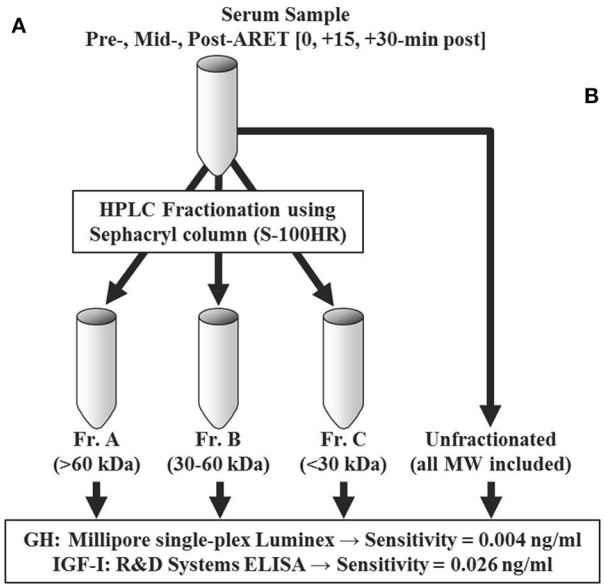
In this study, we examined the total MW content of the fractionated serum samples via HPLC for both GH and IGF-I to determine hierarchical placements and relationships as they relate to sex. We also examined the unfractionated immunoreactivity in each of these samples to determine if the fractions were consistent with known antibody reactivity shown in prior work, and to determine if the patterns of responses to acute resistance exercise were similar between men and women with the desire to gain more information and understanding of this important metabolic and anabolic hormonal axis.

GH

Fractionated GH via HPLC

We observed a main effect ($p \leq 0.01$) on all GH fractions across time and sex, demonstrating differences among the abundance of all fractions in unexercised adults, and independent of sex where Fr.C (<30 kDa; 14.3 ± 11.5 ng) > Fr.A (>60 kDa; 9.6 ± 9.1 ng) > Fr.B (30–60 kDa; 5.2 ± 4.1 ng). There was a significant fraction × time interaction ($p \leq 0.01$) effect observed on the exercise-induced GH responses, independent of sex. In response to exercise, all GH isoforms increased over baseline values ($p \leq 0.01$), and significant differences were observed between all fractions ($p \leq 0.03$) through +30-min of recovery.

We further identified a significant interaction between fraction, time, and sex. For instance, women had a greater



B **Molecular weight isoforms found within pooled fractions:**

	GH	IGF-I
Fr. A (> 60 kDa)	GH+GHBP Complex; GH Oligomers	IGF-I+BP-3+ALS (Ternary complex)
Fr. B (30-60 kDa)	GH Dimers (e.g., homo- and hetero-dimers)	IGF-I+BP's1-6 (Binary complex)
Fr. C (< 30 kDa)	GH Monomers (e.g., 20-, 22-kDa GH)	Free IGF-I
Unfractionated	All above complexes	All above complexes

FIGURE 1 | Size-excluded proteins via HPLC were pooled into three molecular weight (MW) ranges **(A)**. Details and examples of the corresponding MW isoforms expected to be found among fraction pools are provided in **(B)**. An additional aliquot was left unfractionated for recovery purposes. See Materials and Methods for immunoassay details.

abundance of Fr.A GH compared to men Pre- and Mid-exercise ($p \leq 0.03$). Within women, resistance exercise-induced increases in Fr.A GH ($P \leq 0.03$) represented by a baseline-to-peak 2.0 fold-change immediately post-exercise. Within men, Fr.A GH did not increase above baseline until post-exercise ($p \leq 0.01$), with a baseline-to-peak 5.2 fold-change +15-min post-exercise, and remained elevated through +30-min of recovery ($p \leq 0.01$) (**Figure 2A**). Fr.B isoforms were also higher in women than men ($p \leq 0.02$) through mid-exercise. In women, Fr.B GH was elevated with exercise ($p \leq 0.01$), with a baseline-to-peak 2.2 fold-change immediately post-exercise, and returned to baseline ($P > 0.12$) within +15-min of recovery. Men demonstrated a delayed increase ($P \leq 0.01$) in Fr.B GH isoforms, where Fr.B GH was represented by a baseline-to-peak 7.7 fold-change +15-min post-exercise and remained elevated immediately post-exercise through +30-min of recovery (**Figure 2B**). Fr.C GH isoforms were also higher in women than men at Pre- and Mid-exercise ($P \leq 0.03$). More specifically, Fr.C GH isoforms in women were elevated above baseline, represented by a baseline-to-peak 2.9 fold-change immediately post-exercise and remained elevated through +15-min following exercise. Men once again demonstrated a delayed and prolonged increase in Fr.C GH, represented by a baseline-to-peak 7.1 fold-change +15-min post-exercise and remained elevated through +30-min of recovery ($P = 0.01$) (**Figure 2C**).

When made relative to the total GH collected during fractionation, Fr.A GH ranged between 27 and 36%, Fr.B ranged between 15 and 20%, and Fr.C represented the highest abundance collected between 44 and 54%. Similar responses were observed for men and women (data not shown).

Unfractionated GH

Unlike MW-fractionated GH, unfractionated GH results did not present any main effects or interactions with sex as a factor. We only observed a significant main effect of time (data are not shown), which demonstrated that GH concentrations were increased Mid-exercise, and remained elevated through +30-min of recovery ($P \leq 0.03$). **Table 1** displays the relative recovery concentrations from fractionated compared to unfractionated GH.

IGF-I

Fractionated IGF-I via HPLC

Similar to GH, we observed a main effect across all time points and sex, with differences among all IGF-I fractions collected ($P \leq 0.05$), where Fr.A (>60 kDa; 76.6 ± 27.6 ng) > Fr.B (30–60 kDa; 11.6 ± 6.8 ng) > Fr.C (<30 kDa; 2.6 ± 3.6 ng) ($P \leq 0.01$). Unlike GH, when IGF-I fractions are collapsed across sex, there were no significant exercise-induced changes over time within the three MW fraction pools ($P > 0.05$).

Taking into account the observed interaction between fraction, time, and sex for IGF-I, the ARET induced a small but significant increase in Fr.A ($P \leq 0.05$) only in men (**Figure 3A**), which represented a baseline-to-peak 1.1 fold-change immediately post-exercise. There were no exercise-induced changes at any time point in men or women for Fr.B IGF-I ($P > 0.05$) (**Figure 3B**). In contrast to Fr.A IGF-I, the ARET induced a significant increase in Fr.C IGF-I only in women, with a baseline-to-peak 3.9 fold change immediately post-exercise and remained elevated through +30-min of recovery ($P \leq 0.05$) (**Figure 3C**).

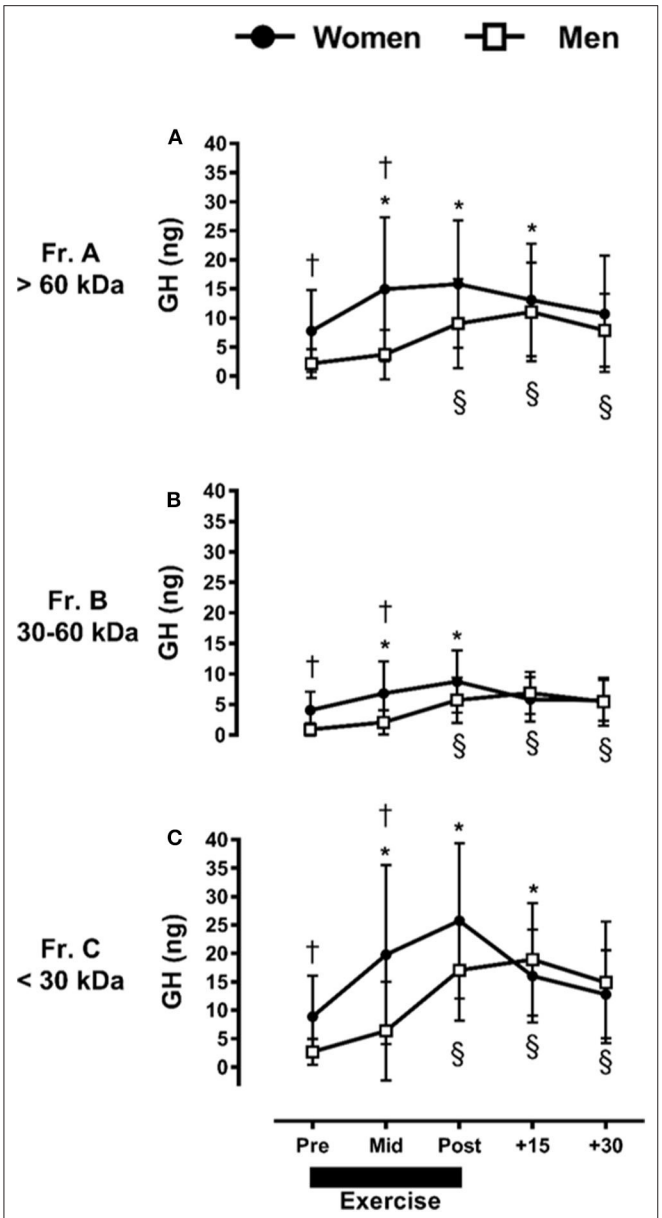


FIGURE 2 | Sex-dependent exercise-induced responses in GH separated by MW size [Fractions (A–C) presented in **(A–C)**, respectively]. *$P \leq 0.01$ vs. Pre-exercise within women; §$P \leq 0.01$ vs. Pre-exercise within men. †$P \leq 0.03$ vs. men at time point within fraction.

When made relative to the total IGF-I collected with HPLC fractionation, Fr.A IGF-I ranged between 80 and 88%, Fr.B ranged between 10 and 17%, and Fr.C ranged between 1 and 5%. Similar responses were observed for men and women (data not shown).

Unfractionated IGF-I

We observed a main effect for sex between unfractionated IGF-I concentrations (women > men, $P \leq 0.05$), although no significant differences between men and women were observed at any specific time point (data not shown). **Table 1** displays recovery concentrations compared to unfractionated IGF-I.

TABLE 1 | Recovery of GH and IGF-I molecular weight isoforms following HPLC fractionation compared to unfractionated.

	Summed fractionated GH recovery		Summed fractionated IGF-I recovery	
	ng/mL	Fold difference	ng/mL	Fold difference
Pre				
Women	41.28 ± 33.73	22.0	176.93 ± 67.66	1.05
Men	11.45 ± 11.23	49.8	171.06 ± 68.14	1.26
Mid				
Women	82.93 ± 64.84	13.3	190.12 ± 59.91	1.01
Men	24.23 ± 29.14	22.0	188.87 ± 69.9	1.31
Post				
Women	100.58 ± 57.10	11.4	190.50 ± 60.44	0.99
Men	63.63 ± 34.49	16.1	192.60 ± 74.49	1.39
Post+15				
Women	69.80 ± 40.57	12.8	171.30 ± 65.50	0.99
Men	73.69 ± 35.69	12.8	184.79 ± 60.90	1.36
Post+30				
Women	58.26 ± 40.18	16.3	176.66 ± 65.74	1.07
Men	56.35 ± 35.22	15.7	173.83 ± 51.79	1.36

Summary of GH and IGF-I recovered following sample fractionation via HPLC Values are the mean ± SD (summed recovery) or mean only (fold difference) calculated from the individual recoveries. Recovery was determined as the sum of all collected protein fractions where concentration was corrected for HPLC dilution, and Fold difference was the sum of all fractions made relative to the unfractionated sample (ng/mL) at the same time point.

DISCUSSION

Using HPLC fractionation of the serum along with immunoreactivity of the associated samples we observed sex-specific differences for different MW isoforms and an exercise-induced perturbation. The primary findings of the study were: (1) there were clear hierarchies in GH and IGF-I MW isoforms confirming the heterogeneous nature of this hormonal axis in men and women, and (2) fractionated GH and IGF-I responses to acute resistance exercise appear to be sex-dependent. These exercise-induced endocrine responses aid in the understanding of sex-specific hormonal control and underlying complex cascades partially mediating physiological adaptations from resistance exercise.

