

# Long-term impact of caloric restriction on liver health and cell survival in a mouse model

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## Research Article

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# Abstract

**Objective:** This study investigated the effects of chronic and intermittent caloric restriction (CR) on apoptotic and anti-apoptotic signaling in the liver of aging male C57BL/6 mice.

**Methods:** A total of 97 mice were assigned to Baseline, Ad Libitum (AL), Chronic Caloric Restriction (CCR; 25% restriction), and Intermittent Caloric Restriction (ICR; alternating 3-week periods of AL feeding and 50% restriction) groups. Liver tissue samples were collected at predefined time points, and BCL-2 and caspase-3 immunoreactivity in hepatocytes and Kupffer cells was evaluated immunohistochemically using a semi-quantitative H-score (0–300). Group differences were analyzed using ANOVA, Kruskal–Wallis, or Mann–Whitney U tests as appropriate.

**Results:** At 49 weeks, hepatocyte caspase-3 expression was significantly higher in CCR and ICR-RF than in AL (Kruskal–Wallis,  $p=0.0023$ ; adjusted  $p=0.0096$  and  $p=0.0151$ , respectively), whereas no significant differences were observed at 85 weeks ( $p=0.34$ ). At 85 weeks, Kupffer-cell BCL-2 expression was significantly higher in CCR than in AL and ICR-RF (ANOVA,  $p<0.001$ ), and ICR-RF was also higher than AL.

**Conclusion:** CR modulates hepatic apoptotic and anti-apoptotic signaling in an age- and regimen-dependent manner, with distinct responses in hepatocytes and Kupffer cells.

**Innovation:** This study provides a cell-specific comparison of chronic and intermittent CR across different stages of aging. These findings provide a basis for understanding how different CR regimens may influence hepatic cellular adaptation during aging and support further studies evaluating their relevance to age-related liver health.

## Introduction

Caloric restriction (CR), defined as the reduction in energy intake by a 10–40% without nutrient deficiency, is a well-documented intervention shown to extend lifespan and health span across various animal models. CR promotes healthy aging by delaying molecular and cellular decline. These include neuroendocrine adaptations such as increased insulin sensitivity, reduced anabolic and metabolic hormone levels, and the prevention of inflammation (Pignatti et al. 2020). Additionally, CR enhances DNA repair mechanisms, aids in the removal of damaged proteins and oxidized lipids and improves autophagy and apoptosis while suppressing growth factors and inflammatory cytokines. Studies have demonstrated that CR effectively prevents or delays chronic diseases including cancer and diabetes, and alters the crucial biological pathways involved in aging (Colman et al. 2009; Colman et al. 2014). In rodent studies, the short term and long term effects of CR has been shown to reduce the incidence of tumor development, serum pro-inflammatory cytokines, and IGF-I levels, and has been correlated with increased levels adiponectin signaling molecule, specifically adiponectin (Dogan et al. 2017; Rogozina et al. 2011). In primates, CR has been associated with lower rates of glucose intolerance, cardiovascular disease, and cancer, as well as a reduction in age-related issues such as sarcopenia and cognitive decline (Colman et al. 2008; Colman et al. 2009; Colman et al. 2014). Historical population studies

highlight the relationship between forced CR during periods of scarcity and improved mortality rates. Although long-term CR in humans may not conclusively extend maximal lifespan, it induces advantageous metabolic and hormonal changes conducive to overall health improvement (Ravussin et al. 2015). The liver is a major target organ for the metabolic effects of caloric restriction because of its central role in nutrient sensing, glucose and lipid metabolism, energy homeostasis, and adaptation to metabolic stress. Aging is associated with progressive alterations in hepatic metabolism, mitochondrial function, inflammatory responses, and cellular homeostasis, which may increase susceptibility to steatosis, fibrosis, and other age-related liver abnormalities. CR may counteract several of these changes by shifting cellular activity from growth and proliferation toward maintenance, stress resistance, and repair. In the liver, these adaptations include enhanced autophagic recycling and modulation of apoptosis-related pathways (Ando et al. 2002; Teofilović et al. 2022). In particular, early-onset CR has been reported to attenuate age-associated hepatic steatosis, inflammation, and fibrosis while preserving mitochondrial homeostasis and ATP production in aged mice (Chiang et al. 2023).

Hepatocytes and Kupffer cells are particularly relevant for evaluating these hepatic adaptations because they represent two functionally distinct but closely interacting cellular compartments. Hepatocytes constitute the major parenchymal cell population of the liver and are responsible for most hepatic metabolic functions; consequently, the balance between hepatocyte survival and apoptosis is critical for maintaining liver homeostasis during aging. Previous experimental evidence indicates that aging and lifelong CR can alter the expression of apoptosis-related genes in the liver, supporting the relevance of evaluating cell-survival pathways in hepatocytes (Ando et al. 2002). Kupffer cells, the resident macrophages of the liver, are key regulators of hepatic immune surveillance, inflammatory responses, tissue homeostasis, and communication with hepatocytes. Age-related alterations in hepatic inflammatory responses may contribute to chronic low-grade inflammation and liver dysfunction, and these processes can be modulated by caloric restriction (Chiang et al. 2023; Teofilović et al. 2022). Therefore, assessing hepatocytes and Kupffer cells separately may provide complementary information regarding the effects of CR on both the parenchymal and resident immune compartments of the aging liver.

