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The effects of gradual vs. rapid weight loss on serum concentrations of myokines and body composition in overweight and obese females

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Abstract

Context: Research has shown the modulations of Follistatin (FST) and Myostatin (MST) following weight loss

Objective: We evaluated the effects of gradual weight loss (GWL) and rapid weight loss (RWL) on serum MST, FST concentrations, and body composition in overweight and obese females.

Materials and Methods: Thirty-six overweight and obese females successfully completed the study interventions: GWL (n = 18) or RWL (n= 18). Serum MST and FST concentrations, as well as anthropometric measurements, were collected at baseline and at the conclusion of each weight loss intervention.

Results: MST significantly ($p<0.05$) concentration decreased in the GWL; while FST, body fat percentage and skeletal muscle mass significantly declined in both conditions. The loss in skeletal muscle mass was significantly greater in RWL relative to GWL.

Discussion and Conclusion: GWL was more effective than RWL in preserving skeletal muscle mass in overweight and obese females. Moreover, GWL leads to declines in MST concentrations.

Keywords: body composition, diet, insulin resistance, obesity, weight loss.

Introduction

Excess caloric intake along with increased sedentarism have been reported as the most immediate contributors to the increasing occurrence of obesity in society, as well as the subsequent development of several non-communicable diseases such as metabolic syndrome, type II diabetes mellitus, cardiovascular diseases, and specific types of cancers(Middelbeek & Breda, 2013). Given the importance of obesity-related health conditions, numerous strategies have been implemented aiming to reduce fat mass (FM). Dietary interventions leading to body mass (BM) loss have been suggested as the first stage to alleviate obesity and allied health risk factors (Jakicic et al., 2001). Correspondingly, diverse dietary interventions have been proposed for FM loss(Freire, 2020). Most of the proposed strategies are based off the division of macronutrients (e.g. low-carbohydrate/high-fat, high-carbohydrate/low-fat, or low-carbohydrate/high protein diet) (Floegel & Pischon, 2012; Yancy, Olsen, Guyton, Bakst, & Westman, 2004), manipulation of total energy balance to induce either gradual weight loss (GWL), or rapid weight loss (RWL) (Ashtary-Larky et al., 2018). Even though RWL approaches may be attractive to obese individuals, it has been theorized that GWL may yield finer alterations in body composition and anthropometric variables and be better maintained chronically (Ashtary-Larky et al., 2018). Indeed, prior work (Hill, 2008; Lutes et al., 2008; Sbrocco, Nedegaard, Stone, & Lewis, 1999) has advised a GWL method, contending that GWL may generate superior long-term FM levels compared with RWL, which is unlikely to be maintained. However, others (Astrup & Rössner, 2000; Carels, Cacciapaglia, Douglass, Rydin, & O'Brien, 2003; Elfhag & Rössner, 2005; Nackers, Ross, & Perri, 2010) have pointed out that larger calorie deficits and subsequent RWL are more likely to reinforce the BM-change process and produce superior long-term FM loss results. Accordingly, ambiguity continues concerning the optimal energy intake and rate of weight loss demanded most favorable for obesity management.

Some myokines such as myostatin (MST) and follistatin (FST) have been reported to be affected by both BM and FM alterations in humans (Flanagan et al., 2009; Hittel, Berggren, Shearer, Boyle, & Houmard, 2009; Milan et al., 2004). MST serves as a negative regulator of skeletal muscle mass (SMM) growth and differentiation, while FST acts as the regulator of MST through binding to the active form of MST and inhibiting the binding of MST to the activin IIB receptor(Amthor et al., 2004). Consequently, FST over-expression and MST inhibition is associated with increased SMM (Wagner, Liu, Chang, & Allen, 2005; Wagner, McPherron, Winik, & Lee, 2002) and decreased FM (McPherron & Lee, 1997; Yang et al., 2001). On the contrary, increased MST concentration have been reported to increase FM (Allen, Hittel, & McPherron, 2011; Reisz-Porszasz et al., 2003). FST was found to be more expressed in lean compared with obese females, and weight loss resulted in increased FST in obese females (Flanagan et al., 2009). For instance, some studies have reported MST (Allen, Cleary, Lindsay, Loh, & Reed, 2010; Allen et al., 2008; Gonzalez-Cadavid et al., 1998; Milan et al., 2004; Park, Berggren, Hulver, Houmard, & Hoffman, 2006) and FST (Allen et al., 2008; Flanagan et al., 2009) concentration to be changed following weight loss. Nevertheless, most studies investigated the intracellular expression (Allen et al., 2010; Allen et al., 2008; Gonzalez-Cadavid et al., 1998; Milan et al., 2004; Park et al., 2006) or protein(Allen et al., 2010) in animals (Allen et al., 2010; Allen et al., 2008), and only two studies conducted on obese humans(Milan et al., 2004; Park et al., 2006). Although these previously mentioned studies were conducted on weight loss, fewer studies have examined the alterations of FST and MST concentrations following the rate of weight loss. Only Motevalli et al. (2015) reported a significant increase in MST and a decrease in FST concentration following RWL compared to GWL in competitive wrestlers(Motevalli et al., 2015).

