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Article

Regulation of Metabolic Aging Through AMPK and mTOR: A Comparative Study of Intermittent Fasting Variations in Obese Young Women

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Abstract

Background/Objectives: Obesity accelerates metabolic aging through oxidative stress, inflammation, and mitochondrial dysfunction. AMP-activated protein kinase (AMPK) and mammalian Target of Rapamycin (mTOR) are nutrient-sensing pathways that regulate metabolism. AMPK promotes energy metabolism and autophagy, while excessive mTOR activity contributes to aging. Intermittent fasting (IF), including Time-Restricted Feeding (TRF) and Alternate Day Modified Fasting (ADMF), may improve metabolic regulation, but their effects on AMPK, mTOR, and metabolic age remain unclear. **Methods**: This study compared the effects of TRF and ADMF on metabolic age, AMPK, and mTOR levels in young obese women. Twenty participants (mean age: 21.29 ± 1.76 years; body fat: 36.92 ± 3.18%; BMI: 29.68 ± 3.70 kg/m²) were divided into three groups: Control, TRF, and ADMF. Participants in the TRF and ADMF groups underwent fasting interventions for 20 days. **Results**: Statistical analysis showed a significant decrease in AMPK levels in the ADMF group ($p = 0.043$), while no significant changes were observed in the TRF and control groups. mTOR levels tended to decrease but were not significant ($p > 0.05$). Metabolic age also showed no significant variation across groups ($p > 0.05$). **Conclusions**: Twenty days of IF had no significant effect on AMPK, mTOR, or metabolic age in obese women. However, TRF may more effectively enhance AMPK and reduce mTOR, while ADMF appears more effective in reducing metabolic age. These findings suggest that short-term IF may have distinct metabolic effects, warranting further studies with longer interventions to assess potential clinical benefits.

Keywords: AMPK, mTOR, metabolic age, obesity, intermittent fasting

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1. Introduction

Obesity is a condition characterized by the accumulation of excess body fat due to an imbalance between energy intake and expenditure, which directly contributes to various serious health issues [1]. Obesity has become an increasingly serious global health crisis, with prevalence continuously rising worldwide, including in middle- and low-income countries. WHO data shows that in 2022, there were 2.5 billion adults with overweight, and the global prevalence of obesity has more than doubled between 1990 and 2022 [2]. In Indonesia, the prevalence of obesity among adults has doubled in the past two decades, reaching 21.8% in 2022 [3]. Vulnerable groups, such as young women aged 18–25 living in big cities, face a higher risk of obesity due to sedentary lifestyles and the consumption of processed foods high in fat and sugar [1]. With the increasing number of people with overweight and obesity worldwide, the health burden has also significantly increased [4]. Obesity and age-related diseases are estimated to cost the global economy more than US$4 trillion by 2035 [5]. This indicates that obesity has now become a serious global health issue [6].

Obesity has serious health impacts by accelerating the aging process through various biological mechanisms, including increased oxidative stress, systemic inflammation, and metabolic dysfunction, all of which exacerbate cellular damage similar to the natural aging process [7]. Research on humans shows that obesity causes dysfunction of adipose tissue, which acts as an endocrine organ and produces pro-inflammatory adipokines such as TNF-α, leptin, and resistin. This imbalance of adipokines can accelerate aging by triggering chronic inflammation and metabolic dysfunction. Additionally, individuals with obesity tend to experience insulin resistance and energy homeostasis disturbances, which contribute to accelerated cellular aging [8]. Another study also shows that individuals with obesity tend to experience telomere shortening, which is the end part of chromosomes that plays a role in maintaining genomic stability, potentially accelerating cellular aging and increasing the risk of age-related chronic diseases such as type 2 diabetes, cardiovascular diseases, and neurodegeneration [9]. Additionally, other studies show that the expression of longevity genes such as Forkhead Box O Transcription Factors (FOXO3a) is lower in obese individuals, which can accelerate the aging process by reducing the cells' ability to cope with oxidative stress and increasing the risk of degenerative diseases [10]. The relationship between obesity and aging not only reduces life expectancy but also shortens the healthy lifespan free from disease [7,11]. Therefore, an approach is needed to immediately reduce the rates of obesity and aging in order to prevent premature aging and reduce the economic and social burden caused by age-related diseases.