We observed a hierarchy among all GH MW fractions investigated via HPLC fractionation, which is in line with well-known GH heterogeneity, including nearly 100 different isoforms (5). From prior work, this hierarchy of difference is consistent and shows important internal validity of the HPLC analytical approach (33). In addition to 22-kDa GH, typically measured by most immunoassays, other GH MW isoforms include a 20-kDa mRNA splice variant, disulfide-linked homo- and hetero-dimers, glycosylated GH, high MW oligomers (up to pentamers), receptor-bound GH, and GH fragments

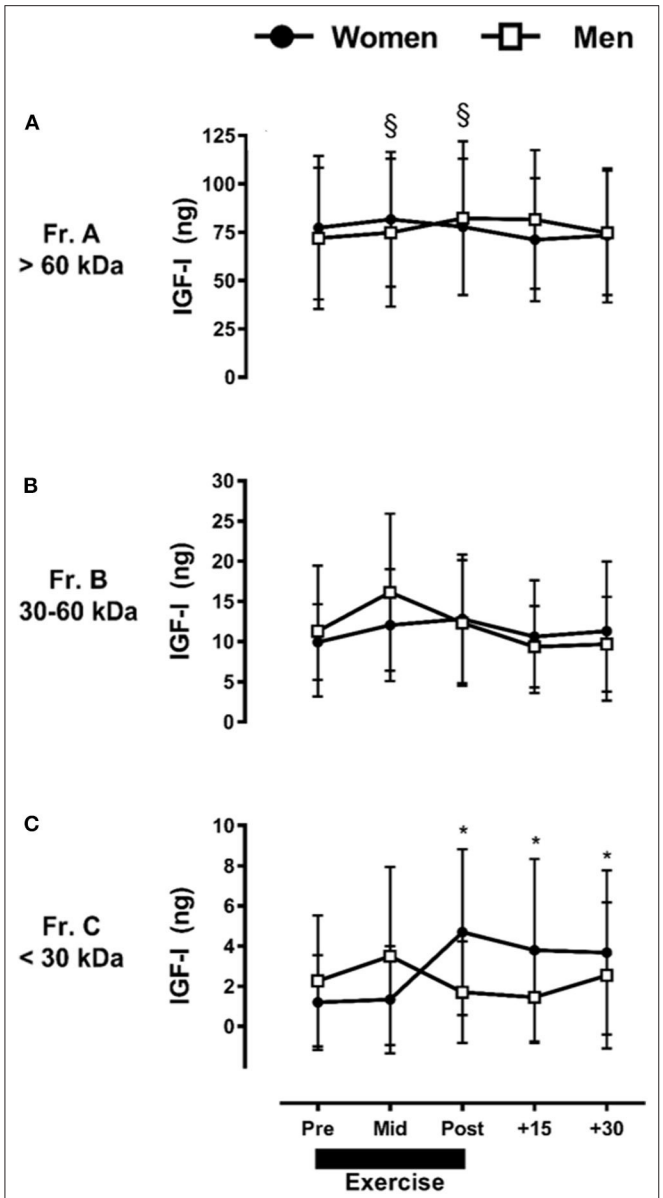


FIGURE 3 | Sex-dependent exercise-induced responses in IGF-I separated by MW size [Fractions (A–C) presented in **(A–C)**, respectively]. Data are mean ± SD. *$P \leq 0.05$ vs. Pre-exercise within women; §$P \leq 0.05$ vs. Pre-exercise within men.

(5, 32, 34–36). Distribution estimates of specific isoforms are known to vary extensively; however, the largest proportions are accounted for by monomeric 22- and 20-kDa GH (∼70–85% of circulating GH), while heterodimeric and homodimeric GH account for ∼10–20%, and oligomeric and GH bound to GHBP account for ∼5–10% (5, 25, 36). Our results partially align with these pooled estimates, where the largest proportion of GH collected across all time points and sex in our study was Fr.C (<30 kDa), which includes the major circulating constituents: 20- and 22-kDa GH as well as segments and fragments of those monomers.

The second most abundant fraction collected in our study was Fr.A (>60 kDa), which would include high-molecular weight oligomers and GH-GHBP complexes. Although reports from both Baumann and Hymer suggest that Fr.A constituents are the least abundant (5, 25), it is possible that the subjects in our study had a higher abundance of oligomeric and/or GH-GHBP complexes in the circulation than previous estimates. In fact, higher GH concentrations in women might also be expected to lead to increased GHBP concentrations, given the dependency between GH and GHBP as demonstrated in female rats (37) and women (38). The investigation closest to ours in study design (25), which examined acute heavy resistance exercise in women, presented data within reasonable proximity to our Fr.C immunoreactive GH estimates (having accounted for ∼50% of GH collected across all time points examined). This further suggests that the 191 amino acid, 22-kDa GH isoform detected by most GH immunoassays most likely accounted for the majority of our study's most abundant MW fraction, Fr. C (<30 kDa).

Men and women experienced exercise-induced increases among all GH MW isoform pools, but these responses were delayed and sustained longer in men. Our findings corroborate a recent study that demonstrated dimeric GH was higher in women, but sustained longer into recovery in men following resistance exercise (39). Additionally, Wallace et al. reported that a pituitary-derived GH assay (22-, 20-kDa, and other modified GH forms), as well as an exclusive 20-kDa assay, demonstrated an extended disappearance half-time following acute aerobic exercise in men (23). Those authors pointed out that their pituitary GH assay had a high affinity for dimeric GH, the presence of which could also help to explain prolonged elevations. In the context of our study, we observed sustained increases in all three GH fraction pools, which would cover monomeric 20-kDa GH, homo- and hetero-dimeric 20- and 22-kDa GH, GH-GHBP and oligomeric GH complexes, all of which possess increased half-lives when compared to monomeric 22-kDa GH (23, 40). We speculate that these sex-specific alterations in GH responses could relate to sex differences in GH's hypothalamic stimuli such as GHRH or somatostatin, leading to or inhibiting its pituitary release, respectively, and is likely under control of one or more sex steroid neuroendocrine loci including both estrogen and testosterone (41).

IGF-I MW fractions displayed clear differences across all time points and sex (main fraction effect) using HPLC fractionation, demonstrating a similar isoform hierarchy as observed with GH. Accordingly, we noted that the largest abundance of assayed IGF-I resided in Fr.A (>60 kDa), followed by Fr.B (30–60 kDa), and lastly by Fr.C (<30 kDa). These findings correspond with prior estimates of IGF-I existing primarily in ternary complexes (≥ 75%), followed by binary complexes (∼20–25%) and finally in free form (<1%) (8). Similar to GH half-life extension through interactions with its binding protein, GHBP (40), the half-life of IGF-I complexes are altered depending on the isoform. Guler et al. (42) estimated that the half-life of the 150-kDa ternary IGF-complex (IGF-I+BP-3+ALS) is markedly increased (e.g., 12–15 h) over that of either binary-complexed (IGF-I + one of 6 BPs) (e.g., 20–30 min) or free-IGF-I (e.g., 10–12 min). Given our observation that the most abundant

isoform pool was Fr.A (>60 kDa), it is reasonable to expect that IGF-I half-life would be extended considerably through the majority of IGF-I being sequestered in this MW fraction pool. The second largest abundance of IGF-I components was observed in Fr.B. Combined with Fr.A isoforms, these two MW pools (binary/ternary complexed IGF-I) accounted for ∼97% of measured IGF-I.

We did not detect acute exercise-induced alterations in IGF-I isoforms over time when our data were collapsed by sex; however, a significant main effect of sex (women > men) was observed across all IGF-I MW fraction pools, and significant increases were apparent in two of three IGF-I MW fractions in a sex-dependent manner (Fractions A, C, **Figures 3A,C**, respectively). Thus, we only observed differences between the isoforms or over time within specific isoforms when sex was taken into account.

Regarding the sex-dependent IGF-I system exercise effects, we observed a significant increase in Fr.A (>60 kDa) with resistance exercise only in men, which indicates that only larger MW IGF-I isoforms (e.g., ternary complexes) were increased in men. Alternatively, we observed that Fr.C (< 30 kDa) IGF-I molecules were increased following exercise and remained elevated throughout 30 min of recovery only in women. To our knowledge, we are the first to present this sexual dimorphism in the IGF-I system. Although we are unable to fully explain these observations other than lending further credence to the regulatory complexity provided through the family of 6 IGFBPs (8), it is possible that alterations in the IGFBP's affinity for IGF-I are also likely sexually-dimorphic, leading to our observed divergent responses in men and women.

There is a lack of congruence in the literature with the acute IGF-I response to exercise. This is in stark contrast to exercise-induced GH responses, and it is possible some of our limitations noted below may have led to some of these differences. With regard to GH, we recognize that using gel fractionation to create a <30 kDa pool limits our ability to distinguish 20- vs. 22-kDa GH; however, discerning between these two specific isoforms was not a main focus of the current investigation. With regard to IGF-I, we cannot differentiate between locally vs. systemically-derived IGF-I. For instance, local IGF has emerged as a contributor to the adaptations of exercise training; however, researchers have questioned the functional importance of IGF-I to post-natal skeletal muscle growth resulting from physical exercise (43–45). Further, our observation of heightened Fr.C IGF-I (<30 kDa) during and following exercise in women vs. men should be interpreted with caution, as there were several samples that did not meet the sensitivity of the assay employed. Of the total number of samples in Fr.C, only 43% of all samples met the sensitivity of the assay, and it should be noted that this phenomenon occurred more frequently in women (48% of samples) than in men (38% of samples). Also, more samples in post-exercise (0, +15, and +30 min post) met the assay sensitivity, and once again this was observed more frequently in women (63%) than in men (37%). Since free/unbound IGF-I demonstrates the lowest abundance among the IGF-I MW variants (accounting for <1% of all IGF-I) (8), and given sample dilution using HPLC fractionation, this was not a completely surprising observation. Finally, we did not specifically measure sex steroids and only relied on verbal reports of menstrual cycle timing when the participants performed the acute exercise test. Given that GH responses can vary directly (and vary indirectly via negative feedback from IGF-I) according to estrogen concentrations (41), it may be important to measure sex steroid concentrations concomitantly with systemic measures of the GH-IGF-I axis to more adequately characterize these responses. Future research should aim to confirm our findings given the above limitations.

Finally, while the sex-specific physiological significance of our findings are unclear, it is possible that the differential isoform responses observed currently may help to explain phenotype outcomes resulting from chronic exercise training. For instance, different exercise training regimens result in divergent tissue and organ adaptations (e.g., metabolic adaptations, cardiovascular improvements, bone and muscle tissue accumulation, etc.), which are clearly linked to the mode of training. Previous data suggested that chronic resistance training increased the GH bioactivity (as measured in the tibial line bioassay) among all three MW fractions in college-aged women, despite no consistent changes observed in immunoreactive GH (46). This has not been examined in long-term aerobic exercise training or in men to our knowledge, which could provide greater support to this speculation. Likewise, conducting a future similar study of IGF-I examined across MW isoforms and different exercise regimens coupled with a bioactive assay [e.g., kinase receptor activation (KIRA)] (47) could eluciate additional mechanisms by which this hormonal axis contrbutes to sex- and mode-specific chronic exercise-induced adaptations.

In conclusion, we observed sexually-dimorphic patterns in the GH and IGF-I hormonal axis. With regard to GH, women had higher abundances of GH in all three pooled fractions before and during exercise, but men experienced a delayed but sustained appearance of all three GH MW fractions following exercise and into recovery. Within the IGF-I family, men experienced acute increases in Fr.A (largest MW isoform pool mostly accounted for by ternary complexed IGF-I), while women experienced acute and sustained increases in Fr.C (smallest MW isoform pool mostly accounted for by free/unbound IGF-I). Once again, the changes in Fr.C IGF-I may have to be confirmed in future investigations using more sensitive techniques. We believe that the observed changes in GH and IGF-I MW isoform distributions are among factors explaining sex-specific phenotypic outcomes and metabolic alterations resulting from exercise.