Combined, these studies suggest a relationship between BM and/or FM reductions and the MST signaling pathway. However, little attention has focused on MST and FST following weight loss in sedentary females. Given the effects of MST and FST in regulating SMM and FM, we hypothesized that FST and MST could be affected by the rate of weight loss. Accordingly, as the primary purpose, this paper aimed to evaluate alterations in MST and FST concentrations following different models of FM loss (RWL vs GWL) in overweight and obese females. As a secondary aim, we assessed body composition changes after RWL and GWL.

Materials and Methods

Participants

Thirty-six overweight and obese (body mass index, BMI >25 kg/m^2) female participants who were referred to our nutrition clinic [(Ahvaz, Iran); (age: 35 ± 11 y, weight: 83.7 ± 13.6 kg, BMI: 33.1 ± 6.9 kg/m^2)] successfully completed this study. The study protocol was approved by the Ethics Committee of Jundishapur University of Medical Sciences and conducted in accordance with the Declaration of Helsinki and was registered in Iranian Registry of Clinical Trials (IRCT2016010424699N2). Procedures were explained to participants and written informed consent was obtained before participation. Inclusion criteria were as follows: not to contribute in any physical activity (PA) at least in the last year (self-reported), not to use alcohol or smoke, not to use herbal supplements and vitamins, and a lack of BM changes in the last 6 months (self-reported). Exclusion criteria were non-willingness to continue nutritional protocols, participation in other dietary weight loss procedures, pregnancy, or breastfeeding, use of effective drugs on metabolism, lipid and glycemic profile, eating disorder, diabetes, cardiovascular disease, kidney problems, thyroid, digestive, and respiratory diseases. In addition, participants consuming more than 300 mg of caffeine daily (described as caffeine users (Shirali et al., 2016)) were excluded from the study.

Study design

This double-blinded clinical trial was conducted on overweight and obese female participants. All participants were initially screened for enrolment by phone and social media (Instagram or Telegram) and scheduled for a preliminary visit at nutrition clinic (preliminary visit). The preliminary session included measures of height, weight, waist circumference and questionnaires that confirm their eligibility for the study. Subsequently, Participants were randomly assigned into a GWL (n = 18; 34.4 ± 7.4 y, 83.6 ± 13.5 kg, 44 ± 6.4 body fat percentage [BFP]) or RWL (n= 18; 33.1 ± 11.7 y; 83.7 ± 14.1 kg; 40.8 ± 9.6 BFP) condition. Participants were asked not to change their PA levels throughout the study.

Diet protocol

The methods and design of were previously reported in detail elsewhere (Ashtary-Larky et al., 2017). Briefly, RWL and GWL, based on the lost weight (at least 5 %), were defined over a period of 5 weeks and 15 weeks, respectively. Dietary restrictions were calculated from individual daily energy expenditure requirements. Total energy expenditure was estimated from the Dietary Reference Intake (DRI) for non-obese adult women(Table, 2005). The DRI prediction formula requires an estimate of the level of PA, age, weight, and height. To achieve and maintain the dietary energy restriction, the food exchange system from the Academy of Nutrition and Dietetics and American Diabetes Association was used(Wheeler, Daly, & Evert, 2014). The prescribed calorie-restricted diet contained 15% protein, 30% to 35% fat, and 50% to 55% carbohydrate, on average, in order to provide weight loss. In general, the meal plans included 3 main meals (breakfast, lunch, and dinner) and three snacks (mid-morning, mid-afternoon, and bedtime), and low saturation and trans fats, cholesterol, salt (sodium), and added sugars. All diets were designed according to Dietary Guidelines for Americans, 2010 (Motevalli et al., 2015). Low-calorie diets produced an energy deficit of 500 to 750 for the 15-week duration GWL, and 1000 to 1500 kcal/d for the 5-week duration for the RWL.