Although chronic caloric restriction (CCR) has been extensively investigated and has shown beneficial effects on several age-related hepatic alterations, considerably less is known about the long-term hepatic cellular responses to intermittent caloric restriction (ICR), particularly during restriction and refeeding phases. Furthermore, whether CCR and ICR differentially modulate apoptotic and anti-apoptotic signaling in hepatocytes and Kupffer cells during aging remains insufficiently characterized. Therefore, the present study aimed to compare the effects of CCR and ICR on liver cell-survival responses by evaluating caspase-3 and BCL-2 expression separately in hepatocytes and Kupffer cells at different stages of aging.

## Material and Methods

### Animals and Diet

A total of 97 male C57BL6 mice were used in this study. After 10 weeks of age male C57BL6 mice were randomly assigned to one of the following experimental groups: 1. Baseline (BL, n = 5 mice, with sampling at week 10), 2. Ad libitum (AL, n = 22 mice, 4 subgroups, with sampling at weeks 46, 49, and 85), 3. Chronic Caloric Restriction (CCR, n = 38 mice, 4 subgroups, with sampling at weeks 46, 49, 82, and 85), 4. Intermittent Caloric Restriction (ICR, n = 32). Mice in the AL group had free access to diet throughout the study. Chronic caloric restriction (CCR) was implemented as a 25% reduction relative to age-matched AL controls, corresponding to 75% of AL caloric intake, based on daily food consumption measurements. After 10 weeks, the ICR group of mice had 3 weeks of AL feeding (at a rate matching 100% of age-matched AL consumption), followed by 50% CR relative to age-matched AL controls, formulated to maintain isocaloric values. The ICR group was further divided into two subgroups: Mice that were dissected at the end of the restriction period is referred as Intermittent Caloric Restriction-Restricted (ICR-R, n = 18 mice, with sampling at weeks 46 and 82). Mice that were dissected at the end of the refeeding period are referred as Intermittent Caloric Restriction-Refed (ICR-RF, n = 14 mice, with sampling at weeks 49 and 85). Information regarding the groups and the weeks at which sampling was conducted is provided in Table 1. The overall experimental design and dietary intervention timeline are summarized in Fig. 1

Table 1  
Distribution of mice by age and caloric restriction protocols

| Group         | Dietary regimen                                     | Intake (% of AL) | Sacrifice age (weeks) | n  |
|---------------|-----------------------------------------------------|------------------|-----------------------|----|
| Baseline (BL) | Standard diet                                       | 100%             | 10                    | 5  |
| AL            | Ad libitum                                          | 100%             | 46                    | 8  |
| AL            | Ad libitum                                          | 100%             | 49                    | 5  |
| AL            | Ad libitum                                          | 100%             | 85                    | 9  |
| CCR           | Chronic caloric restriction                         | 75%              | 46                    | 4  |
| CCR           | Chronic caloric restriction                         | 75%              | 49                    | 12 |
| CCR           | Chronic caloric restriction                         | 75%              | 82                    | 11 |
| CCR           | Chronic caloric restriction                         | 75%              | 85                    | 11 |
| ICR-R         | Intermittent Caloric Restriction, restriction phase | 50%              | 46                    | 10 |
| ICR-RF        | Intermittent Caloric Restriction, refeeding phase   | 100%             | 49                    | 6  |
| ICR-R         | Intermittent Caloric Restriction, restriction phase | 50%              | 82                    | 8  |
| ICR-RF        | Intermittent Caloric Restriction, refeeding phase   | 100%             | 85                    | 8  |

The experimental sampling time points were weeks 46, 49, 82, and 85; however, not all dietary groups were represented at every time point. Specifically, no independent AL cohort was available at week 82. These time points were selected to capture key phases of aging and dietary intervention effects. Week 46 was chosen to assess the immediate effects of ICR at the end of the initial restriction period of middle-age. Week 49 was selected to evaluate the early response to refeeding in the ICR group. Weeks 82 and 85 were chosen to represent late-stage effects and to determine the long-term impact of both CCR and ICR dietary restriction regimens on liver health. All mice utilized the same Altromin 1314 diet sourced from Kobay (Ankara, Türkiye), recognized for its stringent quality control measures that ensure consistent nutritional standards. Animals had free access to water throughout the study. All animal care protocols were approved by the Yeditepe University Animal Ethics Committee, Istanbul, Türkiye, an AAALAC-accredited facility.

### Tissue Sampling and Analysis

At the designated sacrifice ages (10 weeks for BL; 46, 49, and 85 weeks for AL; 46, 49, 82, and 85 weeks for CCR; 46 and 82 weeks for ICR-R; and 49 and 85 weeks for ICR-RF), mice were fasted overnight, and liver tissues were harvested immediately following animal dissection. The liver samples were immediately fixed, embedded, and sectioned for histological analysis.