DATA AVAILABILITY STATEMENT

Data are property of the U.S. Government and are only available through approved data sharing agreements between the government and individual institutions.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Use Review Committee U.S. Army Research Institute of Environmental Medicine. The patients/participants

provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JP, JS, WK, WH, and BN contributed to the conception and design of the study. JA, JS, KR, WK, and BN contributed to the acute resistance exercise data and sample collection. JP, JA, JS, KR, and WH contributed to the HPLC fractionation methods and/or laboratory assays. JP and BM conducted the statistical analyses and wrote the initial draft of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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Impact of a 4-Week Intensified Endurance Training Intervention on Markers of Relative Energy Deficiency in Sport (RED-S) and Performance Among Well-Trained Male Cyclists

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Cyclists often apply block periodization to high training volumes in meso- and macrocycles to optimize training adaptation and to prepare for competition. Body mass influences performance in many sports, including endurance disciplines, and conditions related to the syndrome Relative Energy Deficiency in Sports (RED-S) such as metabolic adaptations and premature osteoporosis have also been reported in male cyclists. This study aimed to determine how a 4-week mesocycle of intensified endurance training designed to increase performance, would affect markers of RED-S in well-trained male cyclists. Twenty-two participants (age: 33.5 ± 6.6 years, height: 181.4 ± 5.2 cm, weight: 76.5 ± 7.4 kg, peak oxygen uptake (VO_{2peak}): 63.5 ± 6.6 mL·kg^{-1}·min^{-1}) were recruited and instructed to maintain their background training load and to follow a supervised training protocol consisting of three high-intensity interval training sessions per week with a work duration of 32 min per session. Protocols included pre- and postintervention assessment of resting metabolic rate (RMR) using a ventilated hood, body composition and bone health by dual-energy X-ray absorptiometry (DXA), blood samples, energy intake, and aerobic performance. The interval training increased participants' aerobic performance—peak power output [4.8%, $p < 0.001$], VO_{2peak} [2.4%, $p = 0.005$], and functional threshold power [6.5%, $p < 0.001$] as well as total testosterone levels [8.1%, $p = 0.011$]—while no changes were observed in free testosterone [4.1%, $p = 0.326$]. Bodyweight, body composition, and energy intake were unchanged from pre- to post-test. Triiodothyronine (T_3) [4.8%, $p = 0.008$], absolute RMR [3.0%, $p = 0.010$], relative RMR [2.6%, $p = 0.013$], and RMR_{ratio} [3.3%, $p = 0.011$] decreased, and cortisol levels increased [12.9%, $p = 0.021$], while no change were observed in the total testosterone:cortisol ratio [1.6%, $p = 0.789$] or the free testosterone:cortisol (fT:cor) ratio [3.2%, $p = 0.556$]. A subgroup analysis of the five participants with the largest increase

in fT:cor ratio, revealed a greater improvement in functional threshold power (9.5 vs. 2.5%, $p = 0.037$), and higher relative RMR (0.6 vs. −4.2% $p = 0.039$, respectively). In conclusion, 4 weeks of intensified endurance interval training increased the athletes' aerobic performance and testosterone levels. However, negative changes in markers related to RED-S, such as a reduction in RMR and T_3, and an increase in cortisol were observed. These results indicate the complexity involved, and that male athletes are at risk of developing clinical indications of RED-S even during a short 4-week endurance training mesocycle.

Keywords: endurance athletes, energy availability, hormonal response, male cyclists, resting metabolic rate, testosterone, training intervention

INTRODUCTION

Preparing for a competitive cycling season often involves high volumes of training, quantified over several periods, ranging over micro-, meso-, and macrocycles, designed to induce specific physiological adaptations (1). If a planned overload is followed by a well-matched recovery strategy, functional overreaching with the intended physiological adaptation occurs (1). However, large training volumes that are combined with insufficient recovery strategies can trigger the development of non-functional overreaching and overtraining syndrome, with symptoms of fatigue and decreased performance (2, 3). Monitoring changes in an athletes' hormone concentrations, including testosterone and cortisol, have previously been used to assess athletes' anabolic-catabolic balance (4). However, monitoring athletes' testosterone-to-cortisol ratio is considered more sensitive to training stress than is merely measuring testosterone and cortisol separately (5).

Several parameters such as body mass and nutritional intake affect cycling performance, and low energy availability is frequently reported among cyclists (6, 7). Energy availability is the amount of energy relative to fat-free mass (FFM) left to support basic body functions after subtraction of the energy used during exercise; energy availability = (energy intake [kcal]—exercise energy expenditure [kcal])/(FFM [kg])/day (8–12). Low energy availability combined with large training volumes, can cause negative consequences such as impaired protein synthesis, impaired hormonal and training response, and increased risk of fatigue; these may lead to performance impairment (5, 9, 10). Research in females has also shown a variety of health parameters being negatively affected by both short- and long-term low energy availability (8–13). Clinical trials exposing eumenorrheic females to low energy availability (<30 kcal·kg^{-1} FFM·day^{-1}) for only 5 days found reductions in levels of insulin-like growth factor-1 (IGF-1), leptin, insulin, triiodothyronine (T_3), and luteinizing hormone (12, 14). Furthermore, long-term low energy availability has been found to increase the risk of premature osteoporosis and to give elevated cardiovascular risk factors (10, 13).

Research reports that male athletes are also vulnerable to the negative health and performance consequences of low energy availability as outlined in the Relative Energy Deficiency in Sports (RED-S) model (10). Not all health and performance aspects of RED-S are, however, fully elucidated, and recent research in male athletes has shown inconsistent findings (15–17). One possible reason for this inconsistency is believed to be the methodological difficulties of assessing energy availability (8, 18). Measurements of resting metabolic rate (RMR), implicating the energy expended on basic bodily functions have therefore been proposed to, and to some extent used as, a potential objective indicator of energy availability (9, 19). However, only two studies to date, have investigated the impact of different training regimens on RMR as a surrogate marker, where one study investigated trained cyclists eliciting overreaching (20), and the other investigated elite rowers during an intensified training period (19). These studies, however, did not include an assessment of hormonal responses when monitoring athlete's responses to changes in energy intake, training regimen or the combination of both.

Therefore, the aim of this study was to determine how a mesocycle of 4 weeks of intensified endurance training designed to increase aerobic performance, would affect RMR, body composition, energy intake, total and free testosterone, cortisol, testosterone:cortisol ratio, T_3, insulin and IGF-1 levels in well-trained male cyclists.

METHODS

Study Design

This prospective intervention study was part of a larger training study (clinicaltrials.gov; NCT04075929) with no control group. The training intervention consisted of three supervised high-intensity interval sessions per week, for 4 weeks. Athletes were instructed to maintain their current background training load while enrolled in the study. Each interval training session contained 20 min of self-regulated warm-up, followed by an interval work period with a total accumulated work duration of 32 min of high-intensity training, followed by a 20-min self-regulated cool-down. The total accumulated amount of exercise during the 4-week training intervention was 384 min of high-intensity training and 480 min of self-regulated warm-up and cool-down. Bone mineral density (BMD) was assessed before the intervention, while RMR, body composition, hormone levels, performance variables (peak oxygen consumption and time trial), and energy intake were assessed before and after the intervention period.

Participants

To be included in the study, participants had to be at least 18 years old but younger than 50 years, with a peak oxygen uptake (VO_{2peak}) $\geq$ 55 mL·kg body mass^{-1}·minutes^{-1} and a training frequency of at least three sessions per week during the last year. Furthermore, absence of disease and injury was required. All participants' were classified at performance level 3–4 (21). Recruitment was accomplished through social media and local online newspapers. Before inclusion, all participants received information about the study and test procedures, and signed an informed consent agreement. The study protocol was approved by the University Faculty Ethics Committee and the Norwegian Center for Research Data (no. 46706). All testing complied with the Declaration of Helsinki. Initially, 22 well-trained male cyclists aged between 22 and 45 years who competed at a regional or national level were recruited (**Figure 1**). Throughout the intervention, two participants were excluded from the analysis: one failed to complete the intervention, and one was excluded because of non-compliance; hence 20 participants were included in the final analysis.

Procedures and Measures

Training volume from the last 4 weeks before pretesting, as well as during the intervention, was collected via written training diaries. During a 2-week period before and after the intervention, participants completed physiological testing, and logged their dietary intake. Participants arrived at the university laboratory during three non-consecutive days for physiological testing. On the first day, participants arrived using motorized transport in a fasted and rested state. RMR, body composition, BMD, and blood sampling were assessed at 06:00–09:00 a.m. to control for diurnal variation. On the second day, participants completed a maximum aerobic exercise testing protocol at 12:00–05:00 p.m. in an unfasted state. On the third test day, the participants performed a 40-min time trial to assess their functional threshold power. During the last week before, and just after the intervention, participants weighed, and registered their dietary intake for four consecutive days (**Figure 2**).

Performance Variables

VO_{2peak} and peak power output measurements were performed on a stationary bike (Excalibur Sport, Lode B.V., Groningen, the Netherlands) starting with 1 min of cycling at a power output of 175 W and increased by 25 W per minute until voluntary exhaustion or failure to maintain a cadence of at least 70 rounds per minute. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) was measured using Oxycon Pro™ with mixing chamber and 30 s sampling time (Oxycon, Jaeger GmbH, Hoechberg, Germany) and was calibrated according to standard laboratory procedures. Mean power during the last minute of the test decided the 'cyclist's peak power output, and the mean from the two highest VO_2 measurements determined VO_{2peak}. Heart rate was measured continuously, and blood lactate was measured 1 min after test completion. Objective criteria, such as plateau of the oxygen uptake, heart rate $\geq$ 95% of known heart rate peak, respiratory exchange ratio $\geq$ 1.10, and blood lactate $\geq$ 8.0 mMol·L^{-1} were used to ensure a valid test (22). To assess functional threshold power, participants performed a seated 40-min all-out test using their own bike on CompuTrainer Lab bike rolls (Race Mate, Seattle, WA, USA). The test started with a 20–30-min warm-up at a self-regulated load, followed by a 40-min ride with the highest possible mean wattage. All participants were blinded for power output and heart rate, with only time remaining and rounds per minutes displayed.

Resting Metabolic Rate

Indirect calorimetry using a canopy hood system was used to assess RMR (Oxycon Pro, Jaeger, Germany), and systems were calibrated before each test according to standard laboratory procedures. Participants rested for 15 min before measurement. VO_2 and VCO_2 were assessed over a 30-min period. The last 20 min of measurements were used to assess RMR as described elsewhere (23). Measured RMR was calculated using the Weir equation (24) (3.94 × VO_2 [ml]) + (1.1 × VCO_2 [ml]) × 1.44. Relative RMR was calculated as measured RMR in kcal·kg^{-1} FFM·day^{-1}. Predicted RMR was calculated using the Cunningham equation (25) (500 + (22 × FFM [kg]), and RMR_{ratio} was calculated as measured RMR [kcal]/predicted RMR [kcal]. Resting heart rate (V800, Polar Elektro Oy, Kempele, Finland) was defined as the lowest heart rate measured during RMR measurement.

Energy Intake and Macronutrients

Participants weighed and registered their dietary intake for four consecutive days using a digital kitchen scale (OBH Nordica 9843 Kitchen Scale Color, Taastrup, Denmark). In-depth oral and written instructions were given before registration, and participants were asked to maintain their habitual dietary patterns and routines during the registration period. All dietary data were logged using software from Dietist Net (Dietist Net, Kost och Näringsdata, Bromma, Sweden) with access to the Norwegian food table and an open Norwegian nutritional information database.

Body Composition and Bone Mineral Density

Height was measured without shoes to the nearest 0.1 cm using a wall-mounted centimeter scale (Seca Optima, Seca, Birmingham, UK). Body weight was measured in underwear to the nearest 0.01 kg with an electronic scale (Seca 1, model 861, Birmingham, UK). Body mass index (BMI) was calculated as weight in kg divided by height squared in meters (kg/m^2). Body composition and BMD were assessed using dual-energy X-ray absorptiometry (DXA) (GE-Lunar Prodigy, Madison, WI, USA, EnCore software version 15). The same technician performed all tests with the same scanner on all participants. BMD was assessed in the lumbar spine (L1–L4), femoral neck, and total hip. Low BMD in athletes was defined as a Z-score of < -1.0 in one of the measured sites, as recommended by Nattiv et al. (11). Body composition was assessed according to a best-practice protocol (26), including assessment of hydration status (USG) before DXA measurement using a digital refractometer (Atago UG-α cat. no. 3464, Atago U.S.A. Inc., Bellevue, WA).

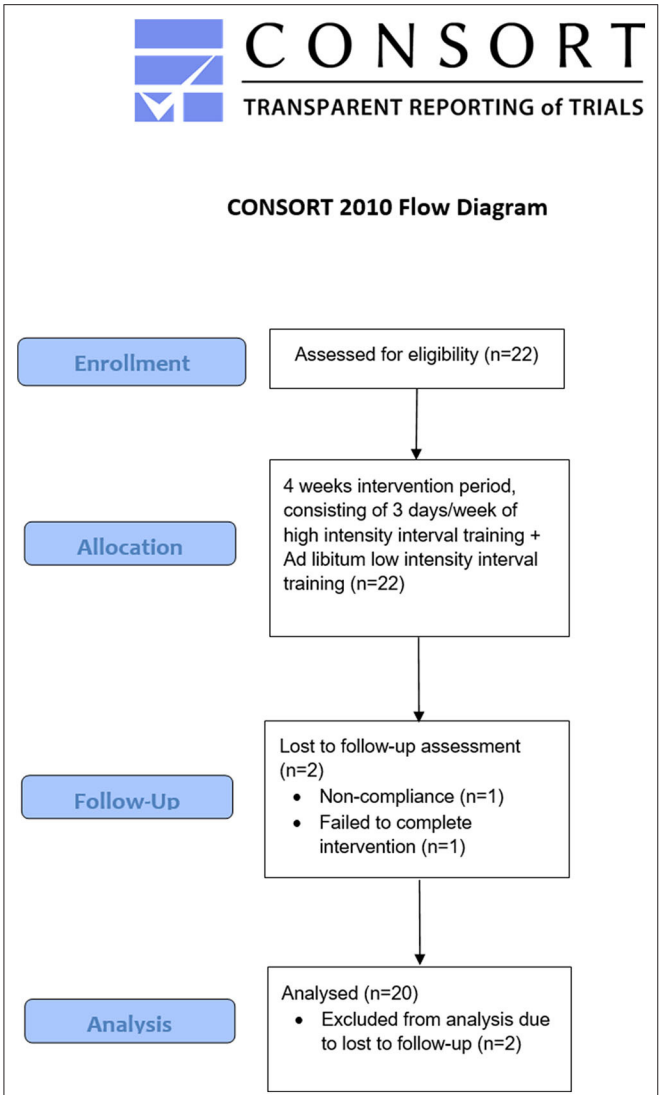

FIGURE 1 | Participation flow. The study was conducted in Kristiansand, Norway.

