TIME-RESTRICTED FEEDING PREVENTS THE DETRIMENTAL EFFECTS OF A HIGH-FAT DIET UNDER BOTH HOMEOSTATIC AND INFLAMMATORY CONDITIONS

PhD thesis

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Budapest, 2025

1. Introduction

In modern societies, irregular and unhealthy eating patterns, such as late-evening meal timing, are increasingly prevalent, contributing to the development of both metabolic and inflammatory disorders. In turn, restricting food access to a specific time window of the day, even without reducing caloric intake, entrains the circadian clocks of peripheral tissues and enhances metabolic rhythm, therefore improving metabolic health. Consequently, timed food intake represents an affordable and well-tolerable approach, which is beneficial for both the prevention and treatment of metabolic diseases. Bidirectional communication between metabolism and immunity suggests that time-restricted eating - through improving metabolic function - may have an inflammation-attenuating effect and serve as a complementary therapy for inflammatory diseases, including rheumatoid arthritis and allergic contact dermatitis.

2. Objectives

Our aim was to investigate the impact of a 4-week time-restricted feeding (TRF) on metabolic and immune parameters under *steady-state* conditions. Furthermore, we aimed to explore how TRF influences the progression of (1) K/BxN serum-transfer arthritis, a murine model of human rheumatoid arthritis, and (2) contact hypersensitivity, a mouse model for allergic contact dermatitis.

Our study aimed to address the following questions:

1. What are the effects of normal versus high-fat diets on metabolic parameters, and does the timing of food intake influence key indicators of the metabolic state?
2. How is the metabolic rhythm modulated by different feeding regimens?
3. Does a high-fat diet alter of neutrophil and monocyte activation under *steady-state* conditions? Is adhesion molecule expression affected by TRF?
4. Can TRF mitigate the severity of inflammatory arthritis despite a high-fat diet?
5. Which immune parameters are influenced by a high-fat diet during a contact hypersensitivity reaction? How does time-restricted feeding attenuate CHS response?

3. Methods

3.1. Animals and diets

In our experiments male C57BL/6N (B6 *wild type*) and BKS.Cg-Dock7m$^{+/+}$Leprdb/J ObRb leptin receptor mutant (*db/db*) mice and their *wild type* (*wt*) controls with BKS background were used. The animals were maintained in a minimal disease animal facility under 12-hour light and 12-hour dark cycles and fed with standard chow diet. At the age of 60-80 B6 *wild type* mice were randomly assigned to a calorie-dense high-fat diet (HF, high-fat, cat.no.: D12230, fat content: 59%) or standard diet (NC, normal chow, cat.no.: S8189, fat content: 17%). Each dietary group was further divided into two feeding regimens: *ad libitum* (AL) and time-restricted feeding (TRF). In the TRF group food availability was restricted to the first 10 hours of the active phase of the animals (Figure 1). Experiments were conducted after a 4-week conditioning period.

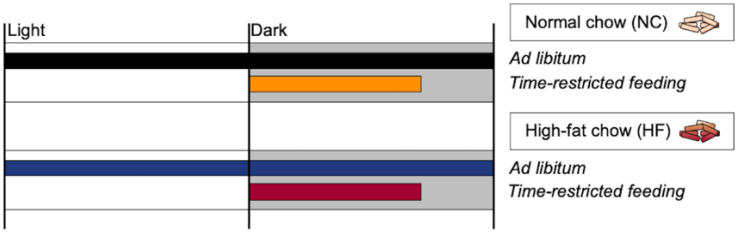

Figure 1. Schematic outline of the feeding regimes.

Study design and experimental procedures were approved by the Animal Experimentation Review Board of Semmelweis University and the Government Office for Pest County (Hungary) (Ethical approvals: PE/EA/1967-2/2017 (KA-2281) and PE/EA/00375-6/2024.

3.2. Blood sampling and flow cytometry

Blood samples were collected at six different time points (ZT1, 5, 9, 13, 17, 21, ZT=*Zeitgeber* time, which refers to time after light onset in hours) through retroorbital bleeding to isolate serum or from tail snip for flow cytometry.

In flow cytometry measurements singlets were gated using FSC-A and FSC-H. Within singlets and the CD45+ gate, CD11b+Ly6G+ (neutrophils), CD11b+CD115+Ly6G- (monocytes and macrophages), and CD11b-Ly6G-SSClow (lymphocytes) populations were determined. Mean fluorescence intensity (geometric mean) of antibody-labeled adhesion molecules was measured. Cell counts were determined using CountBright™ Plus Absolute Counting Beads.

3.3. K/BxN serum-transfer arthritis

K/BxN serum-transfer arthritis (STA) was induced by intraperitoneal injection of 250 µl arthritogenic serum (Day 0). Arthritis severity was assessed on day 6, corresponding to the

peak of disease manifestation. Clinical scoring, ankle thickness measurements with a caliper (Koeplin), and functional, grid-holding ability tests were performed. For further analysis, after termination of the animals, hind limbs were collected, minced and digested.