Anthropometric measurements

Prior to arriving to the laboratory, participants fasted for 12 hours (overnight, with an aim of 8 hours sleep), refrained from consuming alcohol for 48 hours caffeinated beverages, and other diuretics before assessments. The stature was measured with a stadiometer (Race Industrialization, China) to the nearest 0.1 cm. BM, BMI, BFP, waist-hip ratio (WHR), and SMM were evaluated by a multi-frequency bioelectrical impedance device

(Inbody 230, Biospace, Korea) (Bagheri, Rashidlamir, Motevalli, et al., 2019). The test-retest reliability of the bioelectrical impedance method was high (R = 0.95 to 0.98).

Blood sampling and laboratory analysis

Fasting blood samples (5 mL) were taken from the cubital vein using standard procedures following 12-hour overnight fasting (Bagheri, Rashidlamir, Ashtary-Larky, et al., 2019). The initial collection occurred 48 hours before initiation of dieting. Blood samples were clotted and stored for 20 minutes at room temperature before being centrifuged at 3000 revolutions per minute for 20 minutes. Spun serum was removed from the centrifuge and frozen at -80°C. Serum MST (human MST, Zellbio Gmbh, Germany) and FST (human FST, Zellbio Gmbh, Germany) concentration were measured in duplicate using enzyme-linked immunosorbent assay (ELISA) with a microplate reader (GDV, Germany) at a wavelength of 450 nm. The intra-assay and inter-assay coefficient of variation were less than 10% and 12% for MST and FST, respectively.

Nutrient intake and dietary analysis

Participants in RWL submitted 3-day (2 weekdays and 1 weekend) food records at baseline and after week 3 and 5, while participants in GWL submitted their food records at baseline and after week 3, 6, 9, 12, and 15. Each item of food was individually entered into Diet Analysis Plus version 10 (Cengage, Boston, MA, USA) and total energy consumption, and the amount of energy derived from proteins, fats, and carbohydrates was assessed (Bagheri, Rashidlamir, Motevalli, et al., 2019).

Statistical analysis

An a priori sample size calculation was conducted using the G*Power analysis software (Faul, Erdfelder, Lang, & Buchner, 2007). Our rationale for sample size was based on our prior data (Bagheri et al., 2020) and estimated that 15 participants per condition (30 total participants) would provide 80% power (two-sided $\alpha = 0.05$) to detect significant change in FST and MST concentrations. Shapiro-Wilk test was performed to assess the normality of data. Student's t test was performed for condition comparisons at baseline. A 2 × 2 RM ANOVAs [time (pre-intervention vs. post-intervention) × condition (GWL vs. RWL)] with Bonferroni adjustments was used to determine treatment differences. When appropriate, a one-way ANOVA across change scores was used to detect between-condition differences. Following extra sum-of squares F test, Pearson's correlation was performed between body composition and endocrine markers with data treated as one condition or separated by condition (GWL vs RWL) as suitable. Statistical significance was set at $p < 0.05$. Cohen's *d* effect size (ES) was calculated as post effect mean minus pre effect mean/pooled pre effect standard deviation means(Cohen, 1992). An ES of

0.00-0.19 was considered trivial, 0.20-0.49 = small, 0.50-0.79 = moderate, and ≥ 0.80 = large. All analyses were performed using SPSS (version 25.0, IBM; Chicago, IL).

Results

Of sixty-five participants that assessed for eligibility, forty-one participants underwent evaluation. Twenty participants were assigned to RWL and 21 were assigned to GWL. Of these, 36 participants completed the study (18 participants per condition). During the study, two participants in RWL (inability for maintaining diet) and three in GWL (exercising, pregnancy, or medication) were excluded. No adverse events in the two study conditions were reported. Baseline characteristics of RWL and GWL conditions are presented in Table 1. No significant differences were observed at baseline between two conditions for any variable (Table 1).