### Immunohistochemistry

Liver tissue samples were fixed in 10% neutral-buffered formalin, routinely processed, and embedded in paraffin. Sections of 4 µm thickness were obtained from formalin-fixed, paraffin-embedded tissue blocks and mounted on positively charged glass slides. The sections were deparaffinized in xylene and rehydrated through a graded series of ethanol solutions to distilled water. Heat-induced epitope retrieval was performed using citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min.

Immunohistochemical staining was performed to assess apoptotic and anti-apoptotic profiles in liver tissues. Sections were incubated with anti-caspase-3 rabbit polyclonal antibody (Cell Signaling Technology, Cat. No. 9662; 1:500 dilution) and anti-BCL-2 mouse monoclonal antibody (clone 124, Dako, Cat. No. M0887; 1:100 dilution) overnight at 4°C. Following incubation with the primary antibodies and washing, the sections were incubated with an appropriate horseradish peroxidase (HRP)-based polymer detection system according to the manufacturer's instructions. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) as the chromogen. Sections were counterstained with hematoxylin, dehydrated through graded ethanol, cleared in xylene, and mounted.

Immunohistochemical evaluation was performed separately for hepatocytes and Kupffer cells using a conventional light microscope (Olympus BX51).

Immunohistochemical evaluation was performed separately for hepatocytes and Kupffer cells. The entire evaluable liver tissue section was systematically examined for each animal rather than using

selected high-power fields. Hepatocytes and Kupffer cells were distinguished based on morphological criteria, including cell size, nuclear features, cytoplasmic characteristics, and sinusoidal localization. Immunoreactivity for caspase-3 and BCL-2 was evaluated semi-quantitatively using the H-score method. The percentages of cells showing weak, moderate, and strong staining were estimated separately. Staining intensity was categorized as 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong). The H-score was calculated as:  $H\text{-score} = (1 \times \text{percentage of weakly stained cells}) + (2 \times \text{percentage of moderately stained cells}) + (3 \times \text{percentage of strongly stained cells})$ , yielding a score ranging from 0 to 300. All evaluations were performed by an experienced pathologist who was blinded to the experimental group assignments.

## Statistical Analysis

Data was analyzed using JASP (v 0.19.3, NL) and presented as mean  $\pm$  SD. Normality of continuous variables was examined with the Shapiro–Wilk test. H-score data are presented as mean  $\pm$  standard deviation (SD). For group comparisons, statistical analysis was determined by one-way analysis of variance (ANOVA) followed by Bonferroni-adjusted pair-wise t-tests. Non-parametric data was analyzed by Kruskal Wallis test followed by Dunn’s multiple comparison. For two group comparison, statistical analysis was determined by either Student’s t-test or Mann-Whitney U test depending on the normality of the data. Statistical significance was set at  $p < 0.05$  (two-tailed).

Normality of continuous variables was examined with the Shapiro–Wilk test, and equality of variances across groups with Levene’s test. When data satisfied both assumptions, group differences were analyzed with a one-way analysis of variance (ANOVA). Significant ANOVA results were explored by Bonferroni-adjusted pair-wise t-tests. If normality was violated, the non-parametric Kruskal–Wallis test replaced ANOVA. Significant Kruskal-Wallis findings were followed by Dunn’s multiple-comparison test with Nonferrous correction. For two-group comparisons, Student’s t-test was applied when normality was held, whereas the Mann-Whitney U test was used for non-normal data. All calculations were carried out in JASP (v 0.19.3). Statistical significance was set at  $p < 0.05$  (two-tailed). For the Set 5 (old-age, 82–85 week) comparison reported in Results, a preliminary within-arm check compared the CCR group at 82 versus 85 weeks to assess whether adjacent sacrifice weeks could be treated as equivalent; this revealed a significant difference in hepatocyte BCL-2 (Mann–Whitney U,  $p = 0.032$ ). Sacrifice weeks were therefore not pooled within a dietary arm, and each group in Set 5 is represented by data from a single sacrifice week. No independent AL cohort was available at 82 weeks. Therefore, the AL observations included in Set 5 correspond to the same animals evaluated at 85 weeks and were not counted as additional animals in the total study sample.

## Results

Study population and interventions

Results are presented according to predefined age-matched comparisons at 49 and 85 weeks, together with an exploratory late-life comparison (Set 5) using the nearest available cohorts at 82–85 weeks. Statistical significance was interpreted based on post-hoc analyses following omnibus tests, and findings without statistically significant pairwise differences were not overinterpreted.

In this study, short term and long term effects of two different caloric restriction protocols. Three main dietary regimens applied over two different durations (49 weeks and 85 weeks) were compared: Ad libitum (AL), Continuous Caloric Restriction (CCR), and Intermittent Caloric Restriction with Refeeding (ICR-RF; 3 weeks of restriction – 3 weeks of AL periods).