Intermittent Fasting (IF) has become one of the important strategies in reducing obesity and preventing premature aging. Research on humans shows that IF methods such as time-restricted feeding (TRF) and 5:2 fasting can lead to significant weight loss and improvements in metabolic parameters, such as blood pressure and blood glucose levels, although the effects vary depending on the individual [12]. Additionally, IF is also known to contribute to the improvement of cardiometabolic health by modulating epigenetic pathways and enhancing autophagy, both of which are associated with the prevention of premature aging [13,14]. One of the main mediators in this process is

AMPK (AMP-activated protein kinase), the primary energy sensor that is activated during IF when ATP levels decrease. The activation of AMPK plays a role in inducing autophagy, improving mitochondrial function, and reducing oxidative stress, thereby contributing to increased metabolism and slowing down the aging process [8,15]. Nevertheless, research on humans regarding the effects of IF on AMPK still shows varied results. Some studies report that IF can increase AMPK activity, but other studies show that this response does not always occur, especially in individuals with obesity or certain metabolic conditions [16]. In fact, several studies have shown that after longer fasting periods, AMPK activity actually decreases or does not change significantly [17]. Furthermore, recent systematic reviews suggest that the metabolic benefits of IF in humans may be more related to overall calorie restriction rather than direct activation of the AMPK pathway [18].

On the other hand, the mTOR (mammalian target of rapamycin) pathway, which is involved in cell growth and protein synthesis, shows an activity pattern opposite to that of AMPK. mTOR tends to be active when nutrient availability is high, but it can also inhibit autophagy and increase excessive anabolism, which contributes to cellular aging, especially in obese individuals [1,8,15]. A study found that the combination of IF (TRF 16:8) and aerobic exercise can significantly reduce mTOR levels in obese women, which impacts the reduction of inflammation and improvement of cellular metabolism [1]. Additionally, several studies also show that calorie restriction in IF can reduce metabolic age based on epigenetic changes measured through DNA methylation [13]. Because the research results on the effects of fasting on AMPK are still varied, with some studies showing an increase while others report a decrease or no change, and until now, the impact of IF and the differences in its effects on metabolic age and aging, in this case, AMPK and mTOR levels less studied, further exploration is needed.

From the various issues mentioned above, further research is needed to explore the effects of IF variations as a strategy for preventing cellular aging. This study aims to analyze the effects of Time Restricted Feeding (TRF) and Alternate Modified Day Fasting (ADMF) on the reduction of metabolic age, increase in AMPK, and decrease in mTOR as markers of the aging process reduction. In this study, TRF and ADMF were chosen as variations of IF because previous research has shown that TRF and ADMF have a relatively high adherence rate and more significant results in weight loss [19,20]. The type of TRF used is 18:6 to achieve more intensive and significant weight loss and metabolic improvement compared to the 16:8 pattern [21]. Thus, this research can serve as a foundational reference for developing therapies aimed at enhancing the quality of healthy aging.

2. Materials and Methods

2.1 *Participant Characteristic*

The subjects in this study were young 21.29 ± 1.76 years old obese women selected based on inclusion and exclusion criteria. The inclusion criteria for this study included obese women lived in Surabaya with a Body Mass Index (BMI) 29.68 ± 3.70 kg/m² (based on the Asia-Pacific BMI classification, >25 kg/m²), body fat percentage 36.92 ± 3.18% in the high category (>30%), normal Fasting Blood Glucose (FBG) levels and no history of diabetes mellitus, normal Hemoglobin (Hb) levels, and normal blood pressure. During the screening process, potential participants were excluded if they had a history of chronic gastrointestinal disorders (chronic gastritis), cardiovascular conditions such as hypertension and atherosclerosis, as well as any malignancies. Additionally, individuals who reported alcohol consumption, active smoking, or using slimming pills and other routine medications were exclude from this study.

2.2 *Study Design*

This study has been approved by the Ethics Committee of the Faculty of Medicine, Airlangga University (reference number 36/EC/KEPK/FKUA/2024). All participants were provided with written informed consent before their participation into this study.