Energy and macronutrients consumption

Energy and macronutrients of RWL and GWL are presented in Table 2. A significant main effect of time was observed for kilocalories, protein, fat, and carbohydrate consumption in both conditions ($P<0.05$). Participants in RWL condition consumed significantly less kilocalories, protein, fat, and carbohydrates during week 3 and 5 compared to baseline. Participants in GWL condition consumed significantly less kilocalories, protein, fat, and carbohydrates during week 3, 6, 9, 12, and 15, compared to baseline ($P<0.05$). In addition, participants in GWL condition consumed significantly less kilocalories comparted to week 3, 6, and 9 ($P<0.05$). In addition, participants in GWL condition during week 12 consumed significantly less protein compared to week 3. Moreover, participants in GWL condition during week 12 consumed less carbohydrate compared to week 6 and 9 ($P<0.05$).

Rate of weight loss alters body composition

A significant condition × time interaction was detected for BFP [($p = 0.002$), (Figure 1B)] and SMM [($p < 0.001$), (Figure 1C)]. BFP [GWL = -2.8 % (95% CI= -3.5 to -2.1), (d= 1.9) and RWL = -1.2 % (95% CI, -1.9 to -0.5), (d= 0.9)] and SMM [GWL = -0.4 kg (95% CI= -0.7 to -0.1), (d= 0.7) and RWL = -1.4 kg (95% CI, -1.7 to -1), (d= 2)] significantly decreased in both conditions over time. No condition × time interaction was noted for BM ($p = 0.637$), BMI ($p= 0.403$) or WHR [p= (0.202), (Figure 1A)]. Each variable was noted to be significantly decreased with time BM ($p < 0.001$, $\eta2 = 0.961$), BMI, ($p < 0.001$, $\eta2 = 0.957$), BFP ($p < 0.001$, $\eta2 = 0.679$), WHR ($p < 0.001$, $\eta2 = 0.573$), and SMM ($p < 0.001$, $\eta2 = 0.680$); (Table 1). When SMM was examined as change in (post-pre, ΔSMM), significantly greater loss in SMM was noted in RWL relative to GWL ($p < 0.001$, Figure 1D).

GWL condition alters MST and FST while RWL only alters FST concentration

A significant condition × time interaction was detected for MST [($p = 0.011$), (Figure 1E)], FST [($p = 0.036$), (Figure 1F)], and FST/MST ratio [($p = 0.006$), (Figure 1G)]. MST only decreased in GWL [GWL = -44.4 ng/l

(95% CI= -67.4 to -21.5), (d= 0.9)] while FST [GWL = -3 ng/ml (95% CI= -4.6 to -1.5), (d= 0.3) and RWL= -5 ng/ml (95% CI, -6.2 to -3.9), (d= 2.1)] significantly decreased in both conditions. In addition, FST/MST ratio [RWL= -0.01 (95% CI, -0.02 to -0.006), (d= 0.9)] significantly diminished in RWL.

Change in FST associates with change in BFP independent of weight loss model

Following sum-of-square F-test, weight loss condition was either ignored (Figure 2C and 2D), or considered (Figure 2B) when examining changes in endocrine markers as a function of change in body composition. The alteration in FST (ΔFST) was differentiated between GWL and RWL (p = 0.013), and the change in BFP (ΔBFP) in both conditions was associated with ΔFST (GWL $r^2 = 0.531$, $p = 0.025$; RWL $r^2 = 0.342$, $p = 0.011$; Figure 2B). No other condition was differentiated by sum-of-squares F test, nor considered to be associated by Pearson's correlation when considered as one condition (Figure 2A) $r^2 = 0.023$, $p = 0.382$; Figure 2C), $r^2 = 0.098$, $p = 0.063$; Figure 2D), $r^2 = 0.020$, $p = 0.413$).