Blood Sampling

Blood samples were drawn from the cephalic vein of participants 5 min after completion of DXA. Two 5 mL Vacuette Z Serum Sep clot activators (BD, Plymouth, UK) were filled and centrifuged at 3,100 × g for 10 min (Statspin Express 4, Beckman Coulter, Inc. 250 S. Kraemer Blvd. Brea, CA, USA) within the limit of at least 30 min but <60 min. Five 2 mL Cryotube vials (VWR International, Radnor, Penn, USA) were filled with serum and frozen to −80°C. Serum was analyzed at Sørlandets Hospital, Kristiansand and analyzed for testosterone (analytic CV: 6.7 %), sex hormone-binding globulin (SHBG 4.0%), T_3 (6.9%), cortisol (8.2%), insulin (21.1%), and IGF-1 (8.0%). Free testosterone was calculated by dividing total testosterone with SHBG.

Statistics

Data were analyzed using Statistical Package for the Social Sciences (SPSS) for Windows (v. 25; IBM Corp., Armonk, NY, USA). The dataset was controlled for missing data and signs of non-normality using histograms as reference, and the assumption of normality of variance was found to be satisfied. Difference and relative changes between pre- (PRE) and posttest (POST) were assessed using paired-samples *t*-test (POST-PRE), generating mean, standard deviation of difference, and 95% confidence interval including percent change. Changes between groups were found using independent sample *t*-test. Effect size (ES) was calculated to interpret the meaningfulness of results using Cohen (27) criteria (0.2 = small effect, 0.5 = medium effect, 0.8 = large effect). Statistical significance level was defined as $p < 0.05$. A priori power analysis was calculated based on an expected standard deviation of 2.0 (19), and we had 80% power to detect a true mean group difference at 1.9 kcal·kg^{-1} FFM·day^{-1} in relative RMR with a minimum of 11 participants (alpha: 0.05; two-tailed).

RESULTS

Descriptive characteristics of the participants are presented in **Table 1**. Comparing total accumulated training load (including background training) from before pretesting with total accumulated training during the intervention, no significant increase was found ($p = 0.497$).

Performance

Athletes improved their VO_{2peak} (2.4%, $p < 0.01$), peak power output (4.8%, $p < 0.001$) and functional threshold power (6.5%, $p < 0.001$) from pre- to post-test (**Table 2**).

Resting Metabolic Rate, Energy Intake, Body Composition, and Bone Health

A 3.0% reduction was found in both absolute RMR, relative RMR, and RMR_{ratio} from pre- to post-test ($p < 0.05$) (**Table 3**). No changes were observed in energy intake (kcal/day) or intake of macronutrients (g per kg, E%). Body weight, fat mass, and FFM was assessed during stable urine specific gravity (0.001 ± 0.006 kg.m^3, $p = 0.420$), and did not differ from pre- to post-test (**Table 3**). L1-L4 average Z-score was 0.1 ± 1.1, while average femoral neck and total hip Z-scores were 0.2 ± 1.0 and 0.3 ± 0.9, respectively. Three athletes (15%) had low BMD in either L1-L4, femoral neck, or total hip, respectively: participant 1 (Z-scores: −3.6, −2.2, and −2.0, 26 years old, 17.4% fat); participant 2 (+0.2, −1.1 and −1.1, 36 years old, 15.9% fat); and participant 3 (−1.8, +1.2, and +0.9, 43 years old, 24.7% fat).

Blood Markers

Total testosterone increased 8.1% ($p = 0.011$) from pre- to post-test while no significant changes in free testosterone (4.1%, $p = 0.326$) were found. Cortisol levels increased 12.9% ($p = 0.021$) while total testosterone:cortisol ratio (1.6%, $p = 0.789$), and free testosterone:cortisol ratio (−3.2%, $p = 0.556$) remained unchanged from pre- to post-test. Mean T_3 levels decreased 4.8% ($p = 0.008$) while no significant changes

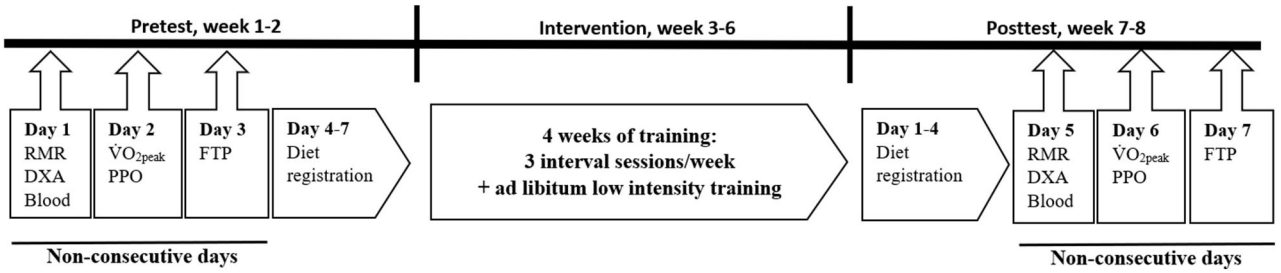

FIGURE 2 | Schematic overview of study design. Pre- and posttest measures included rested and fasted resting metabolic rate (RMR), dual-energy X-ray absorptiometry (DXA), and blood sampling. An incremental exercise test for determination of peak oxygen uptake (VO_{2peak}) and peak power output (PPO) as well as a 40-min functional threshold power-test (FTP) were performed in an unfasted state. Diet registration were performed during four consecutive days.

TABLE 1 | Descriptive characteristics of athletes included in the final analysis.

Variables	All ($n = 20$)
Age (years)	33.3 ± 6.7
Height (cm)	180.8 ± 4.9
Weight (kg)	75.8 ± 7.3
BMI (kg/m^2)	23.2 ± 1.9
Body fat (kg)†	11.1 ± 4.5
Body fat (%)†	14.9 ± 5.2
FFM (kg)†	65.5 ± 5.2
Resting HR (beats/minute)	48.0 ± 8.0
VO_{2peak} (mL.kg^{-1}.minute^{-1})	63.5 ± 6.6
VO_{2peak} (L.minute^{-1})	4.8 ± 0.4
Exercise (h/year)	395 ± 171
Active within cycling (years)	12.9 ± 9.7

Data are presented as mean ± SD. †measured by DXA. BMI, body mass index; DXA, dual-energy X-ray absorptiometry; FFM, fat-free mass; HR, heart rate; VO_{2peak}, peak oxygen uptake.

were observed in insulin and IGF-1 levels from pre- to post-test (**Table 4**).

A subanalysis that included the five participants with the largest increase and the five participants with the largest decrease in their free testosterone:cortisol (fT:cor) ratio from pre- to post-test revealed quantitatively quite similar changes in both total and free testosterone. In the group with an increased fT:cor ratio, there was a pronounced increase in testosterone (19 and 25%, total and free testosterone, respectively) and a 7% decrease in cortisol. This contrasted the group with decreased fT:cor ratio, where there was a decrease in total and free testosterone of 4 and 8%, respectively, combined with a 32% increase in cortisol. Furthermore, a greater improvement in functional threshold power was observed in the high fT:cor ratio group vs. the low fT:cor ratio group (9.5 vs. 2.5%), and similarly a higher relative RMR (0.6 vs. −4.2%, respectively) (**Table 5**). No differences were found when comparing training volume before or during the intervention in the high vs. low fT:cor ratio subgroups ($p = 0.609$).

DISCUSSION

In this study, we have demonstrated that 4 weeks of high-intensity training for 32 min, three times a week, superimposed on the athletes' background training, resulted in increased aerobic peak power output, VO_{2peak}, functional threshold power, as well as increased testosterone levels. In contrast, markers associated with low energy availability such as decreased RMR, lowered T_3, and increased cortisol, were found. Thus, our findings suggest positive performance responses of the exercise program used in the present study, however negative responses related to health, potentially caused by lowered energy availability were observed. This is a worrying sign, that a relative short period of 4 weeks can induce such changes, and athletes need to take this seriously.

Resting Metabolic Rate, Energy Intake, Body Composition, and Bone Mineral Density

In the present study, cyclists undertook a 4-week intensified endurance training intervention, which without any apparent increase in their energy intake, led to reduced energy availability, and a 3% reduction in RMR. This is similar to the findings of other studies (19, 20); indeed a 5% decrease in RMR was reported by Woods and co-workers (19) when elite male and female rowers undertook 4 weeks of heavy endurance training, without dietary compensation. Meanwhile, the same group reported that male cyclists achieved a state of overreaching and reduced RMR when 6 weeks of intensified training was undertaken without adjustment of energy intake (20). Meanwhile, other studies of increases in training workloads in endurance-trained male cyclists (28) or healthy males undertaking resistance and endurance training (29) reported an increase, or no change in RMR, respectively. However, in these studies, energy intake was either not assessed (28), or measured using a suboptimal protocol of a 3-day recall (29), and it is uncertain whether energy compensation accounted for the divergent results. RMR is mostly affected by body composition, with FFM as the largest determinant accounting for up to 70% of the individual variation in RMR, and is considered one of the largest components of total

TABLE 2 | Aerobic performance variables at pre- and posttest. Results from paired-sample *t*-tests (post-pre).

Outcome measure	Pre	Post	Mean ± SD of difference	95% CI	*P*-value	Δ Post-Pre (%)	ES
PPO (Watt)	397	416	18.5 ± 12.4	12.7–24.3	<0.001	4.8	1.49
VO_{2peak} (mL.kg^{-1}.minute^{-1})	63.5	65.0	1.5 ± 2.1	0.5–2.5	0.005	2.4	0.72
VO_{2peak} (L.minute^{-1})	4.8	4.9	0.1 ± 0.2	0.01–0.2	0.026	2.1	0.54
FTP (Watt)	261	278	17.0 ± 11.8	11.5–22.5	<0.001	6.5	1.44
FTP (Watt/kg)	3.5	3.7	0.2 ± 0.2	0.2–0.3	<0.001	6.9	1.48

Data are presented as mean ± SD of difference, 95% CI, percent change from pre to posttest and effect size (Cohen's D). PPO, aerobic peak power output; VO_{2peak}, peak oxygen uptake; FTP, functional threshold power.

TABLE 3 | RMR, energy intake, macronutrients, and body composition at pre- and posttest. Results from paired-sample *t*-tests (post-pre).

Outcome measure	Pretest	Posttest	Mean ± SD of difference	95% CI	*P*-value	Δ Post-Pre (%)	ES
Absolute RMR (kcal·day^{-1})	1,768	1,716	−52 ± 81	−90.3 to −14.1	0.010	−3.0	0.64
Relative RMR (kcal·kg^{-1} FFM·day^{-1})	26.9	26.2	−0.8 ± 1.2	−1.3 to −0.2	0.013	−2.6	0.67
RMR_{ratio}	0.91	0.88	−0.03 ± 0.04	−0.1 to 0.0	0.011	−3.3	0.75
Energy intake (kcal)	3,015	3,021	5.6 ± 560.6	−256.7 to 268.0	0.965	0.2	0.01
Carbohydrate intake (g)	332	338	5.2 ± 74.2	−30.6 to 40.9	0.766	1.8	0.07
Relative carbohydrate intake (g/kg)	4.4	4.5	0.1 ± 1.1	−0.4 to 0.6	0.607	2.2	0.09
Protein intake (g)	124	128	4.7 ± 25.0	−7.4 to 16.7	0.428	3.2	0.19
Relative protein intake (g/kg)	1.6	1.7	0.07 ± 0.4	−0.1 to 0.2	0.388	6.3	0.17
Fat intake (g)	123	125	1.7 ± 29.8	−12.6 to 16.1	0.803	1.6	0.06
Relative fat intake (g/kg)	1.6	1.7	0.03 ± 0.5	−0.2 to 0.2	0.741	1.8	0.06
Body weight (kg)†	75.8	75.7	−0.16 ± 0.7	−0.5 to 0.2	0.342	−0.1	0.23
FFM (kg)†	65.5	65.5	−0.05 ± 0.8	−0.4 to 0.3	0.764	0.0	0.06
Fat mass (kg)†	11.1	11.0	−0.09 ± 0.7	−0.4 to 0.2	0.563	−0.9	0.13

Data are presented as mean ± SD of difference, 95% CI, percent change from pre- to post-test and effect size (Cohen's D). †measured by DXA. DXA, dual-energy X-ray absorptiometry; FFM, fat-free mass; RMR, resting metabolic rate.

energy expenditure (30). In the present study, body composition, including FFM, remained unchanged from pre- to post-test and was therefore unlikely to contribute to the reduced RMR, despite several RED-S-related hormonal indications of a more catabolic state. It is unclear whether the increase in testosterone levels, possibly triggered by the endurance interval training, acted as a protective mechanism to prevent increased proteolysis. Hence, the lowered RMR might be a protective mechanism to prevent weight reduction and changes in body composition. Similar findings have been reported in elite male endurance athletes with low energy intake compared with athletes with adequate energy intake, where RMR was calculated to be 8% lower in the low energy intake group, suggesting an energy-conserving mechanism for maintaining body function and stable body weight (31).