This study was designed as a quasi-experimental investigation employing fasting interventions in humans using a Pretest-Posttest Control Group Design. Subjects who met the inclusion criteria underwent a matching process based on their BMI for their allocation into groups. Those with the highest BMI values were ranked in descending order and subsequently allocated into Control Group (Control Group, n=8), the Time-Restricted Feeding Group (TRF Group, n=8), and the Alternate-Day Modified Fasting Group (ADMF Group, n=8). The fasting intervention was carried out over a period of 20 days, whereas the Control Group were instructed to maintain their diet habit.

Anthropometric data, body fat measurements, and blood samples were collected both before (pretest) and after (posttest) the intervention. If the subject was absent during screening, pre-test, and post-test; or experienced severe illness during the study and could not continue the fasting intervention for at least 80% of the total fasting, then the subject was dropped out from the study. Pretest data were gathered prior to initiating the fasting intervention, and posttest data were obtained one day after the final fasting session. Before each measurement, all subjects were instructed to fast for 8 hours (with their last meal consumed at 8:00 PM on the previous day until the time of blood sample collection). The measurements were conducted between 07:00 and 10:00 PM. Blood samples were drawn from the median cubital vein and placed in an icebox at approximately 0°C until they were analyzed for AMPK and mTOR levels using ELISA.

2.3 *Intermittent Fasting Protocol*

In this study, two types of intermittent fasting were implemented: Time-Restricted Feeding (TRF) and Alternate Day Modified Fasting (ADMF). TRF was defined as an 18:6 protocol, whereby subjects fasted for 18 consecutive hours and consumed all calories within a 6-hour feeding window. The fasting period commenced at 20:00 and extended until 14:00 the following day, with the feeding window spanning from 14:00 to 20:00.

During the fasting period, subjects were permitted to consume only plain water, devoid of flavorings or sweeteners, while during the feeding period they were allowed to eat ad libitum without any caloric restrictions. This regimen was maintained continuously for 20 consecutive days.

ADMF was defined as a 24-hour fasting period during which subjects received less than 25% of their calculated daily energy needs—determined by a nutritionist using the Harris-Benedict formula [22] and provided by the researchers via a specialized catering service. On the subsequent day, subjects were allowed unrestricted ad libitum eating. Throughout the intervention phase, subjects were asked to record daily food using an approximated food record method, having received standardized instructions on accurate recording prior to the commencement of the study.

2.4 Outcomes measurement

2.4.1 Body Composition Assessment

During the screening process, haemoglobin, fasting blood glucose, heart rate, and blood pressure were measured. Haemoglobin were measured by finger prick test using hemoglobinometer (Accu Chek Performa; Roche Diabetes Care, Switzerland) while blood glucose was measured using glucometer (Easy Touch GCU ET322; Zhejiang Easy Touch Medical Instruments Co., Ltd, China). Heart rate and blood pressure were measured using the digital sphygmomanometer (Omron HEM-8712; Omron Healthcare Co., Ltd, Japan). Body composition was measured using BIA (Omron HBF-375 Karada Scan Body Fat Composition Analyzer; Omron Healthcare Co., Ltd, Japan).

2.4.2 Blood Sampling and Biochemical Analysis

A blood sample was taken from the median cubital vein between 07:00-10:00 am using a 10 ml Terumo syringe with a 20 G needle. The collected blood was stored in a BD vacutainer SST II Advance Plus Blood Collection Tube. The blood sample was centrifuged at 1000 rpm (5 minutes, 23°C) to separate the red blood cells and serum. This procedure is performed within <20 minutes of blood collection from the subject. The separated serum is used for the analysis of AMPK and mTOR levels. Measurement of AMPK levels using the Human Phosphorylated Adenosine Monophosphate Activated Protein Kinase ELISA kit (catalogue No.E0746Hu, Bioassay Technology Laboratory, Shanghai Korain Co., Ltd) with AMPK sensitivity of 0.28 ng/mL and detection range of 0.5-200 ng/ml. Measurement of mTOR levels using the Human Mammalian Target of Rapamycin ELISA kit (catalogue No.E3693Hu, Bioassay Technology Laboratory, Shanghai Korain Co., Ltd) with mTOR sensitivity of 0.28ng/mL and detection range of 0.5-200 ng/ml.