3.4. Contact hypersensitivity

3.4.1. Acute and subacute model

In the acute model, mice were sensitized with 100 µl 3% TNCB (2,4,6-trinitrochlorobenzene) solution applied on the shaved abdomen. In the elicitation phase, ears of all animals were treated with 20 µl 1% TNCB solution on day 6. 24 hours later, the severity of contact hypersensitivity (CHS) was assessed at the phenotypic and cellular levels. In the subacute model, severity was analyzed after three consecutive TNCB challenges.

3.4.2. Administration of leptin receptor antagonist

Ears of the HF-AL or *db/db* sensitized animals were treated with intradermal injection of leptin receptor antagonist, Allo-aca (10 ng/g body weight) or with vehicle on day 6. Four hours later, both ears were TNCB challenged. 24 hours later, the impact of Allo-aca treatment was assessed.

4. Results

4.1 Impact of a high-fat diet and timed food intake on metabolic and immune parameters

4.1.1. Metabolic parameters under different feeding conditions

Caloric intake and weight gain were monitored throughout the 4-week conditioning. Weekly caloric intake in the HF groups was significantly higher than that in the NC groups. Interestingly, TRF did not result in a reduction of caloric intake compared to the corresponding *ad libitum* group, indicating that TRF does not cause caloric restriction. Additionally, TRF groups gain significantly less weight than the AL groups. HF-AL had significantly more daytime caloric intake than NC-AL, indicating an increased metabolic disruption in HF-AL animals.

4.1.2. Indicator of the metabolic rhythm: leptin oscillation in the white adipose tissue and serum

Leptin is clock-controlled in the white adipose tissue (WAT), resulting in a *leptin* expression rhythm that reflects the metabolic rhythm in response to dietary changes. To follow the consequences of the diets on the rhythmic function of WAT, *leptin* expression was investigated. HF-AL increased *leptin* expression and phase-advanced its rhythm. However, in TRF

mice, *leptin* expression was significantly lower compared to the AL-fed animals and peaked at night, which suggests a compensatory effect of timed eating. Parallel with gene expression, leptin levels in NC-AL serum samples showed no significant rhythm, while in the NC-TRF group lowered serum leptin levels with robust rhythmicity were detected. The HF-AL group had significantly elevated and phase shifted leptin levels compared to the NC groups, whereas HF-TRF prevented this negative effect and stabilized the rhythmicity. Besides indicating the metabolic rhythm of white adipose tissue, leptin is an important immune modulator. It acts via cytokine receptors, positioning it as a potential link between metabolic regulation and immune responses; hence, this interaction may be modulated by time-restricted feeding.

4.1.3. Assessing the activity state of the circulating myeloid cells under homeostatic conditions

Expression of the activation marker CD11b, was determined on neutrophils and monocytes at six time points after the 4-week conditioning. In the HF-AL group, the daily average of CD11b expression was increased on neutrophils, which was prevented by TRF. Similarly, HF-AL significantly elevates CD11b on the surface of the monocyte population and TRF prohibited this effect. These data support the idea that even a short-term HF diet

promotes priming and facilitates migration of myeloid cells, whilst TRF has a preventive effect under homeostatic conditions.

4.2 TRF improves inflammation outcomes

4.2.1. Effect of TRF and HF diet on the development of K/BxN serum-transfer arthritis

To investigate how HF diet affects arthritis severity, STA was induced in NC-AL (Control), HF-AL and HF-TRF groups. At the peak of the arthritis symptoms (day 6), disease severity was assessed using clinical scoring and ankle thickness measurement. Additionally, a functional grid-holding ability test was performed. There were no differences in the phenotype between HF-AL and NC-AL mice, whereas HF-TRF reduced all parameters below NC-AL and HF-AL values. HF-AL feeding slightly increased leukocyte counts and significantly elevated the counts of infiltrated neutrophils compared to the NC-AL group. This finding indicates that neutrophils could be the main contributors to the inflammation-worsening effect mediated by HF diet. However, in HF-TRF mice, significantly decreased synovial leukocyte count, myeloid leukocyte count, leptin and IL-1ß levels were detected. These data suggest that anti-

inflammatory mechanism of TRF may partially be mediated through leptin reduction.

4.2.2. Investigation of diets' effects on acute and subacute contact hypersensitivity

TNCB-induced contact hypersensitivity, the mouse model of human allergic contact dermatitis (ACD), was performed. NC-TRF showed no beneficial effect compared to NC-AL. The largest normalized ear thickness was detected in the sensitized HF-AL group, accompanied by elevated IL-1ß and CXCL2 levels (key mediators of the CHS model) and increased neutrophil accumulation in the inflamed tissue. Intriguingly, HF-TRF prevented these effects.