Poor bone health develops over a long period with several influential factors, where a lack of loading due to the mode of exercise and poor nutrition are key factors (8, 10, 11). It is well-documented that long-term low energy availability is linked to poor bone health in both male and female athletes (8, 10, 11, 13), and road cycling does not induce significant osteogenic benefits compared with weight-bearing sports (32). Olmedillas et al. (33) reported lower BMD in young cyclists compared with recreationally active age-matched controls, and a recent Norwegian study showed that as many as 53% of elite cyclists had low BMD in the lower extremities, despite reporting regular resistance training (34). In our study 15% of the athletes had low BMD in either the lumbar spine, femoral neck, or total hip. Despite not having information on our athletes previously athletic history, it still raises concerns of athletes being unaware of the potentially negative effects of the lack of bone-loading involved in non-weight-bearing exercise, not performing high-load exercise that dampens the effects of bone loss as well as the intake of insufficient amounts of macro- and micronutrients.

The etiology of low energy availability is complex and may include excessive exercise, "making weight" before a competition, eating disorders, or unintentional mismatch between energy expenditure and energy intake resulting from a lack of appetite, poor nutrition knowledge, or lack of time to plan and prepare meals (11). In the present study, carbohydrate intake was lower and protein intake was higher than recommended (35, 36) and remained unchanged from pre- to post-test. In weight reduction periods, a higher protein intake at the expense of carbohydrates has been shown to improve the amount of fat loss and to preserve lean tissue (37), and this may contribute to the explanation of maintained FFM, despite unchanged energy intake during the intensified endurance training period in the present study. Nonetheless, the importance of periodizing energy intake to make changes in nutritional demands during different phases of training has previously been demonstrated (19); this should be emphasized to help support and enhance endurance training adaptations, especially

TABLE 4 | Blood markers at pre- and posttest. Results from paired-sample *t*-tests (post-pre).

Outcome measure	Pretest	Posttest	Mean ± SD of difference	95% CI	*P*-value	Δ Post-Pre (%)	ES
Total testosterone (nmol/L) [7.2–24.0]	17.4	18.8	1.35 ± 2.13	0.35–2.35	0.011	8.1	0.63
Free testosterone (nmol/L) [0.168–0.607]	0.459	0.478	0.020 ± 0.087	−0.021 to 0.060	0.326	4.1	0.23
Cortisol (nmol/L) [138.0–690.0]	381.1	430.3	49.25 ± 87.31	8.39–90.11	0.021	12.9	0.56
Free testosterone:cortisol ratio	0.00125	0.00121	0.0001 ± 0.0003	−0.0001 to 0.0001	0.556	−3.2	0.06
Total testosterone:cortisol ratio	0.047	0.046	0.001 ± 0.012	−0.007 to 0.005	0.789	1.6	0.06
SHBG (nmol/L) [8.0–60.0]	39.7	40.2	0.45 ± 4.23	−1.53 to 2.43	0.640	2.8	0.11
T_3 (nmol/L) [1.2–2.8]	2.1	2.0	−0.12 ± 0.18	−0.02 to −0.04	0.008	−4.8	0.67
Insulin (pmol/L) [≤160.0]	34.7	31.0	−3.70 ± 10.20	−8.48 to 1.08	0.121	−10.6	0.36
IGF-1(nmol/L) [17.0–63.0]	18.1	18.0	−0.16 ± 2.06	−1.12 to 0.81	0.740	−0.6	0.08

Data are presented as mean ± SD of difference, 95% CI, percent change from pre to post-test and effect size (Cohen's D). [xx-xx] indicates reference values. IGF-1, insulin-like growth factor-1; SHBG, sex hormone-binding globulin; T_3, triiodothyronine.

TABLE 5 | Relative changes in hormonal and performance variables from pre- to post-test in participants with highest increase vs. highest decrease in free testosterone:cortisol ratio. Results from independent sample *t*-test.

Relative change (%)	Highest increase ($n = 5$)	Highest decrease ($n = 5$)	*P*-value
Total testosterone	18.7 ± 20.6	−4.1 ± 8.9	0.053
Free testosterone	24.7 ± 26.0	−7.8 ± 9.0	0.030
Cortisol	−6.9 ± 17.7	32.3 ± 15.5	0.006
SHBG	−4.1 ± 6.1	4.2 ± 7.4	0.088
T_3	−10.0 ± 3.5	−11.0 ± 7.4	0.803
Insulin	−8.1 ± 17.6	−1.3 ± 30.0	0.675
IGF-1	−7.2 ± 3.0	−3.9 ± 6.8	0.352
PPO	6.3 ± 4.1	4.2 ± 3.2	0.388
VO_{2peak}	3.8 ± 3.8	2.4 ± 3.8	0.557
FTP	9.5 ± 5.4	2.5 ± 3.1	0.037
Training volume (h/week)	−5.1 ± 31.5	2.7 ± 18.9	0.650
Energy intake	6.6 ± 22.6	10.2 ± 38.2	0.860
Relative RMR	0.6 ± 2.8	−4.2 ± 3.3	0.039
Body weight	−0.4 ± 0.7	−0.5 ± 1.2	0.901
FFM	−0.7 ± 1.1	0.3 ± 0.6	0.126

Data are presented as mean ± SD. FFM, fat free mass; FTP, functional threshold power; IGF-1, insulin like growth factor-1; PPO, peak power output; relative RMR (kcal/kg FFM/day), resting metabolic rate; SHBG, sex hormone-binding globulin; T_3, triiodothyronine; VO_{2peak}, peak oxygen uptake.

when athletes undergo strenuous meso- and macrocycle training (38).

Blood Markers

We observed an increase in total testosterone levels from pre- to post-test, presumably as a positive response from the intensified endurance training protocol. We measured testosterone directly, and calculated free testosterone as well, by dividing total testosterone with SHBG. Based on the old free hormone hypothesis, free testosterone should be the bioavailable form of testosterone. However, this hypothesis has been debated for three decades, without definite conclusion. Thus, a recent comprehensive review by Goldman et al. (39) concluded that no measure of testosterone is ideal, and that both total and free testosterone should be considered. The calculated free testosterone, found by dividing total testosterone with SHBG, is also hampered by assumptions of association constants, and further accuracy and precision are affected negatively by the need for using two analyses. In the literature on the effect of training on testosterone levels, most studies have reported only total testosterone levels. Therefore, we chose to report both measures. The increase in total testosterone observed, could partly be a result of an observed small, however insignificant increase in SHBG. Testosterone, an anabolic steroid, stimulates growth, increases protein synthesis, and controls the development and maintenance of the secondary sex characteristic. Previous studies have demonstrated acute changes in testosterone in resistance training and high volumes of exercise using large muscle mass (40). Severe reductions in testosterone have been reported in male soldiers undergoing prolonged starvation (41), while Koehler et al. (16) found no reduction in testosterone when males were exposed to short periods of very low energy availability (∼15 $kcal \cdot kg^{-1}$ $FFM \cdot day^{-1}$) for 4 days.

In the present study, cortisol increased by 12.9% from mean values of 381–430 nmol/L. Cortisol is likely to contribute to increased adiposity during energy abundance, and is an important catabolic hormone secreted to ensure glucose homeostasis during prolonged exercise, glycogen depletion, stress, and starvation (13, 42). A meta-analysis of human studies by Nakamura et al. (43) investigating fasting and severe caloric restriction found increased cortisol levels followed by a long-term normalization, while another study by Kyrolainen et al. (17) found increased cortisol as a response to heavy prolonged physiological stress in soldiers, followed by an immediate reduction when soldiers experienced a stress reduction. However, in a study comparing nine long-distance male runners with low energy availability with eight non-athletes with optimal energy availability, cortisol was not different between the groups (44). The increase in cortisol in our athletes could, therefore,

be a combination of a natural response to a sudden increase in high-intensity training as well as an increased need to catabolize alternate energy sources and preserve glycogen, as shown previously (17).

Increased training volumes, combined with insufficient recovery strategies, increases the risk of non-functional overreaching (2, 3). A decrease in the testosterone:cortisol ratio of 30% has been suggested as an indicator of poor recovery (45, 46) and a catabolic status (4, 5), while a value of 0.35×10^{-3} has been suggested as a threshold of overtraining (46). In the present study 50% of the participants increased their free testosterone:cortisol ratio during the intervention period, while 50% had a reduced free testosterone:cortisol ratio, including two athletes who had a decrease of > 30%. In the exploratory subanalysis where we looked at the five athletes with the highest increase and five athletes with the greatest decrease from pre- to post-test values of the fT:cor ratio, the changes in total and free testosterone were quantitatively similar, and the ratio was not affected by changes in SHBG to a major degree. Of particular interest, we also found a greater improvement in functional threshold power in those with the highest fT:cor ratio increase compared with those with the largest decrease. Due to a combined increase in free testosterone and decrease in cortisol levels and maintained RMR, this indicates a highly improved anabolic state from pre- to post-test in our study. Although no differences were found in the changes in energy intake between the groups, a > 4% reduction in RMR indicates low energy availability in the participants with the largest decrease the in fT:cor ratio (19). Interestingly, none of the participants in this group showed any signs of preexisting low energy availability on endocrine markers, body composition, BMD, or markers related to RMR; hence, the changes in RMR could potentially be linked to inadequate recovery in this group. Unfortunately, no information regarding heredity of low BMD, history of earlier eating disorder behavior, or what type of training the athletes did before starting being active within cycling were available. Furthermore, when examining the training diaries from before the intervention with the diaries from during the intervention, we found no differences between subgroups. Unfortunately, we do not know the exact distribution of their low- and high-intensity training before the intervention, due to low compliance regarding intensity distribution.

IGF-1 is a pro-insulin-like structure with broad anabolic properties, and low levels are linked to starvation and chronic undernutrition (47). Insulin is a metabolic hormone involved in energy balance, and insulin secretion is correlated with visceral fat in humans, and particularly in males (48). In an eight-week military-exercise study with extreme starvation, Friedl et al. (41) reported a 50% reduction in both IGF-1 and insulin, suggesting improved insulin sensitivity, with a normalization of IGF-1 after a refeeding period halfway through the intervention; however, IGF-1 returned to its declining trajectory when energy again was restricted. Research by Koehler and co-workers (16) showed no reduction in IGF-1, while a decrease in insulin of 36% was observed during short periods of severe low energy availability. These results are similar to the findings in the present study, although we found a non-significant decrease in insulin of 11%. It is possible that the athletes' energy deficiency was not large enough to initiate significant changes, or that the athletes in our study were able to refeed and recover in the week between the last exercise bout and testing.

We did, however, observe a reduction in T_3, an important hormone for growth, reproduction, and metabolism (13), and a suggested surrogate marker of low energy availability, widely associated with suppressed RMR (49, 50). However, in the study by Koehler et al. (16) where they exposed males to very low energy availability, they found no reduction in T_3, and therefore questioned whether exercising men are more robust to short bouts of low energy availability compared with sedentary and exercising women. A reduction in T_3 among soldiers experiencing prolonged starvation has been reported (41), and a recent study reported lower T_3 levels in males with testosterone levels within the lowest quartile of the reference range compared with males with testosterone levels above this threshold (51).

In the present study, the intensified endurance training protocol could potentially have induced the increase in testosterone levels, while subclinical low energy availability could have induced the lowered RMR and T_3 and increased cortisol levels. Although indications of low energy availability with an increased catabolic state and a less positive response of the training intervention were found in a subgroup of participants, it is possible that the intensified mesocycle superimposed on their habitual training load, was not strenuous enough to induce widespread hormonal changes. Other reasons could be that the participants' overall energy deficit was not large enough to induce the severe endocrine changes associated with clinical low energy availability in males earlier reported (16, 41). Unfortunately, we were not able to obtain a thoroughly detailed training load from the participants' habitual training pattern, or energy availability, since the details of the participants' training diaries were of inadequate quality to distinguish between high-intensity and low-intensity training.