2.5 Statistical Analysis

The sample size was calculated using software G power, resulting sample size of 4 subjects. Then, a sample size correction was performed using the Higgins and Kleinbaum formula. According to the formula, the proportion of the sample that dropped out using previous research [1] was found to be n=8 in each group with a statistical power of 80% and a significance level of 0.05, resulting in a total of 24 subjects across the 3 groups. All

statistical analyses were calculated using IBM SPSS Statistics Software version 27. Normality tests were examined using the Shapiro-Wilk test. If the data was normally distributed, the pre-test and post-test means of one group were compared using the paired t-test, and the mean values between groups were compared using One-way ANOVA, followed by the Turkey's HSD post hoc test. But if the data is not normally distributed, the data were analysed using the Wilcoxon Signed Rank Test and Kruskal-Wallis Test. Data will be presented with mean ± standard deviation (SD) or Median (Interquartile Range). All statistical data analyses used a level of significance at $p<0.05$.

3. Results

3.1. Participant Characteristics

Of the 24 participants in this study, 4 samples were excluded from the analysis due to identified using SPSS as an outliers. These excluded data included 1 sample from the Control Group (AMPK measurement) and 3 samples from the ADMF Group (2 from mTOR measurement and 1 from AMPK measurement) because their ELISA results far exceeded the highest standard curve, leaving 20 subjects for statistical analysis. We found no significant differences in age, BMI, heart rate, blood pressure, fasting blood glucose (FBG), fat mass and visceral mass at baseline (all p>0.05) (Table 1). The detailed of subject's characteristics is shown in Table 1.

Table 1. Participants baseline characteristics

	Control (n=7)	**ADMF (n=5)**	**TRF (n=8)**	***P* value**
Age, years [a]	20.60 ± 1.82	21.20 ± 2.28	20.4 ± 1.14	0.877
Blood pressure, mmHg				
Sistolik [a]	114.40 ± 10.26	110.60 ± 8.65	122.20 ± 15.88	0.185
Diastolik [a]	80.20 ± 8.32	77.60 ± 2.70	84.40 ± 11.41	0.471
Fasting Blood Glucose, mg/dL [b]	101 (98-139.5)	102 (92-120)	95 (88.5-118.5)	0.878
Haemoglobin, mg/dL [a]	12.46 ± 1.54	13.2 ± 2.41	12.80 ± 1.39	0.920
Weight, kg [a]	76.49 ± 12.03	73.46 ± 11.06	79.19 ± 5.73	0.591
Height, cm [a]	157.80 ± 6.51	155.7 ± 6.91	161.30 ± 2.71	0.397
BMI, kg/m^2 [a]	30.58 ± 5.25	30.08 ± 3.44	30.48 ± 2.28	0.824
Fat mass, % [a]	37.16 ± 3.86	36.86 ± 4.69	37.58 ± 2.57	0.880
Visceral Fat, % [b]	9 (7.25-16)	9.5 (7.75-13)	10 (8.25-12)	0.319
AMPK, ng/mL [b]	35.29 (29.22-43.30)	45.64 (36.75-66.83)	44.29 (29.97-50.92)	0.546
mTOR, ng/mL [b]	1029 (7.25-10.61)	8.22 (4.89-9.21)	8.14 (6.72-9.07)	0.751
Metabolic age, years [a]	44.20 ± 6.18	44.20 ± 4.44	45.40 ± 4.16	0.484

Description: **ADMF**, Alternate-day modified fasting; **TRF**, Time Restricted Feeding; **BMI**, Body Mass Index; **AMPK**, AMP-activated protein Kinase; **mTOR**, mammalian Target of Rapamycin. Representative of data with mean±SD or median (IQR1-IQR3. [a] *p* value was obtained by One Way ANOVA. [b] *p* value was obtained by Kruskal Wallis

Before and after of weight, BMI, fat mass and visceral fat mass between groups, is shown in Table 2. A downward trend in body weight and BMI was observed in the ADMF Group; however, it was not statistically significant (body weight p=0.086; BMI p=0.052).