Discussion

The major findings were as follows: GWL resulted in a significant decrease in MST concentration, an effect not seen in the RWL group. In addition, both GWL and RWL resulted in a significant decrease in FST concentration. The FST/MST ratio significantly diminished in RWL. In regards to body composition, BFP significantly decreased in both conditions. Moreover, significantly greater loss in SMM was noted in RWL relative to GWL. Collectively, our results suggest a more catabolic environment in the RWL than the GWL condition.

MST is a member of transforming growth factor-beta (TGF-β) family member which inhibits muscle differentiation and growth(Allen et al., 2011). Mice in which MST processing or signaling is disrupted exhibited muscle mass gains (Matsakas et al., 2009; Yang et al., 2001), while MST over-expression resulted in a significant decrements of muscle mass(Reisz-Porszasz et al., 2003). These results confirm MST's critical role in inhibiting muscle mass gains. Follistatin is a member of the TGF-β superfamily (Görgens et al., 2013), which is ubiquitously expressed in all tissues of the human body, including skeletal muscle, and has both paracrine and autocrine influences. FST has shown to bind MST and inhibit its activity(Nakatani, Kokubo, Ohsawa, Sunada, & Tsuchida, 2011), but also can bind and inhibit other TGF-β family members (Tsuchida et al., 2000), suggesting a more diverse physiological role. Obesity is associated with increased MST expression in both adipose and skeletal muscle tissues(Allen et al., 2011), and MST mRNA levels decreased during weight loss following daily injection of recombinant leptin in mice (Allen et al., 2008). Similarly, circulating FST concentration is also elevated in obese individuals relative to normal weight controls (Maïmoun et al., 2020), concentration of which is subsequently reduced following bariatric surgery-induced weight loss (Wiewiora et al., 2020). These results highlight a role of weight loss to reduce MST and FST concentrations.

To date, only one study has been conducted comparing GWL and RWL on serum MST and FST concentrations. Motevalli et al. (2015) examined 8 weeks of weight loss in male competitive wrestlers, serum MST and FST concentrations remained unchanged in the GWL condition while a significant increase in serum MST and a significant decrease in FST concentration was observed in the RWL condition. In their study, serum MST/FST was significantly increased in the RWL condition (Motevalli et al., 2015), suggesting a more catabolic environment was present in the RWL compared to RWL condition, similar to the findings of the results we present herein.

At the end of our study, GWL experienced 711 kcal/d and 13.6 g/d of protein reduction in daily energy intake while RWL experienced a quite severe 1339 kcal/d and 44.2 g/d of protein reduction. Moreover, FST/MST was decreased following RWL, indicating a drop in positive muscle growth leading to their deficiency in the homeostatic balance of the muscle. Considering the losses in SMM observed our RWL participants, ultimately these changes may have led to a greater catabolic condition in RWL compared to GWL. The main mechanism of this catabolic condition could be due the activation of AMP-activated protein kinase (AMPK), which is an intracellular energy sensor and the activation of AMPK occurs when the ratio of ATP/ADP decreases in result of caloric restriction. The activation of AMPK can directly activate Tuberous Sclerosis 2 (TSC2) signaling, which is an antagonist of mTORC1 activation(Laplante & Sabatini, 2009), involved in the regulation of MST signaling(Elliott, Renshaw, Getting, & Mackenzie, 2012). It could be speculated that these mechanisms are responsible, at least in part, for inducing a catabolic intracellular condition witnessed herein; however, more invasive measures such as muscle biopsies would be required to confirm this mechanism.