Limitations

To minimize limitations in this study, we used strict best-practice protocols developed for RMR and body composition assessments, including urine specific gravity tests to secure reliable results for comparison (26, 52, 53). Furthermore, we had an appropriate number of participants to gain sufficient statistical power, and we used a 4-day consecutive dietary record period mirroring participants typical food patterns, including weighed dietary records to assess energy intake. Although most assessments in the present study were performed in a controlled laboratory-based setting, some limitations must still be acknowledged. First, the sample was classified as having some convenience sample characteristics. Second, we acknowledge that RED-S is a complex field of research, and the results presented in this study should be interpreted with care, given that: (1) various individual responses to intensive exercise occurs, (2) the cyclists were not matched according to training/hormonal status, (3) the study design did not include measurement of energy intake, exercise energy expenditure, and changes in RMR during the intervention period, only pre- and posttest, (4) we experienced low compliance regarding the details of training diaries, making it difficult to assess total accumulated high- and low-intensity training prior to and during the intervention, and

(5) we had no control group. We acknowledge that the lack of a control group makes it difficult to conclude with certainty that the changes are due to the intervention. We therefore also chose a more exploratory approach, by investigating the participants with the highest increase/decrease in fT:cor ratio, aiming at generating new hypotheses. Regarding the analysis of testosterone, it should be emphasized that this analysis has inherent and until now unsolved problems, as discussed earlier. Finally, we also acknowledge that the participants in this study were free-living, well-trained athletes, not elite athletes. This makes them prone to stresses outside of our control, including those associated with obligations to family and friends, work and study loads, as well as lifestyle factors that may have influenced their training load.

NOVELTY STATEMENT

Periods with increased training loads are common as part of an attempt to increase aerobic performance. Today, only a few studies have examined how various intensified endurance training regimens expose male athletes to the risk of RED-S, and this study contributes to new knowledge on a group of athletes not previously investigated; it also uses blood markers, as called for in recent studies (19, 20). The present study demonstrated that 4 weeks of high-intensity endurance training superimposed on their regular training increased athletes' aerobic performance and testosterone levels. However, adverse changes in markers related to low energy availability, such as a reduction in RMR and T_3, and an increase in cortisol were observed. It is, however, unclear whether these changes resulted from a lack of increase in energy intake *per se,* if the length of the intervention period was too short to identify more severe clinical changes in markers of low energy availability, or a combination of both. It is worrying, that negative changes in RED-S-related parameters were observed after only 4 weeks of intensified endurance training, and our findings substantiate the importance of further understanding and monitoring RED-S in male athletes undertaking intensified endurance training regimens, as well as increased awareness and education among athletes and coaches.

PRACTICAL IMPLICATIONS

The present study indicates that well-trained male athletes seem to underestimate the importance of matching their energy intake when undertaking a mesocycle of intensified endurance training. This is challenging, and practitioners should be aware that male athletes are also prone to develop indications of RED-S even during a short intensified 4-week endurance mesocycle. Investigating and understanding RED-S, especially in male athletes, is a complex and difficult task. Several markers exist to help researchers and practitioners to interpret energy availability among athletes. The use of blood markers as one of several measures should be included in future research to better understand how males respond to various levels of endurance exercise regimens in combination with assessing their RMR and energy availability. Furthermore, to prevent RED-S-associated conditions in athletes, established and available tools, such as the RED-S clinical assessment tool (RED-S CAT), may be of value for practitioners and health personnel (8, 54).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University Faculty Ethics Committee and the Norwegian Centre for Research Data (No. 46706). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The study was conceptualized and designed by TS, MT, and AM. Data were collected and analyzed by TS. Contribution were made to materials analysis by TS, MT, JF, and AM. Visualization was performed by TS. Writing of the original draft was performed by TS. Reviewing and editing were done by TS, MT, JF, and AM. All authors approved the final version of the paper.

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Performance and Health Decrements Associated With Relative Energy Deficiency in Sport for Division I Women Athletes During a Collegiate Cross-Country Season: A Case Series

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The purpose of this case series was to evaluate the presence of low Energy Availability (EA) and its impact on components of Relative Energy Deficiency in Sport (RED-S) in a population of female collegiate runners. Seven female NCAA Division I athletes (age: 22.3 ± 1.5 yrs; height: 169.7 ± 5.7 cm; weight: 58.3 ± 4.1 kg) were tracked from August until February, covering the beginning (Pre XC), end (Post XC) of their competitive cross country season, and beginning of the following track season (Pre Track). The athletes were assessed for female athlete triad (Triad) risk, energy availability, body composition, resting metabolic rate (RMR), nutritional intake, and blood markers (including vitamin D, ferritin, and triiodothyronine (T3)). From Pre XC to Post XC there were no significant differences in body mass, fat free mass or body fat percentage. At Pre XC, mean EA was 31.6 ± 13.3 kcal/kg FFM·d^{-1}. From Post XC to Pre Track, there was a significant increase in body mass (59.1 ± 5.1 to 60.6 ± 5.7 kg, p<0.001,d=0.27). From Post XC to Pre Track, there was a significant increase in RMR (1466 ± 123.6 to 1614.6 ± 89.1 kcal·d^{-1}, p<0.001, d=2.6). For 25(OH) vitamin D, there was a significant reduction from Pre XC to Post XC (44.1 ± 10.6 *vs* 39.5 ± 12.2 ng·mL^{-1}, p=0.047,d=-0.4), and a significant increase from Post XC to Pre Track (39.5 ± 12.2 *vs.* 48.1 ± 10.4 ng·mL^{-1}, p=0.014,d=0.75). For ferritin, there was a trend towards a decrease from Pre XC to Post XC (24.2 ± 13.2 *vs.* 15.7 ± 8.8 ng·mL^{-1}, p=0.07, d=-0.75), as well as a trend toward an increase from Post XC to Pre Track (15.7 ± 8.8 *vs.* 34.1 ± 18.0 ng·mL^{-1}, p=0.08, d=1.3). No differences in T3 were observed across time points. Average Triad risk score was 2.3 ± 1.4. Notably, 5 of 7 athletes met criteria for moderate risk. Despite many athletes meeting criteria for low EA

and having elevated Triad risk assessment scores, most were able to maintain body mass and RMR. One athlete suffered severe performance decline and a reduced RMR. Surprisingly, she was the only athlete above the recommended value for ferritin. Following increased nutritional intake and reduced training volume, her performance and RMR recovered. Changes in body mass and body composition were not indicative of the presence of other concerns associated with RED-S. This exploratory work serves as a guide for future, larger studies for tracking athletes, using RMR and nutritional biomarkers to assess RED-S.

Keywords: relative energy deficiency in sport, female athlete triad, body composition, endurance athletes, resting metabolic rate, iron, vitamin D

INTRODUCTION

The consequences of low energy availability (EA) in female endurance athletes have now been known for decades, which initially included menstrual dysfunction and a reduction in bone mineral density (BMD). The interrelationship of these factors became known as the Female Athlete Triad (Triad) with the American College of Sports Medicine (ACSM) publishing position stands in 1997 (1) and 2007 (2) to help clinicians recognize, treat, and prevent these clinical conditions. More recently, a new term, Relative Energy Deficiency in Sport (RED-S) was proposed (3, 4) as an extension of Triad, with the intent to additionally include male athletes, as well as many other aspects of impaired physiological function, including metabolic rate, immunity, protein synthesis, and cardiovascular health (4). Further, it was specifically stated that this clinical phenomenon of low EA is not only a 'triad' of three entities but a syndrome that may influence multiple aspects of health and performance (4).

EA is defined as energy intake (kcal/day) minus exercise energy expenditure (kcal/day), normalized to fat free mass (kg), thereby representing the energy available to support basic physiologic function outside exercise. Low EA has been defined as below 30 kcal·kg^{-1} of fat free mass per day after it was demonstrated that under this threshold, the negative health outcomes began to emerge (5) and is more common in athletes participating in endurance sports, such as running (6). Some suggest that 45 kcal·kg^{-1} of fat free mass per day may be optimal as energy intake for the expenditure expected in athletes (2, 7).

While less well studied, low EA may result in reductions of resting metabolic rate (RMR), and has been noted in both the RED-S (4) and Triad literature (7). In order to assess whether RMR has been reduced in anorexic women, studies have compared measured RMR to predicted RMR (RMRratio) utilizing established prediction equations, and have demonstrated ratios as low as 0.60-0.84 reported (8, 9). This practice was extended to exercising women, and those with a high drive for thinness demonstrated a RMRratio of 0.85, significantly lower than that of exercising women with a normal drive for thinness of 0.9 (10). Thus, it has now been suggested that a ratio less than 0.9 be used as a marker for low EA (7). This reduction in RMR is thought to be a reflection of adaptations that act as an energy-conserving mechanism, where the effects are translated to changes in metabolic rate (10).

One factor tied to metabolic rate in anorexic populations is triiodothyronine (T3), with changes in T3 showing associations with changes in RMR. This was demonstrated both by significantly lower T3 and resting energy expenditure (REE) when comparing underweight individuals with anorexia to normal weight women, as well as concomitant increases in T3 and REE as women with anorexia were treated and gained body mass (11). In exercising women with a high drive for thinness, significantly lower RMRratios were seen in conjunction with a reduced T3 when compared to exercising women without a high drive for thinness, as determined by the Eating Disorder Inventory (12). Thus, T3, in addition to RMR and RMRratio should be explored as a means of assessing adaptation to chronic energy deficiency (7). Collectively, these markers can be used to screen athletes to identify those who may be at risk for low EA or RED-S.

If low EA is occurring as a result of low total energy intake, naturally there may be concerns about meeting macronutrient and micronutrient recommendations. To provide adequate fuel for intense aerobic exercise, high quantities of carbohydrate intake are typically encouraged, with the International Olympic Committee recommending 6-10 g·kg^{-1}·d^{-1} for moderate to high intensity exercise of 1-3 h·d^{-1} (13). In addition, to support recovery from exercise, adequate protein intake is also advised, with recommendations from ACSM ranging from 1.2-2.0 g·kg^{-1} ·d^{-1} (14). ACSM also advocates maintaining an intake of fat of at least 20 percent of overall intake, to meet the lower end of each macronutrient recommendation. As such, a 60-kg athlete would need to consume a minimum of 2,100 kcal·d^{-1}. Additionally, in terms of micronutrients, impairment in iron status can reduce performance at serum ferritin concentrations of less than 25 ug·L^{-1} (15), which is frequent in endurance athletes (16). Low vitamin D is also a common concern in athletic populations, and is of particular importance in athletes with potential bone health issues due to its role in calcium regulation (17). In addition to bone, previous reports have shown detrimental effects on muscle function with 25(OH) vitamin D levels of less than 30 nmol·L^{-1} (12 ng·mL^{-1}) (18). Ultimately, if overall energy intake is low, the consequences of low total calories, essential nutrients, and

supporting macronutrient and micronutrient intake may lead to compromised fuel availability, recovery, oxygen transport and bone health, in addition to other concerns associated with low EA.

To date, limited prospective studies have been conducted in exercising women to define characteristics of low EA and changes to markers of metabolism and nutrition. Studies of performance in the setting of low-EA are also limited. Therefore, the purpose of this study was to evaluate the presence of low EA in a population of female collegiate runners with the consideration of each individual athlete in a case series approach. We hypothesized that components of RED-S would be observed in athletes with low EA, including markers of malnutrition (reduced ferritin, vitamin D status), suppressed RMR and observed reduced performance.

MATERIALS AND METHODS

An entire cross country team of seven female NCAA Division I distance runners (age: 22.3 ± 1.5 years; height: 169.7 ± 5.7 cm; weight: 58.3 ± 4.1 kg) were studied from August until February, covering the beginning (Pre XC) and end (Post XC) of their competitive cross country season, as well as the beginning of the subsequent track season (Pre Track). The athletes were assessed for a variety of factors that pertain to RED-S, including their Triad risk, metabolic rate, body composition, nutritional intake and blood markers (**Figure 1**). All athletes were fully informed of the protocol design and associated risks of this investigation before signing an informed consent document approved by Jacksonville University Institutional Review Board for use of human subjects.

RED-S CAT

Athletes were scored according to the RED-S Clinical Assessment tool (19). Briefly, 'red' corresponds to an athlete who is high risk due to the presence of an eating disorder or other serious medical condition related to low EA. 'Green' is low risk, with appropriate physique and healthy eating habits. 'Yellow' is between the other two levels, characterized by factors such as abnormally low body fat, prolonged low EA, amenorrhea, menarche after age 15 years, low bone mineral density as well as others. These are athletes who may participate in exercise, but would benefit from monitoring.

Triad Risk

A menstrual history questionnaire was used to formulate each individual athlete's Triad Cumulative Risk Assessment score, which was interpreted as follows: 0-1 points = low risk; 2-5 points = moderate risk; ≥6 points = high risk (7). This assessment took place at the Pre XC time point.