The following groups were analyzed for each duration: Set 1 (49 weeks, AL – CCR – ICR-RF). Set 2 (85 weeks, AL – CCR – ICR-RF). Furthermore, pairwise comparisons were conducted for the ICR group between the ICR-R (Intermittent Caloric Restriction-Restricted) animals dissected at weeks 46 and 82, at the end of the restriction period, and the ICR-RF animals dissected after refeeding. Set 3 compared ICR-R animals at 46 weeks with ICR-RF animals at 49 weeks, whereas Set 4 compared ICR-R animals at 82 weeks with ICR-RF animals at 85 weeks. These comparisons were designed to evaluate the restriction and subsequent refeeding phases within the ICR regimen and therefore involved adjacent, rather than identical, sacrifice ages. Because an independent AL cohort was not available at 82 weeks, an exploratory four-group old-age comparison (Set 5) was constructed using the nearest available late-life cohorts: AL and ICR-RF animals at 85 weeks and CCR and ICR-R animals at 82 weeks. The AL data included in Set 5 were the same 85-week AL data used in the 85-week analysis and did not represent an additional group of animals. Therefore, Set 5 was considered an exploratory late-life comparison rather than a strictly age-matched comparison. Hepatic BCL-2/Caspase-3 data for the AL, CCR, and ICR-R cohorts at 46 weeks were not available at the time of this analysis; the Set 1 adult-stage comparison (49 weeks) is therefore limited to AL, CCR, and ICR-RF, and no adult-stage comparison is reported for ICR-R. Immunohistochemical expression of BCL-2 and caspase-3 was quantified using H-scores ranging from 0 to 300.

### BCL-2 Levels in Hepatocytes

At 49 weeks, the Kruskal–Wallis test indicated a near-threshold omnibus effect ( $p = 0.051$ ); however, post-hoc Dunn's tests revealed no statistically significant pairwise differences among groups. At 85 weeks, although the omnibus test reached statistical significance ( $p = 0.020$ ), none of the post-hoc comparisons remained significant after adjustment. Therefore, no definitive group-wise differences in hepatocyte BCL-2 expression could be established at either time point. Hepatocyte BCL-2 immunoreactivity across age and dietary groups is summarized in Fig. 4.

### BCL-2 Levels in Kupffer Cells

Set 1 (49 weeks): One-way ANOVA:  $p = 0.17 \rightarrow$  not significant. Set 2 (85 weeks): One-way ANOVA:  $p < 0.001$ ; post-hoc Pairwise t-test: AL vs CCR:  $p = 8.9 \times 10^{-7}$  ( $p < 0.001$ ); AL vs ICR-RF:  $p = 4.9 \times 10^{-3}$  ( $p < 0.01$ ); CCR vs ICR-RF:  $p = 1.99 \times 10^{-4}$  ( $p < 0.001$ ). The CCR group was dramatically higher compared to both AL

and ICR-RF. At 85 weeks, CCR was associated with a marked increase in BCL-2 expression in Kupffer cells compared with both AL and ICR-RF groups, indicating a robust anti-apoptotic adaptation of hepatic macrophages under long-term continuous restriction. Representative BCL-2 immunostaining patterns at 85 weeks are shown in Fig. 3. In the same Set 5 (old-age, four-group) comparison, the omnibus effect for Kupffer-cell BCL-2 was borderline (Kruskal–Wallis,  $p = 0.050$ ), but no individual pairwise comparison remained significant after Bonferroni correction. Kupffer-cell BCL-2 immunoreactivity across age and dietary groups is summarized in Fig. 6.

### Caspase-3 Activity in Hepatocytes

At 49 weeks, hepatocyte caspase-3 expression differed significantly among groups (Kruskal–Wallis,  $p = 0.0023$ ), with both CCR and ICR-RF showing higher levels compared with AL in post-hoc analyses. Representative caspase-3 immunostaining patterns at 49 weeks are shown in Fig. 2. At 82 weeks, a significant difference between CCR and ICR-R was observed (Mann–Whitney U,  $p = 0.013$ ). In contrast, at 85 weeks no significant group differences were detected (Kruskal–Wallis,  $p = 0.34$ ). Collectively, these findings indicate a transient increase in hepatocyte apoptotic signaling at mid-life that is not sustained at advanced age. When the AL and ICR-RF groups (85 weeks) were combined with the CCR and ICR-R groups (82 weeks) into a single four-group “old-age” comparison (Set 5), hepatocyte caspase-3 expression again differed significantly (Kruskal–Wallis,  $p = 0.0005$ ). Dunn’s post-hoc tests (Bonferroni-corrected) showed the ICR-R group was significantly higher than AL ( $p = 0.0056$ ), CCR ( $p = 0.0163$ ), and ICR-RF ( $p = 0.0010$ ), while ICR-RF did not differ from AL. In this exploratory late-life comparison, higher hepatocyte caspase-3 expression was observed in the ICR-R group than in the other dietary groups, suggesting an association between the active restriction phase and increased apoptotic signaling. However, this finding should be interpreted cautiously because the compared groups were sampled at adjacent rather than identical ages. Hepatocyte caspase-3 immunoreactivity across age and dietary groups is summarized in Fig. 5.