Table 2. Before and after of weight, BMI, fat mass and visceral fat mass

Parameter	Group	Pre-Test	Post-Test	*p*-value
Weight, kg	Control (n=7) [a]	76.49 ± 12.03	76.25 ± 11.87	0.656
	ADMF (n=5) [a]	73.46 ± 11.06	72.38 ± 10.82	0.086
	TRF (n=8) [a]	79.19 ± 5.73	79.57 ± 5.24	0.877
BMI, kg/m²	Control (n=7) [b]	27.10 (25.10 – 29.78)	27.55 (25.1 - 29.1)	0.786
	ADMF (n=5) [a]	30.38 ± 3.52	29.94 ± 3.61	0.052
	TRF (n=8) [a]	30.14 ± 2.54	30.21 ± 2.58	0.351
Fat Mass, %	Control (n=7) [a]	37.16 ± 3.86	37.34 ± 3.82	0.907
	ADMF (n=5) [a]	36.86 ± 4.69	37.26 ± 3.87	0.698
	TRF (n=8) [a]	37.58 ± 2.57	37.58 ± 2.42	0.464
Visceral	Control (n=7) [b]	9 (7.25-16)	9 (7.25-15.5)	0.655
Fat, %	ADMF (n=5) [a]	10.20 ± 2.73	9.10 ± 3.05	0.605
	TRF (n=8) [a]	10.13 ± 2.25	10.19 ± 3.58	0.961

Description: **ADMF**, Alternate-day modified fasting; **TRF**, Time Restricted Feeding; **BMI**, Body Mass Index; **AMPK**, AMP-activated protein Kinase; **mTOR**, mammalian Target of Rapamycin. Representative of data with mean±SD or median (IQR1-IQR3. [a] p value was obtained by One Way ANOVA. [b] p value was obtained by Kruskal Wallis

3.2. Effects on Intermittent Fasting on AMPK levels, mTOR levels and metabolic age

Table 3 summarizes the comparison of AMPK levels, mTOR levels and metabolic age between pre-test and post-test for each group. AMPK levels in the Control Group, we didn't observe no significant change (p = 0.364). In the ADMF Group, AMPK levels significantly decreased (p = 0.043). Similarly, the TRF Group exhibited a downward trend; however, it was not statistically significant (p = 0.744). In the mTOR assessment, a downward trend was observed in all groups; however, it was not statistically significant (p>0.05). Meanwhile, in the metabolic age measurement, the mean values remained stable with no significant differences (p>0,05). In keeping with this, the statistical analysis of changes in AMPK, mTOR, and metabolic age were not significant (Table 4).

Table 3. Comparison of AMPK levels, mTOR levels and metabolic age between pre-test and post-test for each group

Variable	Group	Pre-Test	Post-Test	*p*-Value
AMPK levels, ng/mL	Control (n=7) [a]	36.07 ± 7.75	41.63 ± 8.24	0.364
	ADMF (n=5) [b]	45.64 (36.75-66.83)	37.81 (31.95-56.71)	0.043*
	TRF (n=8) [a]	41.22 ± 12.05	39.45 ± 11.42	0.744
mTOR levels, ng/mL	Control (n=7) [b]	10.29 (7.25-10.61)	6.12 (2.94-7.64)	0.128
	ADMF (n=5) [a]	7.28 ± 2.3	6.42 ± 2.1	0.621
	TRF (n=8) [a]	7.94 ± 1.18	4.32 ± 1.26	0.499
Metabolic age, ng/mL	Control (n=7) [a]	44.20 ± 6.18	44.40 ± 5.73	0.604
	ADMF (n=5) [a]	44.20 ± 4.44	44.20 ± 4.44	1.000
	TRF (n=8) [a]	45.40 ± 4.16	45.40 ± 3.85	0.598

Description: **ADMF**, Alternate-day modified fasting; **TRF**, Time Restricted Feeding; **BMI**, Body Mass Index; **AMPK**, AMP-activated protein Kinase; **mTOR**, mammalian Target of Rapamycin. Representative of data with mean±SD or median (IQR1-IQR3). *significant at Control, ADMF or TRF ($p<0.05$). [a] *p* value was obtained by paired-t test. [b] *p* value was obtained by Wilcoxon sum rank test.