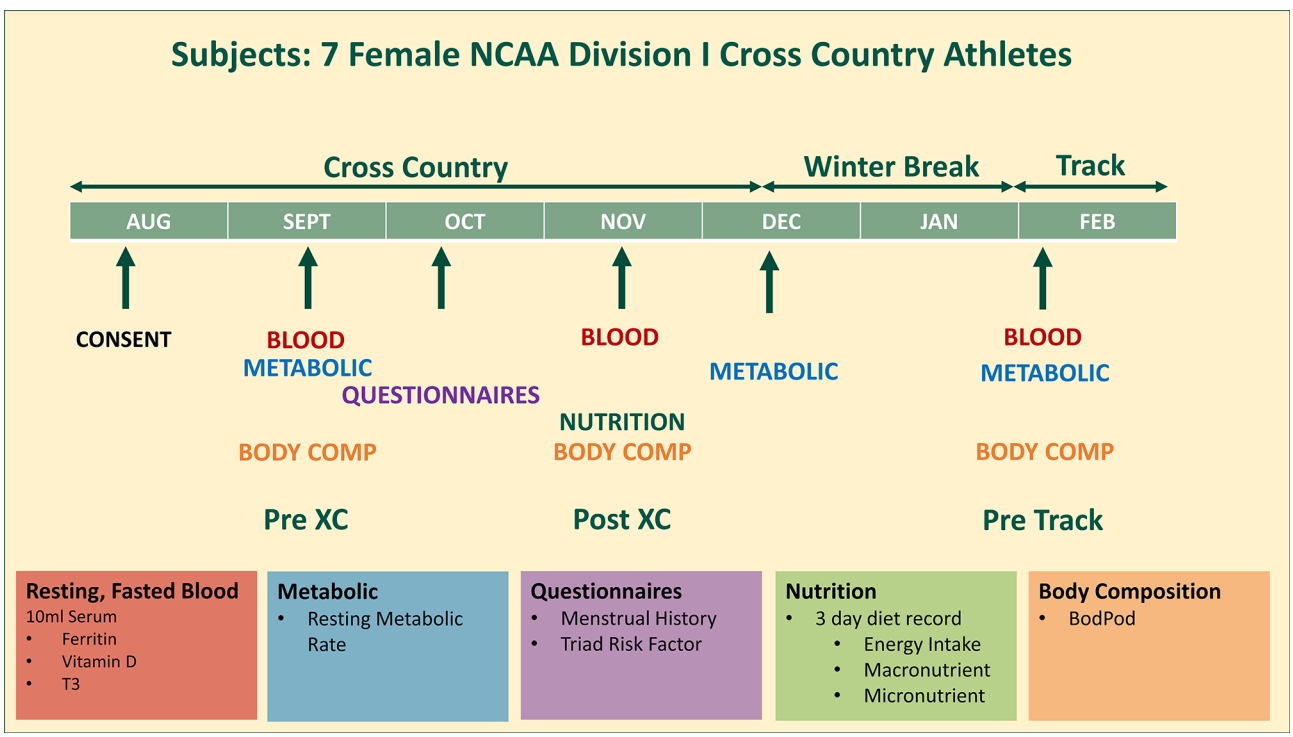

FIGURE 1 | Study design. 7 female NCAA Division 1 collegiate cross country and track and field athletes complete testing before (Pre XC) and after (Post XC) cross country season, and before track (Pre Track) season.

Resting Metabolic Rate

Athletes arrived at the Exercise Physiology Laboratory following an overnight fast and free from any strenuous physical activity in the last 24 hours. RMR was measured *via* indirect calorimetry using a Parvo TrueOne 2400 (Parvo Medics, Sandy, UT) metabolic cart. Athletes were supine with their heads covered fully with a canopy. Each testing session lasted 30 minutes, with the first 10 minutes of data removed from analyses and an average of the final 20 minutes of data collection used to produce a RMR. Further, any single minute average where the fraction of end tidal carbon dioxide ($FECO_2$) concentration was not between 0.8 and 1.2 was removed from analyses. This measured RMR was compared to the predicted RMR as a means of assessing whether RMR was within the expected range (RMRratio) using the Harris-Benedict equation (20). Relative RMR, expressed as RMR per total body mass, was also calculated.

Body Composition

Body composition, including fat mass (FM) and fat-free mass (FFM) was assessed by whole body densitometry using air displacement *via* the Bod Pod® (Life Measurements, Concord, CA) in accordance with the manufacturer's instructions. During testing, athletes wore only tight fitting clothing (e.g., swimsuit, single-layer compression shorts, or undergarments) and an acrylic swim cap. Body composition assessments took place at Pre XC, Post XC and Pre Track.

Nutritional Intake

Nutrition intake was assessed by a 3-day diet record, with the athletes logging all food, drink, and supplement intake over the course of 2 weekdays and 1 weekend day at Pre XC. Subjects were encouraged to complete the logs during the day after each intake. The team dietitian reviewed the 3-day diet recall and met with team members individually to discuss their current intake from food and beverages vs. estimated energy needs. To estimate energy requirements to establish appropriate energy availability, the dietitian used calculated RMR plus an activity factor (based on student athlete reported activity) plus estimation of energy expended for training. If energy availability was deemed suboptimal (less than 45 $kcal \cdot kg^{-1}$ of fat free mass per day), the dietitian made recommendations that would allow the student athlete to achieve adequate EA. The nutrition logs were analyzed and reviewed by a registered dietitian using Nutritionist Pro software (Axxya Systems, Redmond, WA).

Blood Markers

Athletes arrived at the Exercise Physiology Laboratory following an overnight fast. Blood was drawn from an antecubital vein by a trained phlebotomist into a serum vacutainer (10mL). The serum was then separated by centrifuge at 1500×g for 15 min and subsequently stored at -80 °C until it was analyzed in batch. Samples were thawed once only and analyzed in duplicate by enzyme-linked immunoassay (ELISA) (CALBiotech, Spring Valley, CA) for ferritin, 25(OH) vitamin D and T3, with sensitivities of 2.5 $ng \cdot mL^{-1}$, 2.5 $ng \cdot mL^{-1}$ and 0.05 $ng \cdot mL^{-1}$, respectively. All inter-plate and intra-plate coefficients of variance were under 10%.

Statistical Analyses

In this case series, individual athlete data point changes were assessed by visual inspection and in the case of blood variables, compared to known reference ranges. All group data were assessed for normal distribution utilizing the Shapiro-Wilk method and all data were revealed to be normally distributed. Therefore, for each dependent variable, a repeated measures ANOVA was performed to assess changes in each of the dependent variables across time points (Pre XC, Post XC, Pre Track). When a significant ANOVA was reported, post-hoc comparisons were made using dependent t-tests with a Bonferroni correction factor applied to multiple comparisons. Missing data points were replaced with the mean value at the corresponding time point. Less than 10% of the data was replaced. For blood, if the measured value was below the detectable range for that particular assay, the lowest value within the detectable range was used. Statistical significance in this investigation was set at $p \leq 0.05$. To determine the magnitude of change, a Cohen's d effect size was performed. The criteria used to interpret the magnitude of the effect size were 0.2 small, 0.5 medium, and 0.8 large (21). All data were analyzed using Statistical Package for the Social Sciences (version 25.0, IBM, Chicago, IL).

RESULTS

RED-S CAT

The RED-S CAT classification for each athlete is shown in **Table 1**. Three athletes were categorized as 'Green' and 4 athletes were categorized as 'Yellow'.

Triad Risk

The Triad risk score and classification for each individual athlete, along with her respective EA is shown in **Table 1**. Average Triad risk score was 2.3 ± 1.4. Notably, 5 of 7 athletes met criteria for

TABLE 1 | Individual athlete RED-S Clinical Assessment Tool risk, menstrual status, Triad risk, Triad risk classification and energy availability of 7 NCAA Division 1 cross country and track and field athletes assessed prior to cross country season.

Athlete	RED-S CAT	Menses (In Past 12 Months)	Triad Risk	Triad Risk Classification	Energy Availability (kcal/kg FFM·d^{-1})
1	Green	≥ 12	0	Low Risk	Unable to obtain
2	Yellow	≥ 12	2	Moderate Risk	24.4
3	Yellow	9-11	3	Moderate Risk	25.5
4	Green	≥ 12	1	Low Risk	55.9
5	Green	6-8	3	Moderate Risk	29.4
6	Yellow	6-8	4	Moderate Risk	18.2
7	Yellow	0-2	3	Moderate Risk	36.3

Risk scores and classifications based on prior research (7, 19).

moderate risk. We were unable to obtain EA for 1 athlete. Four of the 6 athletes with known EA were below 30 kcal/kg-FFM/day.

Resting Metabolic Rate

There were no significant differences in RMR from Pre XC to Post XC (Pre XC: 1410.4 ± 66.1 *vs.* Post XC: 1466.0 ± 123.6 kcal·d^{-1}, p=0.248, d=0.56). There was a significant increase in RMR from Post XC to Pre Track (Post XC: 1466.0 ± 123.6 *vs.* Pre Track: 1614.6 ± 89.1 kcal·d^{-1}, p<0.001, d=2.6) (**Figure 2**). There were no significant differences in RMRratio at any time point. Only one athlete was below the threshold of 0.9 at Post XC (**Figure 3**). There were no significant differences in relative RMR from Pre XC to Post XC (Pre XC: 24.3 ± 1.8 *vs.* Post XC: 24.9 ± 2.7 kcal·d^{-1}·kg BM^{-1}, p=0.39, d=0.27). There was a significant increase in relative RMR from Pre XC to Pre Track (Pre XC: 24.3 ± 1.8 *vs.* Pre Track: 26.8 ± 2.6 kcal·d^{-1}-kg BM^{-1}, p<0.001, d=1.13)

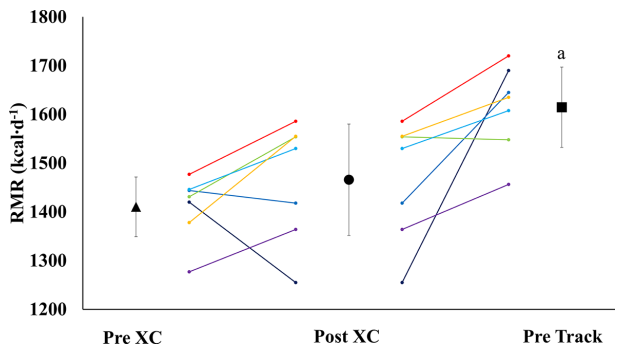

FIGURE 2 | Resting Metabolic Rate (RMR) (mean ± 95% CI) of 7 female NCAA Division 1 collegiate cross country and track and field athletes before (Pre XC) and after (Post XC) cross country season, and before track (Pre Track) season. a= statistically significantly (p<0.05) different from Pre XC.

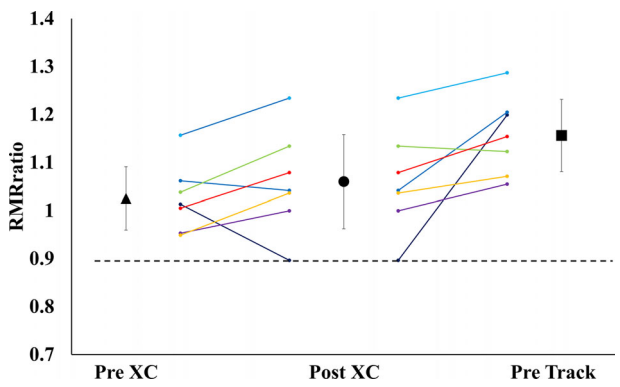

FIGURE 3 | Measured Resting Metabolic Rate to Predicted Resting Metabolic Rate Ratio (RMRratio) (mean ± 95% CI) of 7 female NCAA Division 1 collegiate cross country and track and field athletes before (Pre XC) and after (Post XC) cross country season, and before track (Pre Track) season.

Body Composition

There were no significant differences in body mass, fat free mass or body fat percentage from Pre XC to Post XC. There was a significant increase in body mass from Post XC to Pre Track (Post XC: 59.1 ± 5.1 *vs.* Pre Track 60.6 ± 5.7 kg, p<0.001, d=0.27) (**Figure 4**). There were no other significant differences in fat free mass or body fat percentage from Post XC to Pre Track.

Nutritional Intake

Descriptive data pertaining to nutritional intake are displayed in **Table 2**. Athletes were below recommended carbohydrate intake, but were within protein recommendation guidelines.