### Caspase-3 Activity in Kupffer Cells

Caspase-3 immunoreactivity in Kupffer cells was absent or near the detection threshold in most groups and time points. At 82 weeks, low-level caspase-3 immunoreactivity was observed in the CCR group ( $1.3 \pm 3.5$ ), but this did not differ significantly from the ICR-R group (Mann–Whitney U,  $p = 0.31$ ). Overall, Kupffer-cell caspase-3 expression remained minimal across the experimental groups. Kupffer-cell caspase-3 immunoreactivity across age and dietary groups is summarized in Fig. 7. Comparisons between the restriction and subsequent refeeding phases (ICR-R at 46 weeks vs. ICR-RF at 49 weeks, and ICR-R at 82 weeks vs. ICR-RF at 85 weeks) revealed no significant differences in BCL-2 or caspase-3 expression (Mann–Whitney U tests,  $p > 0.05$ ), suggesting comparable apoptotic and anti-apoptotic responses between these phases of the cyclic ICR regimen. These comparisons involved adjacent, rather than identical, sacrifice ages. The results are summarized in Tables 2 and 3.

Table 2

Hepatocyte and Kupffer-cell BCL-2 and caspase-3 H-scores across dietary groups and time points.

| Time            | Marker        | Cell Type            | AL<br>(mean $\pm$<br>SD, n)      | CCR<br>(mean $\pm$<br>SD, n)  | * ICR-<br>R/ICR-<br>RF<br>(mean $\pm$<br>SD, n) | Test                  | p-value                |
|-----------------|---------------|----------------------|----------------------------------|-------------------------------|-------------------------------------------------|-----------------------|------------------------|
| BL<br>(10<br>W) | BCL-2         | Hepatocytes          | 14,4 $\pm$<br>20,1, n =<br>5     | –                             | –                                               | –                     | No<br>comparison       |
|                 | Caspase-<br>3 | Hepatocytes          | 16,7 $\pm$<br>15,3, n =<br>3     | –                             | –                                               | –                     | No<br>comparison       |
|                 | BCL-2         | Kupffer cells        | 88,0 $\pm$<br>59,3, n =<br>5     | –                             | –                                               | –                     | No<br>comparison       |
|                 | Caspase-<br>3 | Kupffer<br>hücreleri | 0,0 $\pm$ 0,0,<br>n = 4          | –                             | –                                               | –                     | No<br>comparison       |
| 49 W            | BCL-2         | Hepatocytes          | 44,0 $\pm$<br>20,7, n =<br>5     | 17,9 $\pm$<br>23,8, n<br>= 12 | 28,3 $\pm$<br>17,2, n =<br>6                    | Kruskal–<br>Wallis    | p = 0,051              |
|                 | Caspase-<br>3 | Hepatocytes          | 0,0 $\pm$ 0,0,<br>n = 5          | 28,1 $\pm$<br>18,1, n<br>= 8  | 45,0 $\pm$<br>13,8, n =<br>6                    | Kruskal–<br>Wallis    | p = 0,0023             |
|                 | BCL-2         | Kupffer cells        | 98,0 $\pm$<br>46,6, n =<br>5     | 104,4 $\pm$<br>50,0, n<br>= 9 | 60,0 $\pm$<br>27,6, n =<br>6                    | One-way<br>ANOVA      | p = 0,17               |
|                 | Caspase-<br>3 | Kupffer cells        | 0,0 $\pm$ 0,0<br>(All<br>groups) | 0,0 $\pm$<br>0,0              | 0,0 $\pm$ 0,0                                   | –                     | Test not<br>applicable |
| 82 w            | BCL-2         | Hepatocytes          | –                                | 27,1 $\pm$<br>25,2, n<br>= 8  | 23,6 $\pm$<br>19,7, n =<br>7                    | Mann–<br>Whitney<br>U | p = 0,78               |
|                 | Caspase-<br>3 | Hepatocytes          | –                                | 3,8 $\pm$<br>5,2, n =<br>8    | 34,3 $\pm$<br>12,7, n =<br>7                    | Mann–<br>Whitney<br>U | p = 0,013              |

\* ICR-R values correspond to animals sacrificed during the restriction phase at 82 weeks, whereas ICR-RF values correspond to animals sacrificed following refeeding at 49 and 85 weeks.

H-scores range from 0 to 300, with higher scores indicating greater immunoreactivity. Data are presented as mean  $\pm$  SD unless otherwise indicated.

Data are presented as mean  $\pm$  standard deviation. Statistical analyses were performed using Kruskal–Wallis or one-way ANOVA, followed by appropriate post-hoc tests where applicable. Significance was defined as p < 0.05.