Table 4. Comparison analysis of changes in AMPK, mTOR levels, and metabolic age before and after intervention in the three groups

Group	Δ AMPK, ng/mL[b]	*p* value	Δ mTOR, ng/mL[a]	*p* value	Δ Metabolic Age, years[a]	*p* value
Control (n=7)	5.56 ± 10.79	0.174	-3.75 ± 3.68	0.618	0.20 ± 0.84	0.943
ADMF (n=5)	-7.54 ± 4.49		-0.86 ± 3.61		0.00 ± 1.00	
TRF (n=8)	-1.76 ± 13.83		-3.63 ± 0.43		0.0 ± 0.70	

Description: **ADMF**, Alternate-day modified fasting; **TRF**, Time Restricted Feeding; **BMI**, Body Mass Index; **AMPK**, AMP-activated protein Kinase; **mTOR**, mammalian Target of Rapamycin. Representative of data with mean±SD. [a]*p* value was obtained by One Way ANOVA. [b]*p* value was obtained by Kruskal Wallis.

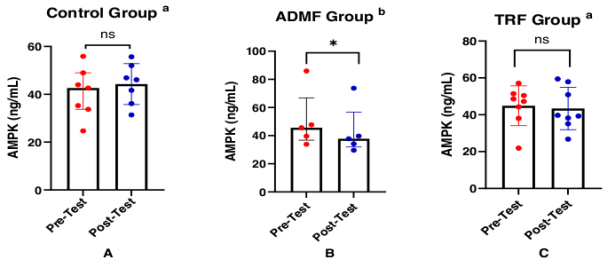

Figure 1. Differences in AMPK levels (ng/mL) between pretest and posttest in the three groups. (**A**) Control Group. (**B**) ADMF Group. (**C**) TRF Group. [a] Representative of data with mean ± SD and *p*-value was obtained by paired sample t-test. [b] Representative of data with median (interquartile range) and *p*-value was obtained by Wilcoxon signed rank test. (ns) Not significant ($p \geq 0.05$). (*) Significant at pretest ($p \geq 0.05$).

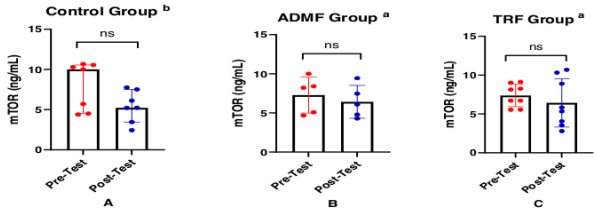

Figure 2. Differences in mTOR levels (ng/mL) between pretest and posttest in the three groups. (**A**) Control Group. (**B**) ADMF Group. (**C**) TRF Group. [a] Representative of data with mean ± SD and *p*-value was obtained by paired sample t-test. [b] Representative of data with median (interquartile range) and *p*-value was obtained by Wilcoxon signed rank test. (ns) Not significant ($p \geq 0.05$).

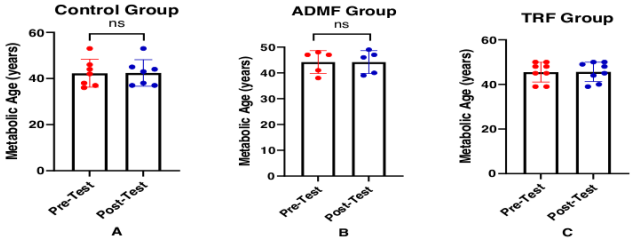

Figure 3. Differences in metabolic age (years) between pretest and posttest in the three groups. (**A**) Control Group. (**B**) ADMF Group. (**C**) TRF Group. Representative of data with mean ± SD. *p*-value was obtained by paired sample t-test. (ns) Not significant ($p \geq 0.05$).

4. Discussion

The results of the analysis of the subject characteristics data show that the three groups are homogeneous in terms of age, BMI, baseline AMPK, mTOR, metabolic age, and other clinical parameters. This indicates that the three groups had controlled variables, allowing for a more accurate interpretation of the results, particularly in comparing the impact of each fasting variation on molecular biomarkers and metabolic age.