To address the recovery of the experimental groups, regeneration kinetics of the acute and subacute models were investigated. In the acute model, animals were sensitized and TNCB-challenged, then the regeneration of the ears was investigated by daily ear thickness measurements during a two-week post-challenge period. Regeneration capacity of NC-AL, NC-TRF, and HF-TRF showed similar kinetics. The regeneration of HF-AL mice was, however, prolonged and no recovery occurred during the observation period.

In the subacute model, TRF significantly shortened the regeneration period even in mice fed with normal chow. HF-AL

feeding not only disrupted recovery but also exacerbated CHS severity. To assess the therapeutic potential of TRF, a group of mice subjected to AL feeding was switched to TRF on day 7. Under both NC and HF conditions, the AL-TRF transition resulted in reduced ear swelling, indicating that TRF has beneficial effects even when applied after disease development.

4.2.3. The role of leptin in CHS

We hypothesized, that diet-induced exacerbation of inflammation is mediated by metabolic reorganization. As *steady-state* leptin levels substantially reflect metabolic state and leptin has multiple effects on immune functions, we measured its levels in ear lysates on day 7. HF diet significantly elevated leptin levels in the ear; however, TRF counteracted this effect.

To investigate the possible role of leptin in CHS development, *db/db* mice were used in the next experiments. NC-AL-fed *db/db* mice, as a model with extremely elevated leptin production, have similar inflammatory processes to those of HF-AL animals, indicating that leptin may contribute to CHS severity, even acting through the short forms of the leptin receptors. Ear thickness of the *db/db* mice was slightly elevated compared to

wild type littermates, and neutrophil counts, CXCL2, and IL-1ß levels were increased.

To further investigate the role of leptin in this inflammatory model, leptin receptor antagonist Allo-aca was applied intradermally to one ear, while vehicle was injected into the other ear. Blockade of leptin receptors resulted in a reduction of ear swelling in *db/db* mice, suggesting that elevated leptin levels might have aggravated CHS.

To assess leptin's effect on allergic responses, CHS was induced in HF-AL mice together with administration of a leptin receptor antagonist. This self-controlled study showed that Allo-aca treatment resulted in a decrease in all measured inflammatory indicators. Ear swelling was significantly decreased, while CXCL2 and IL-1ß levels were tendentiously reduced. Monocyte and neutrophil counts in ear lysates decreased in line with reduced pustule formation. These data also support our hypothesis that leptin contributes to the worsening of symptoms under HF-AL conditions.

5. Conclusions

1. Short-term time-restricted feeding improved metabolic rhythms under both normal and high-fat diets. Remarkably, TRF prevented body weight gain despite similar caloric intake compared to *ad libitum* groups.
2. The metabolic rhythm is reflected by leptin rhythm. NC-TRF group showed significantly lower expression levels of *leptin* in white adipose tissue compared to NC-AL mice. Additionally, HF disrupted, whereas TRF preserved the robust amplitude of *leptin* rhythm. Consequently, serum leptin levels were lower during the day and retained rhythmicity in the TRF groups, whereas AL feeding blunted leptin oscillation.
3. HF diet elevated CD11b levels on the surface of neutrophils and monocytes in the peripheral blood, and the immune cell-activating effect was reversed by TRF.
4. TRF significantly reduced arthritis severity, as evidenced by clinical scores, grip strength tests, and immune cell analysis. Additionally, HF diet increased neutrophil migratory potential.
5. HF worsened, whereas TRF ameliorated acute contact hypersensitivity. Tissue neutrophil count, IL-1β, CXCL2, and leptin levels correlated positively with CHS

severity. We hypothesized that leptin exacerbates inflammation via neutrophil recruitment. Our results show anti-inflammatory effects of leptin receptor blockade in the acute CHS model, both on *db/db* and *wt* HF-AL mice. Furthermore, our study reveals the preventive and therapeutic potential of TRF in both acute and subacute CHS models.

Our findings and their potential translation into clinical practice may provide novel insights into prevention and treatment of rheumatoid arthritis and contact dermatitis.

6. Bibliography of the candidate's publications

Publications related to the thesis:

Bur Z, Vendl B, Lumniczky Z, Farkas B, Szanto CG, Czaran D, Ella K and Kaldi K - Temporal control of feeding attenuates allergic dermatitis via reduced leptin production in mice
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IF: 0

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Front Immunol. 2025, doi: 10.3389/fimmu.2025.1512328
IF: 5.9

Ella K, Sudy AR, **Bur Z**, Koos B, Kisiczki AS, Mocsai A and Kaldi K - Time restricted feeding modifies leukocyte responsiveness and improves inflammation outcome.
Front Immunol. 2022, doi: 10.3389/fimmu.2022.924541
IF: 7.3

∑IF: 13.2