| Time                                                                                                                                                                                                                           | Marker    | Cell Type     | AL<br>(mean $\pm$<br>SD, n)   | CCR<br>(mean $\pm$<br>SD, n) | * ICR-<br>R/ICR-<br>RF<br>(mean $\pm$<br>SD, n) | Test           | p-value             |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|---------------|-------------------------------|------------------------------|-------------------------------------------------|----------------|---------------------|
|                                                                                                                                                                                                                                | BCL-2     | Kupffer cells | –                             | 126,3 $\pm$<br>71,5, n = 8   | 76,7 $\pm$<br>39,8, n = 6                       | Mann–Whitney U | p = 0,22            |
|                                                                                                                                                                                                                                | Caspase-3 | Kupffer cells | –                             | 1,3 $\pm$<br>3,5, n = 8      | 0,0 $\pm$ 0,0, n = 7                            | Mann–Whitney U | p = 0,31            |
| 85 w                                                                                                                                                                                                                           | BCL-2     | Hepatocytes   | 23,9 $\pm$<br>26,4, n = 7     | 71,4 $\pm$<br>44,9, n = 7    | 48,3 $\pm$<br>40,2, n = 6                       | Kruskal–Wallis | p = 0,02            |
|                                                                                                                                                                                                                                | Caspase-3 | Hepatocytes   | 5,7 $\pm$<br>15,1, n = 7      | 10,0 $\pm$<br>26,5, n = 7    | 0,0 $\pm$ 0,0, n = 6                            | Kruskal–Wallis | p = 0,34            |
|                                                                                                                                                                                                                                | BCL-2     | Kupffer cells | 68,6 $\pm$<br>30,8, n = 7     | 231,4 $\pm$<br>29,7, n = 7   | 131,7 $\pm$<br>27,9, n = 6                      | One-way ANOVA  | p < 0,001           |
|                                                                                                                                                                                                                                | Caspase-3 | Kupffer cells | 0,0 $\pm$ 0,0<br>(All groups) | 0,0 $\pm$<br>0,0             | 0,0 $\pm$ 0,0                                   | –              | Test not applicable |
| * ICR-R values correspond to animals sacrificed during the restriction phase at 82 weeks, whereas ICR-RF values correspond to animals sacrificed following refeeding at 49 and 85 weeks.                                       |           |               |                               |                              |                                                 |                |                     |
| H-scores range from 0 to 300, with higher scores indicating greater immunoreactivity. Data are presented as mean $\pm$ SD unless otherwise indicated.                                                                          |           |               |                               |                              |                                                 |                |                     |
| Data are presented as mean $\pm$ standard deviation. Statistical analyses were performed using Kruskal–Wallis or one-way ANOVA, followed by appropriate post-hoc tests where applicable. Significance was defined as p < 0.05. |           |               |                               |                              |                                                 |                |                     |

Table 3

Post-hoc pairwise comparisons of hepatocyte and Kupffer-cell BCL-2 and caspase-3 H-scores across dietary groups.

| Time                                                                                                                                                                                                                                                                          | Marker    | Cell Type     | Comparison    | Raw p                 | Adjusted p            | Interpretation          |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|---------------|---------------|-----------------------|-----------------------|-------------------------|
| 49 w                                                                                                                                                                                                                                                                          | BCL-2     | Hepatocytes   | AL vs CCR     | 0.0383                | 0.115                 | Not significant         |
|                                                                                                                                                                                                                                                                               |           |               | AL vs ICR-RF  | 0.2667                | 0.800                 | Not significant         |
|                                                                                                                                                                                                                                                                               |           |               | CCR vs ICR-RF | 0.1300                | 0.390                 | Not significant         |
| 49 w                                                                                                                                                                                                                                                                          | Caspase-3 | Hepatocytes   | AL vs CCR     | 0.00319               | 0.00956               | p < 0.01, CCR > AL      |
|                                                                                                                                                                                                                                                                               |           |               | AL vs ICR-RF  | 0.00504               | 0.01511               | p < 0.05, ICR-RF > AL   |
|                                                                                                                                                                                                                                                                               |           |               | CCR vs ICR-RF | 0.1469                | 0.441                 | Not significant         |
| 85 w                                                                                                                                                                                                                                                                          | BCL-2     | Hepatocytes   | AL vs CCR     | 0.0238                | 0.0713                | Not significant         |
|                                                                                                                                                                                                                                                                               |           |               | AL vs ICR-RF  | 0.1904                | 0.5713                | Not significant         |
|                                                                                                                                                                                                                                                                               |           |               | CCR vs ICR-RF | 0.3484                | 1.000                 | Not significant         |
| 85 w                                                                                                                                                                                                                                                                          | BCL-2     | Kupffer cells | AL vs CCR     | $2.97 \times 10^{-7}$ | $8.92 \times 10^{-7}$ | p < 0.001, CCR > AL     |
|                                                                                                                                                                                                                                                                               |           |               | AL vs ICR-RF  | $1.64 \times 10^{-3}$ | $4.91 \times 10^{-3}$ | p < 0.01, ICR-RF > AL   |
|                                                                                                                                                                                                                                                                               |           |               | CCR vs ICR-RF | $6.63 \times 10^{-5}$ | $1.99 \times 10^{-4}$ | p < 0.001, CCR > ICR-RF |
| <i>AL: ad libitum; CCR: caloric-restricted; ICR-RF: intermittent caloric restriction–re-feeding.</i>                                                                                                                                                                          |           |               |               |                       |                       |                         |
| Kupffer-cell caspase-3 immunoreactivity was absent or near the detection threshold in most groups and time points; therefore, quantitative comparisons were limited. BCL-2 data are presented as mean ± standard deviation. Statistical significance was defined as p < 0.05. |           |               |               |                       |                       |                         |

As shown in Table 4, AL and ICR-RF values are from animals sacrificed at 85 weeks, whereas CCR and ICR-R values are from animals sacrificed at 82 weeks. No independent AL cohort was available at 82 weeks; therefore, the AL data included in this exploratory Set 5 comparison correspond to the same 85-week AL cohort analyzed elsewhere and do not represent additional animals.