AMPK level analysis showed a significant decrease in the ADMF group and a decreasing trend in the TRF group, while the control group experienced an increase. These results are consistent with previous studies [16,18] which show that fasting does not always consistently activate AMPK, especially in obese individuals. These findings indicate that in individuals with obesity, the metabolic adaptation mechanisms to TRF are likely not mediated by direct activation of the AMPK pathway, but rather through improvements in other metabolic aspects related to reduced calorie intake.

In ADMF, the decrease in AMPK that does not align with the initial hypothesis may be caused by the refeeding phase, the duration and pattern of fasting, and the metabolic status of the subjects [13,23]. The refeeding phase causes the body to switch from a catabolic state to an anabolic state [23]. The insulin spike that occurs during refeeding suppresses AMPK activity through the activation of the PI3K-Akt-mTOR pathway, which is an antagonist of AMPK [15,17]. Nutritional composition also plays a role, where high carbohydrate consumption inhibits the activation of LKB1, an upstream regulator of AMPK [15], while a low-carbohydrate or high-fat diet can support AMPK through ketogenesis [13,15,24]. Non-fasting days on ADMF with ad libitum intake contribute to insulin spikes, increased mTOR activity, and AMPK suppression. TRF shows more controlled metabolic fluctuations due to having a more limited eating window, so the insulin spike is not as strong as in ADMF. The difference in refeeding patterns affects the stability of AMPK activation.

Hormonal factors such as cortisol and IGF-1 also play a role. In ADMF, a more fluctuating eating pattern with alternating fasting and refeeding periods can lead to higher cortisol levels compared to TRF. High cortisol can inhibit AMPK through the activation of SGK1 (serum- and glucocorticoid-regulated kinase-1), which phosphorylates AMPK at its inhibitory residue and suppresses its activity [16,25]. IGF-1 increases during the refeeding phase due to a surge in insulin and higher protein intake. Meanwhile, the increase in IGF-1 can also activate the PI3K-Akt-mTOR pathway, which acts as an inhibitor of AMPK [15]. In ADMF, a longer refeeding period compared to TRF can significantly increase IGF-1, which contributes to a stronger inhibition of AMPK. On the other hand, in TRF, the increase in IGF-1 is more moderate because refeeding occurs within a more limited time window, resulting in a smaller suppressive effect on AMPK.

The duration of fasting that is not long enough can limit AMPK activation. To ensure stable AMPK activation in the body, a consistent adaptation period of intermittent fasting duration over several weeks is needed to create sustainable metabolic adaptation. If the fasting period in the ADMF pattern is not long enough, the cells may not reach the necessary level of metabolic stress to optimally activate AMPK [13,15,25]. The same goes for TRF, where the effects on AMPK are not as pronounced compared to stricter IF, because TRF allows calorie consumption within a certain time frame, which can reduce the potential for significant energy depletion and lead to more subtle metabolic adaptations to significantly activate AMPK. Metabolic adaptation requires an intervention of at least 2-6 weeks. TRF with 16 hours of fasting per day only shows consistent AMPK activation after 2-4 weeks [13,25–27], whereas ADMF requires 4-6 weeks to achieve optimal metabolic effects [15,25,28].

After understanding the role and dynamics of AMPK in responding to intermittent fasting interventions, we will next examine the response of the mTOR pathway as the main metabolic regulatory mechanism. mTOR analysis showed a non-significant decreasing trend, unlike a previous study [1] that used TRF 16:8 for 2 weeks (5 times a week) with an ad libitum diet during the refeeding phase, which showed a significant decrease in mTOR. This difference may be due to the different fasting and refeeding patterns.

According to S. D. Anton et al., (2018)[29], a structured refeeding phase—with a longer