Participants in GWL gradually reduced calories at a rate of 500-750 kcal/d for 15 weeks, while participants in RWL reduced calories at a rate of 1000-1500 kcal/d for 5 weeks. Both conditions significantly decreased BM, BFP, WHR, and SMM. It was not surprising, however, that SMM was decreased to a greater extent in RWL relative to GWL due to the respective reduction of daily protein intake of 44.2 g/d and 13.6 g/d. In a systematic review and meta-analysis study, we have shown the beneficial effects of GWL compared to RWL on BFP in overweight individuals, which is in line with the outcomes of the current study. However, our review also showed that fat-free mass was not different following both rates of weight loss (Ashtary Larky, Bagheri, Abbasnezhad, & M Tinsley, 2020). Additionally, previous studies showed that RWL diets are suboptimal for lean body mass preservation (Ashtary-Larky et al., 2017; Peos, Norton, Helms, Galpin, & Fournier, 2019; Vink, Roumans, Arkenbosch, Mariman, & van Baak, 2016). It has been shown that lean body mass contributes approximately ranged between 20-30% to total weight loss in individuals with overweight or obesity(Tinsley & Willoughby, 2016). Our BFP and SMM results agree with the study of Motevalli et al. (2015) who observed a significant BFP reduction and lean body mass in both RWL and GWL conditions. In our study, GWL experienced a decrease in BFP by 3.2 % more than RWL, likely due to the longer caloric restriction. Indeed, our results revealed an interesting correlation between the changes in BFP and those of FST concentration in both conditions, with a higher r^2 value (= 0.531) with GWL. These findings indicate that participants with smaller deteriorations in FST were the ones with higher decreases in BFP. Although it is not possible to infer causality from correlation, i.e., whether the smaller decrement of FST leads to a higher decrease in BFP or vice versa, the present findings are consistent with the model proposed by Brown et al. (2012) in which the declines in FST concentration may explain increases in fat mass in mice(Brown et al., 2011). The authors proposed that a deterioration in FST concentration could lead to an impaired glucose homeostasis and adipose tissue accumulation due to its important physiological roles in regulating these processes (Brown et al., 2011).

It is important to mention that our investigation possesses several limitations. First, we did not measure tissue level expression of MST and FST, which may give further insight into some of our results if collected at both muscle and sub-cutaneous adipose sites. However, it has been proposed that increases in circulating concentrations of cell-signaling molecules enhances the likelihood of receptor interaction and consequently improve the probability of a physiological effect within these tissues (Kraemer et al., 1990; Patel & Demontis, 2014). Second, we used bioelectrical impedance to measure body composition, which is more feasible in field-based settings but not as accurate as other options such as DEXA. However, previous studies have shown that it is a valid and reliable method (Jackson, Pollock, Graves, & Mahar, 1988; Ling et al., 2011). Since we evaluated MST and FST, as well as body composition markers following RWL and GWL in overweight female participants, our results may not be considered universal to all population types. Finally, we used self-reported dietary intake (via food record), which has been shown to be vulnerable to social desirability bias with under-reporting of energy intake and over-reporting of fruit and vegetable intake(Schoeller, 1995) .

This study was the first to evaluate the effects of GWL and RWL on serum FST and MST in overweight and obese females. Since both options resulted in matched weight loss, and RWL also resulted in reductions in MST and SMM, we would suggest the RWL condition induced a more catabolic environment for SMM and muscle protein. As a result, we conclude that GWL was more effective than RWL in preserving skeletal muscle mass in overweight and obese females. Future efforts should investigate if these differences in serum MST and FST and differences in SMM loss are maintained in the presence of caloric deficit but maintenance of dietary protein intake.

Conflict of interest

The authors declare no conflicts of interest.

Data availability: Data sharing is applicable.