Table 4  
Exploratory comparison of hepatocyte and Kupffer-cell BCL-2 and caspase-3 H-scores among late-life dietary groups (82–85 weeks).

| Marker                                                                                                                                                          | Cell Type     | AL (85w)<br>mean $\pm$ SD,<br>n | CCR (82w)<br>mean $\pm$ SD,<br>n | ICR-R<br>(82w)<br>mean $\pm$ SD,<br>n | ICR-RF<br>(85w)<br>mean $\pm$ SD,<br>n | p-value |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|---------------------------------|----------------------------------|---------------------------------------|----------------------------------------|---------|
| BCL-2                                                                                                                                                           | Hepatocytes   | 23,9 $\pm$ 26,4,<br>n = 7       | 27,1 $\pm$ 25,2,<br>n = 8        | 23,6 $\pm$ 19,7,<br>n = 7             | 48,3 $\pm$ 40,2,<br>n = 6              | 0,506   |
| Caspase-3                                                                                                                                                       | Hepatocytes   | 5,7 $\pm$ 15,1,<br>n = 7        | 3,8 $\pm$ 5,2, n<br>= 8          | 34,3 $\pm$ 12,7,<br>n = 7             | 0,0 $\pm$ 0,0, n<br>= 6                | 0,0005  |
| BCL-2                                                                                                                                                           | Kupffer cells | 68,6 $\pm$ 30,8,<br>n = 7       | 126,2 $\pm$<br>71,5, n = 8       | 76,7 $\pm$ 39,8,<br>n = 6             | 131,7 $\pm$<br>27,9, n = 6             | 0,050   |
| Caspase-3                                                                                                                                                       | Kupffer cells | 0,0 $\pm$ 0,0, n<br>= 7         | 1,2 $\pm$ 3,5, n<br>= 8          | 0,0 $\pm$ 0,0, n<br>= 7               | 0,0 $\pm$ 0,0, n<br>= 6                | 0,475   |
| AL: ad libitum; CCR: caloric-restricted; ICR-R: intermittent caloric restriction, restriction phase; ICR-RF: intermittent caloric restriction, refeeding phase. |               |                                 |                                  |                                       |                                        |         |
| Data are presented as mean $\pm$ standard deviation. Statistical significance was defined as p < 0.05.                                                          |               |                                 |                                  |                                       |                                        |         |

## Discussion

The present study demonstrates that long-term caloric restriction modulates apoptotic and anti-apoptotic signaling in the liver in an age- and regimen-dependent manner. Using immunohistochemical assessment of caspase-3 and BCL-2 expression, we identified a transient increase in hepatocyte apoptotic signaling at mid-life, followed by pronounced anti-apoptotic signaling in Kupffer cells under prolonged continuous restriction. These findings are consistent with previous reports indicating that caloric restriction influences cell survival pathways and stress responses in hepatic tissue during aging (Colman et al. 2014; Fontana 2009; Jadeja et al. 2017). Importantly, the study was designed to characterize cellular responses rather than to dissect upstream molecular mechanisms.

At this juncture, the importance of Sirtuin 3 (SIRT3) should be highlighted. Studies have demonstrated that SIRT3 supports effects of caloric restriction (CR), such as reducing age-related hearing loss and oxidative damage (Someya et al. 2010). SIRT3 has been found to increase oxidative metabolism capacity and extend lifespan in male mice under caloric restriction (Dhillon et al. 2022). Additionally, recent research has also emphasized the role of sirtuins, a class of proteins involved in regulating cellular aging and metabolism, in mediating the beneficial effects of CR on liver health. Specifically, studies have shown that activation of sirtuin 1 (SIRT1) by CR can protect against hepatic steatosis and inflammation (Ding et al. 2017). Although sirtuin signaling has been widely implicated in the cellular adaptations associated with caloric restriction, these pathways were not directly evaluated in the present study. Accordingly, SIRT3-related mechanisms are discussed here solely to provide biological context for

the observed age-dependent apoptotic adaptations, rather than to imply a direct mechanistic link (Dhillon et al. 2022; Someya et al. 2010).

In addition to our findings, other studies have demonstrated the role of oxidative stress in liver diseases like non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease, suggesting that reducing oxidative stress can potentially treat or prevent these diseases (Ceni et al. 2014; Cicekdal et al. 2019). Although antioxidant defenses and oxidative stress-related pathways have been linked to caloric restriction in previous studies, these parameters were not directly measured in the present work.

Previous studies have demonstrated that caloric restriction can influence oxidative stress parameters, lipid metabolism, and mitochondrial function in the liver. For example, chronic CR has been associated with increased antioxidant capacity and altered lipidomic profiles, potentially contributing to improved cellular homeostasis during aging (Cicekdal et al. 2022; Jin 2022; Jové et al. 2014; Mitchell et al. 2015). However, as oxidative stress markers, lipidomics, and mitochondrial function were not directly assessed in the present study, these findings are cited solely to contextualize the observed apoptotic and anti-apoptotic patterns rather than to imply mechanistic causality.