Blood

There was a trend towards a decrease in ferritin from Pre XC to Post XC (Pre XC: 24.2 ± 13.2 *vs.* Post XC: 15.7 ± 8.8 ng·mL^{-1}, p=0.07, d=-0.75), as well as a trend towards increased ferritin from Post XC to Pre Track (Post XC: 15.7 ± 8.8 *vs.* Pre Track: 34.1 ± 18.0 ng·mL^{-1}, p=0.08, d=1.3) (**Figure 5A**). There was a significant reduction in 25(OH) vitamin D from Pre XC to Post XC (Pre XC: 44.1 ± 10.6 *vs.* Post XC: 39.5 ± 12.2 ng·mL^{-1}, p=0.047, d=-0.4). There was also a significant increase in 25(OH) vitamin D from Post XC to Pre Track (Post XC: 39.5 ± 12.2 *vs.* Pre Track 48.1 ± 10.4 ng·mL^{-1}, p=0.014, d=0.75) (**Figure 5C**). There were no significant differences in T3 concentration at any time point.

Performance

Performance level for each athlete is expressed as a percentage of her corresponding lifetime best performance in that particular event. These data are shown in **Table 3**.

DISCUSSION

Overview

The purpose of our investigation was to prospectively evaluate a team of seven collegiate cross-country runners, an endurance sport with elevated risk for low EA (22), and measure prospective changes in health and performance. Despite many athletes meeting criteria for low EA and having elevated Triad risk

TABLE 2 | Nutritional intake of 7 NCAA Division 7 NCAA Division 1 cross country and track and field athletes assessed during competitive cross country season.

Variable	Mean (95% CI)	ACSM Recommendations
Kcal	2146 (1756–2535)	
EA (kcal-kg FFM-d^{-1})	32 (21–42)	>30 kcal/kg FFM/d Aim for 45 kcal/kg FFM/d
CHO (g)	202 (143–262)	
CHO (g·kg BM^{-1})	4.6 (3.5-5.6)	6-10 g/kg/d
Protein (g)	87 (74–100)	
Protein (g·kg BM^{-1})	1.5 (1.3-1.7)	1.2-2.0 g/kg/d
Fat (g)	50 (42–59)	
Fat (%)	13 (11–17)	20-35% total intake

Recommendations provided by American College of Sports Medicine (14).

TABLE 3 | Performance level of each individual athlete during cross country and subsequent track seasons expressed as each athlete's best performance of the corresponding season compared to their personal best time.

Athlete	XC Event	XC SR % of PR	Track Event	SR % of PR
1	5k	87.2	800	98.1
2	5k	105	800	100
3	5k	97.9	3000s	99.7
4	5k	96.6	1500	98.8
5	5k	N/A	N/A	N/A
6	5k	94.9	1500	N/A
7	5k	N/A	10k	N/A

XC, Cross Country; SR, Season Record; PR, Personal Record.

assessment scores, most were able to participate and maintain body mass and RMR throughout the season. Changes in body mass and body composition were not indicative of the presence of other concerns associated with RED-S, such as the observed reduced ferritin and 25(OH) vitamin D concentrations in-season. While both ferritin and 25(OH) vitamin D became reduced (trend) over the cross-country season, these values did appear to increase by the beginning of the following competitive season. These findings suggest that changes observed with low EA may be complex and not possible to evaluate with a single biomarker, and are likely highly individualistic.

Case Observations

Performance Decline

The athlete with the worst in-season performance (Athlete 1, **Table 3**) showed severe reductions in performance during the cross country season, with her season record (SR) performance more than 2.5 minutes slower than her personal record in the 5km event, representing a 12% reduction in performance (**Table 3**). There was no obvious explanation for the notable decline in performance, however, the Head Coach decided to remove the athlete from cross country training and competition and she immediately began training for her track event, the 800m. This resulted in a substantial reduction in training volume. Following a meeting with the team dietitian, the athlete was also encouraged to increase caloric intake. All other athletes were within 5.1% of their respective personal records for the 5k event during the cross country season (**Table 3**).

Reduction in RMR

A proposed indicator of low EA is a reduction in RMR, which can be expressed as a ratio of measured RMR to predicted RMR (RMRratio); DeSouza et al. recommend a threshold of 0.9 as a marker for low EA (7). In the current study, the absolute RMR and RMRratio followed the same pattern as body mass from Pre XC to Post XC (i.e., no significant changes) for the group as a whole; although, Athlete 1 did demonstrate a RMRratio below 0.9 at Post XC (**Figure 3**). The removal of Athlete 1 from cross country training and competition, as previously mentioned, led to a drastic reduction in overall energy expenditure, combined with the recommendation from the team dietitian to increase energy intake, substantially increasing EA. Following the off-season, the athlete increased absolute RMR from 1255 to 1690 kcal·d^{-1} and increased RMRratio from 0.89 to 1.19. This athlete also had a much more successful track season compared to cross country, with a season-best within 3 seconds (2%) of her personal record (**Table 3**). While a case observation, these results show the potential ability of a reduced RMR to detect concerns related to RED-S, with a training and nutritional intervention affecting performance.

Blood Markers

Over the course of 9 weeks from Pre XC to Post XC, body mass and body composition were maintained (**Figure 4**), despite 4 of the 7 athletes at Pre XC demonstrating EA under the threshold of 30 kcal·kg^{-1} of fat free mass (**Table 1**) (2). Nonetheless, there were other concerns for these athletes related to their bone health and

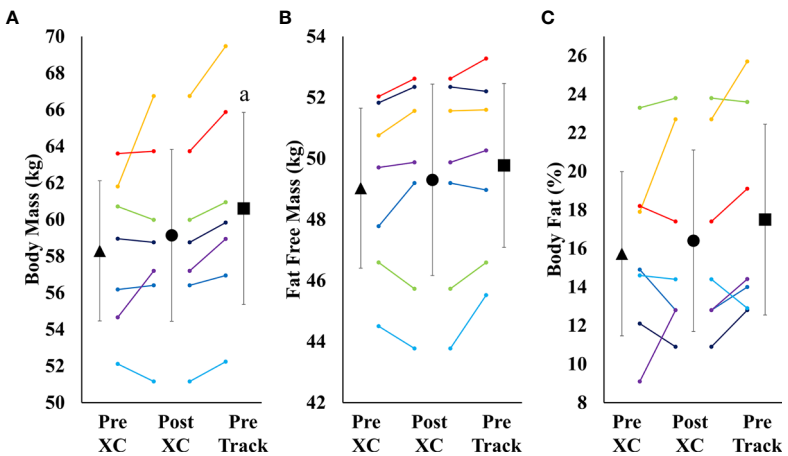

FIGURE 4 | Body Mass **(A)**, Fat Free Mass **(B)** and Body Fat Percentage **(C)** (mean ± 95% CI) of 7 female NCAA Division 1 collegiate cross country and track and field athletes before (Pre XC) and after (Post XC) cross country season, and before track (Pre Track) season. a= statistically significantly ($p<0.05$) different from corresponding Pre XC.

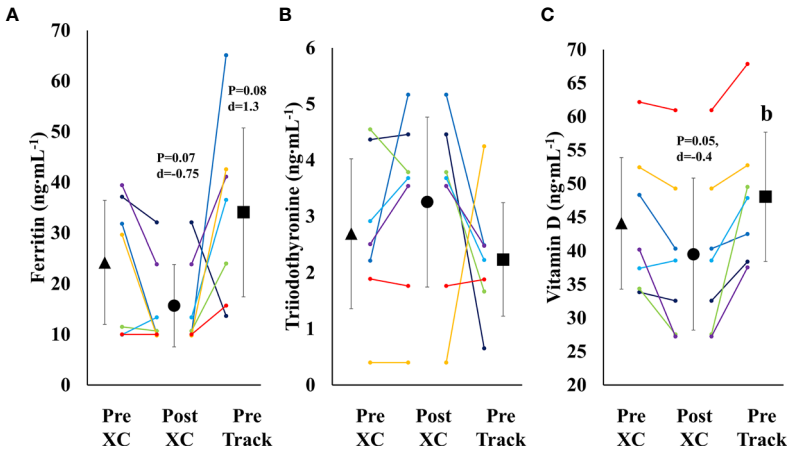

FIGURE 5 | Serum Ferritin **(A)**, Triiodothyronine **(B)** and Vitamin D **(C)** (mean ± 95% CI) of 7 female NCAA Division 1 collegiate cross country and track and field athletes before (Pre XC) and after (Post XC) cross country season, and before track (Pre Track) season. b= statistically significantly ($p<0.05$) different from corresponding Post XC.

performance levels with respect to their blood markers. On average, there were significant reductions in 25(OH) vitamin D concentrations across time. This reduction from Pre XC to Post XC may have been a consequence of the subjects spending less time outdoors during the late fall into the winter months, although this would not explain why the vitamin D concentrations rebounded from Post XC to Pre Track, the time period corresponding to the coldest temperatures and least sun exposure in the region (December-January). A reduction in vitamin D is a significant concern, particularly for athletes susceptible to RED-S, as reduced bone mineral density is an established consequence of low EA (2) and vitamin D plays a critical role in bone health (17).

Ferritin levels were also below target for performance, as only one athlete maintained a ferritin concentration above the threshold of 25 ng·mL^{-1} (15) at Post XC. The group mean for ferritin was well below that threshold (15.7 ng·mL^{-1}). A study of 165 female collegiate rowers found that athletes with a ferritin level above a threshold of 20 ng·mL^{-1} (n=44) had a statistically-significantly improved 2-km rowing time trial performance compared to those below the threshold (n=121) (15). Somewhat surprisingly, the one athlete in our study with a ferritin concentration above the recommended 25 ng·mL^{-1} was Athlete 1, who suffered the substantial performance decline. Ferritin concentration changes in this population were not indicative of changes in performance as has been previously shown, suggesting a complex interaction of multiple influences on performance.

Group Observations

Following the cross country season, the athletes saw statistically significant increases in RMR (**Figure 2**), 25(OH) vitamin D (**Figure 5**), and a trend toward increases in ferritin (**Figure 5**) ($p=0.08$). Although efforts were made to reevaluate EA at this time, unfortunately, it was not possible to obtain adequate dietary records to reflect this period and we cannot objectively illustrate the increase in EA. However, the significant increase in body mass from Post XC to Pre Track (**Figure 4A**) is likely indicative of an increase in EA and a prior systematic review demonstrated female endurance athletes do not typically reduce energy intake in the non-competitive season, despite reducing total energy expenditure (23). Thus, it appears that following the cross country season, the low EA was not as severe and allowed for the increase in body mass. The increase in total body mass could also account for the increase seen in RMR, as both variables significantly increased, as did relative RMR. An increase in EA could be the reason that multiple variables improved between Post XC and Pre Track. It is interesting to note that there was a lot of agreement between the RED-S Clinical Assessment Tool and Triad Risk Classification. In all but 1 case, 'Green' and 'Yellow' in the RED-S Clinical Assessment Tool corresponded to 'Low Risk' and 'Medium Risk' respectively in the Triad Risk Classification (**Table 1**). In the 1 exception, athlete 5 was considered 'Green' in the RED-S Clinical Assessment Tool but 'Moderate Risk' in the Triad Risk Classification. Overall, both scales are extremely user friendly and the use of either scale is highly recommended in practice or in future similar research studies.

Limitations

While every effort was made to maintain scientific rigor, this study is not without limitations. This study used self-reported dietary intake, rather than controlling dietary intake, which could lead to measurement error. In addition, there was no second evaluation of energy availability, where dietary intake could certainly have changed over time. Due to the nature of tracking athletes in their normal environment, testing only occurred when it was convenient to incorporate within their training regimen, thus testing was not standardized to a specific menstrual phase. Finally, while the athletes were encouraged to

arrive to the laboratory hydrated for body composition testing, their hydration was not confirmed by any testing.

CONCLUSIONS

When athletes participate in competitive endurance sports, there are several factors that need to be monitored to help maintain their health and performance, particularly factors associated with RED-S. This study showed changes in body mass and body composition were not indicative of the presence of other concerns associated with RED-S, such as the observed reduced ferritin and 25(OH) vitamin D concentrations in-season, which could be impacted by micronutrient intake and thus should certainly be considered when assessing dietary habits. Therefore, there is a need for ongoing nutrition evaluation, consistent screening for ferritin throughout the competitive season, as well as a need to consider low EA as a possible cause of performance decrement if it occurs. While many studies historically have demonstrated these concerns in a controlled environment, there are few long-term prospective tracking studies in competitive athletes in this area. We propose that future research should explore these findings in larger populations of exercising women, men, and adaptive athletes to provide a new perspective on monitoring of RED-S in athletes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Jacksonville University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. DRH: Study design, data collection, data interpretation, manuscript preparation. JM: Study design, data collection. JTW: Study design, data interpretation, manuscript preparation. KLC: Data collection, data interpretation, manuscript preparation. GGAP: Study design data interpretation, manuscript preparation. KP: Dietary analysis, data collection. CS: Dietary analysis, data interpretation, manuscript preparation. ACH: Data interpretation, manuscript preparation. AST: Study design, data interpretation, manuscript preparation. KEA: Data interpretation, manuscript preparation.

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