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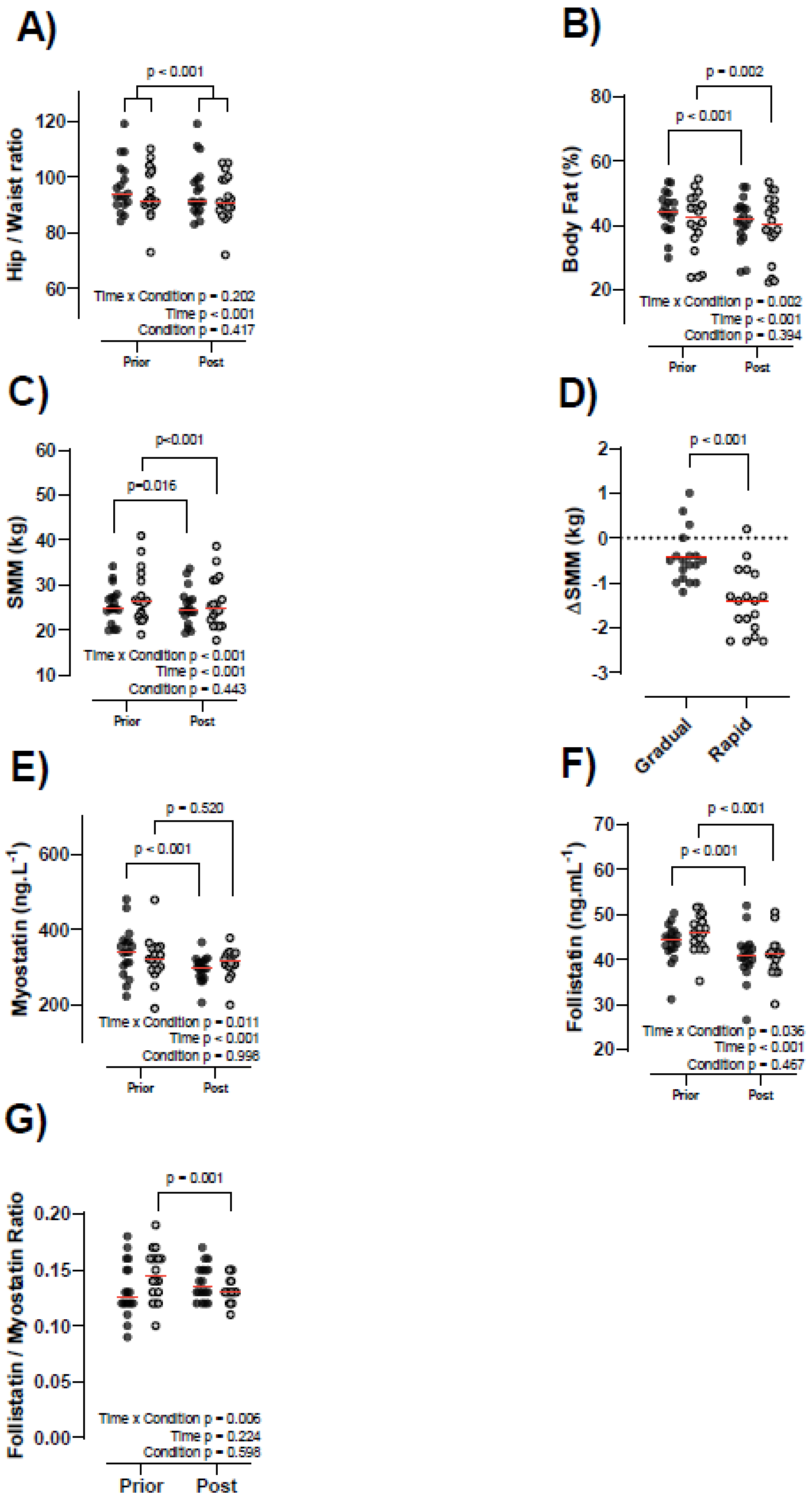

A)
Hip / Waist ratio
p < 0.001
Time x Condition p = 0.202
Time p < 0.001
Condition p = 0.417
Prior
Post
B)
Body Fat (%)
p = 0.002
p < 0.001
Time x Condition p = 0.002
Time p < 0.001
Condition p = 0.394
Prior
Post
C)
SMM (kg)
p<0.001
p=0.016
Time x Condition p < 0.001
Time p < 0.001
Condition p = 0.443
Prior
Post
D)
ΔSMM (kg)
p < 0.001
Gradual
Rapid
E)
Myostatin (ng.L-1)
p = 0.520
p < 0.001
Time x Condition p = 0.011
Time p < 0.001
Condition p = 0.998
Prior
Post
F)
Follistatin (ng.mL-1)
p < 0.001
p < 0.001
Time x Condition p = 0.036
Time p < 0.001
Condition p = 0.467
Prior
Post
G)
Follistatin / Myostatin Ratio
p = 0.001
Time x Condition p = 0.006
Time p = 0.224
Condition p = 0.598
Prior
Post

Figure 1: Alterations in Body Composition and endocrine markers with dietary condition. **A)** Hip / Waist ratio, **B)** BFP (%), **C)** SMM (kg) as a function of time (prior or post). **D)** ΔSMM (kg) by condition (GWL or RWL), **E)** Myostatin (ng.L^{-1}), **F)** Follistatin (ng.mL^{-1}), **G)** Follistatin/Myostatin ratio. Closed black circles indicate GWL and open grey circles indicate RWL. Horizontal red lines indicate condition means. Statistical comparisons and p values as indicated. N = 18 per condition and time point.