eating window (e.g., 8–12 hours) and non-fasting days—can prevent overcompensation, a condition where excessive calorie intake during the eating phase repeatedly causes insulin spikes and reactivation of the mTOR pathway. In my research, a shorter eating window (6 hours) and daily fasting without breaks allowed for the accumulation of refeeding effects, causing repeated insulin spikes that activated mTOR, thus the mTOR inhibition effect was not significantly observed. Additionally, another research [30] also supports that an unstructured refeeding phase can diminish the metabolic adaptation benefits of fasting. Therefore, the impact of IF on mTOR highly depends on the duration of fasting, the intensity of the refeeding phase, and the balance between the fasting and eating phases [31–33]. In the TRF 18:6 protocol, the 6-hour eating phase provides an opportunity for nutrient intake that can increase amino acid and insulin levels, which subsequently reactivates mTOR. Thus, although mTOR is inhibited during the fasting phase, this effect can be compensated by the refeeding period, especially if the food consumed after fasting is high in protein or carbohydrates. Protein, especially amino acids like leucine, are known to be direct activators of mTOR. If the respondents in this study still consume a high amount of protein during the eating window, then the mTOR inhibition effect that occurs during the fasting phase could be quickly compensated by the reactivation of mTOR during the eating phase [24,34], therefore, the differences that take place are not significant enough to be statistically significant [4,30,35]. Thus, although both studies used the TRF approach, differences in the duration of the eating window and refeeding patterns were key factors influencing the significance of mTOR reduction.

In addition, the optimization of IF interventions is also necessary to suppress mTOR, which requires a duration of TRF or ADMF of at least 4-8 weeks [32,36,37] and the implementation of early TRF (08:00-14:00) to align with the circadian rhythm [24].

Subsequent to the analysis of mTOR activity alterations, the following step is to examine its influence on metabolic age, a crucial metric for evaluating metabolic adaption and overall biological health. It is evident from the results that the intermittent fasting intervention in this study did not produce significant differences in metabolic age between the control, TRF, and ADMF groups, although there was a trend of decreasing BMI and body weight in the ADMF group. The 20-day intervention duration is likely not sufficient to induce significant changes in metabolic biomarkers that reflect biological age. Metabolic adaptations, such as increased insulin sensitivity, improved lipid profiles, and reduced inflammation, only become apparent after a longer duration of intervention [38], Thus, it can be claimed that gradual adaptation is necessary for metabolic improvement [11,31].

Furthermore, fat mass as an indicator of metabolic age did not experience significant changes in this study, despite a trend of weight loss and BMI reduction in the ADMF group. This is different from the previous research [39], who reported a significant reduction in fat mass (~9%) in the TRF intervention combined with exercise over 12 weeks. This comparison suggests that a minimum intervention duration of 12 weeks, along with the addition of moderate to high physical activity, may be necessary to achieve optimal metabolic adaptations.

Several studies [40–42] confirm that a 12-week TRF intervention can improve blood pressure, lipid profile, fat mass, and insulin sensitivity, all of which contribute to reducing the risk of metabolic diseases and premature aging. Thus, although IF has the potential to improve metabolic health, the intervention period being too short is insufficient to produce significant changes. Further research is recommended to use an intervention duration of at least 12 weeks with additional physical activity to detect clearer changes and to thoroughly understand the mechanisms of metabolic adaptation.

This study has several limitations that need to be considered. First, the lack of strict control over calorie intake and macronutrient composition during the refeeding phase in the intervention group may affect the AMPK and mTOR metabolic pathways, thereby obscuring the effects of intermittent fasting. Second, the non-uniform environment of the respondents (respondents living in their respective homes) makes it difficult to verify the accuracy of the data, which potentially reduces the significance of the results. Therefore, stricter control of diet and environment, such as establishing dormitories during the study, is necessary to achieve more valid results.

5. Conclusions

The conclusion of this study is that a 20-day intermittent fasting does not increase AMPK levels nor decrease mTOR and metabolic age. Although the results of this study did not show significant differences, Time Restricted Feeding (TRF) has a better tendency to increase AMPK activity and decrease mTOR compared to ADMF. But to lower metabolic age marked by indicators such as body weight, fat mass, and visceral fat, ADMF tends to be more impactful than TRF. Further studies with controlled calorie restriction during refeeding, longer intervention durations to trigger the desired changes, and serial examinations to monitor the metabolic changes that occur are needed.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki. This study was part of study on the "Effect of Various Types of Intermittent Fasting (IF) on Several Markers: Aging, Working Memory, Inflammation and Immune System in Obese Young Women", approved by the Health Research Ethics Committee of Faculty of Medicine, Airlangga University (no. 36/EC/KEPK/FKUA/2024) on June 24, 2024.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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