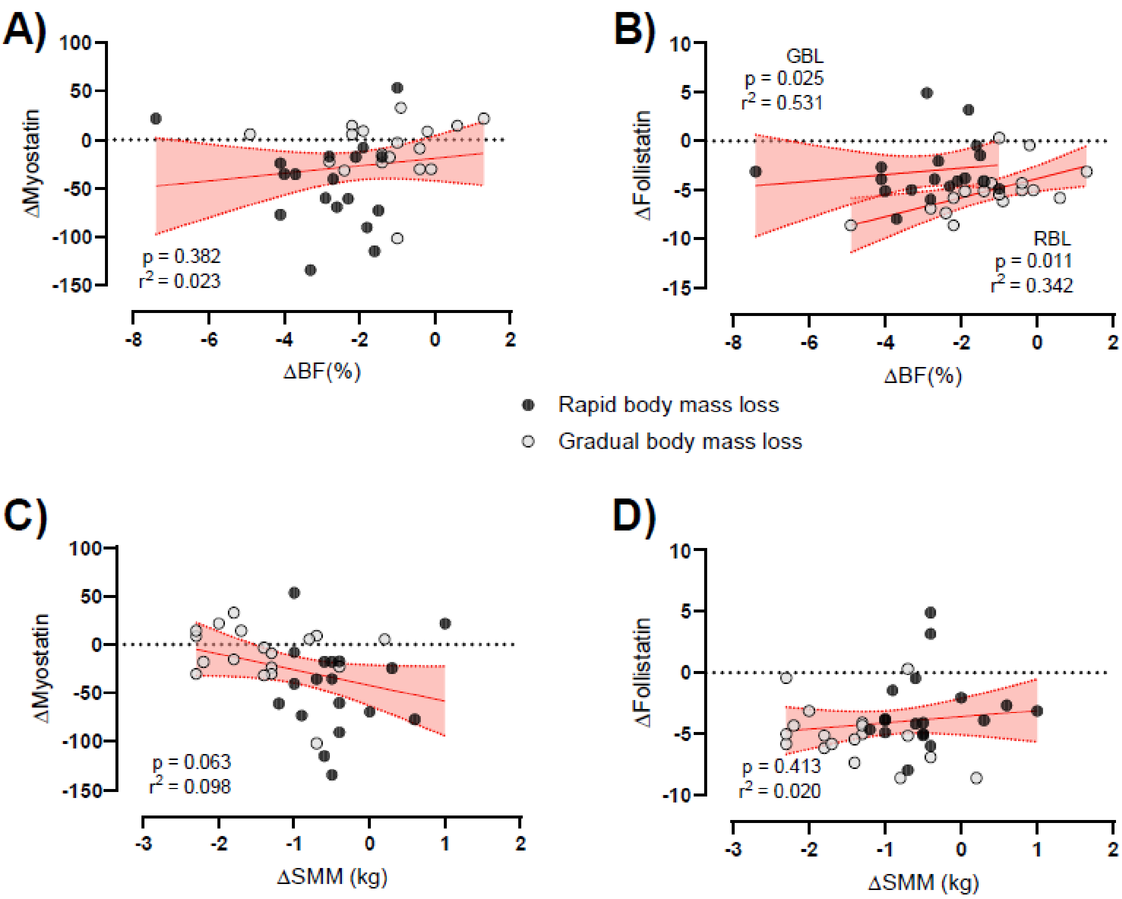

Figure 2: Correlations between change in (Δ) Endocrine marker and Δ body composition. **A)** Δ Myostatin, **B)** Δ Follistatin as a function of ΔBFP, **C)** Δ Myostatin, **D)** Δ Follistatin as a function of ΔSMM (kg). Closed black circles indicate RWL and open grey circles indicate GWL. Solid red line indicates linear regression; shaded red zone indicates 95% confidence interval. Figures **A)**, **C)**, and **D)** treated as one condition (sum-of-squares F test **A)** p = 0.505, **C)** p = 0.177), and **D)** p = 0.063), **Figure B)** treated as two distinct conditions (p = 0.013).