The observed modulation of apoptosis in hepatocytes and Kupffer cells under different CR regimens highlights the complex interplay between cell survival and cell death in the liver. The marked increase in BCL-2 expression in Kupffer cells under continuous restriction (CCR) at 85 weeks is particularly noteworthy. This suggests that long-term CR can induce adaptive mechanisms that promote cell survival in the liver, potentially contributing to the maintenance of liver homeostasis. Given this potential for long-term adaptation, future research should focus on investigating whether this anti-apoptotic response translates to increased resilience against various forms of liver injury or disease. Such studies could employ more sophisticated in vivo models that more closely mimic the complex conditions leading to liver damage in humans, such as NAFLD or toxicant-induced injury. The absence of detectable caspase-3 immunoreactivity in Kupffer cells should be interpreted in the context of the sensitivity limits of immunohistochemistry and the known resistance of hepatic macrophages to classical apoptotic pathways under physiological conditions. Importantly, no overinterpretation of this finding was made, and conclusions were restricted to cell types and markers with robust quantitative signals.

These findings may have potential translational relevance, given the increasing burden of age-related metabolic and liver disorders. However, as the present findings are derived from an experimental mouse model, further studies are required before their clinical relevance can be established.

The present findings contribute to the understanding of how caloric restriction may influence liver cell-survival pathways during aging. Although oxidative stress has been implicated in the hepatic effects of caloric restriction in previous studies, oxidative stress markers were not directly assessed in the present study. Therefore, our findings should be interpreted specifically in the context of the observed apoptotic and anti-apoptotic signaling patterns.

It is instructive to compare our findings with those of Keinicke et al., who examined the role of fibroblast growth factor 21 (FGF21) in dietary interventions and demonstrated its involvement in metabolic regulation and hepatic homeostasis (Turnbaugh et al. 2008). While oxidative stress–related pathways and metabolic mediators such as FGF21 have been linked to the biological effects of caloric restriction in previous studies, these mechanisms were not directly assessed in the present work. Accordingly, such pathways are discussed here solely to situate the observed apoptotic and anti-apoptotic adaptations within the broader literature on dietary interventions and liver aging, without implying mechanistic causality (Jin 2022; Turnbaugh et al. 2008).

The implications of our findings extend to theoretical frameworks around aging and metabolic health. Our findings contribute to the understanding of how CR influences hepatic cell-survival signaling during aging and, when considered together with previous studies on oxidative stress and metabolic regulation, may help provide a broader perspective on age-related liver adaptations. This raises important questions for future research on the mechanistic pathways involved. For example, exploring the interplay between CR and gut microbiota could reveal additional layers of complexity in the relationship between dietary practices and metabolic health, an area highlighted by recent investigations in this domain. Recent studies have indicated that gut microbiota plays a crucial role in metabolic health, and dietary interventions can modulate gut microbiota composition, which in turn influences liver health (Jin 2022). Specifically, studies have shown that CR can alter the gut microbiota composition, leading to reduced intestinal permeability and decreased systemic inflammation, which could contribute to improved liver health [23].

Moreover, while our study highlights encouraging results regarding the benefits of caloric restriction, it is essential to acknowledge the limitations inherent in rodent studies as they may not entirely replicate human physiology. Future studies are needed to better understand how these findings translate to human health. Specifically, well-designed human clinical trials are needed to assess the effects of different CR regimens on liver health, including markers of oxidative stress, inflammation, and fibrosis. This will help illuminate how caloric restriction might translate to human dietary practices and health outcomes. Furthermore, exploring the role of individual genetic factors and gut microbiota composition in mediating the response to CR could provide personalized dietary recommendations for optimizing liver health. Moreover, the study would also benefit from exploring the effect of the same diet to a wider age group and including both males and females to better understand the effects on liver health.

Importantly, the present study is based on histological and immunohistochemical assessments and does not include functional, biochemical, or molecular measurements of liver metabolism or oxidative stress. Therefore, interpretations regarding liver resilience or cellular protection should be viewed as descriptive and limited to apoptotic and anti-apoptotic signaling patterns observed in hepatocytes and Kupffer cells. Additional functional studies will be required to determine whether these cellular adaptations translate into measurable improvements in liver function.

## Conclusion

In conclusion, long-term caloric dietary restriction using the Altromin 1314 diet induces age- and regimen-dependent modulation of apoptotic and anti-apoptotic signaling in the liver. Continuous restriction is associated with a pronounced anti-apoptotic adaptation in Kupffer cells at advanced age, while hepatocyte apoptotic responses appear transient. These findings provide a descriptive framework for liver cell survival under prolonged dietary restriction and support future studies incorporating functional and molecular validation.

## Declarations

### Acknowledgment

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### Declaration of Generative AI and AI-Assisted Technologies

Artificial intelligence (AI)-assisted tools were used solely for language editing, grammatical correction, and improving the clarity and readability of the manuscript. AI was not used to generate scientific content, data, results, analyses, or conclusions. All scientific content was reviewed and approved by the authors, who take full responsibility for the final manuscript.

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This research received no external funding.

### Conflict of Interest

The authors declare that there is no conflict of interest.

### Ethical Considerations

This study was approved by the Yeditepe University Animal Ethics Committee (no. 2021/11-12, İstanbul, Türkiye).

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## Figures

**Figure 1.** Experimental Design and Dietary Intervention Timeline

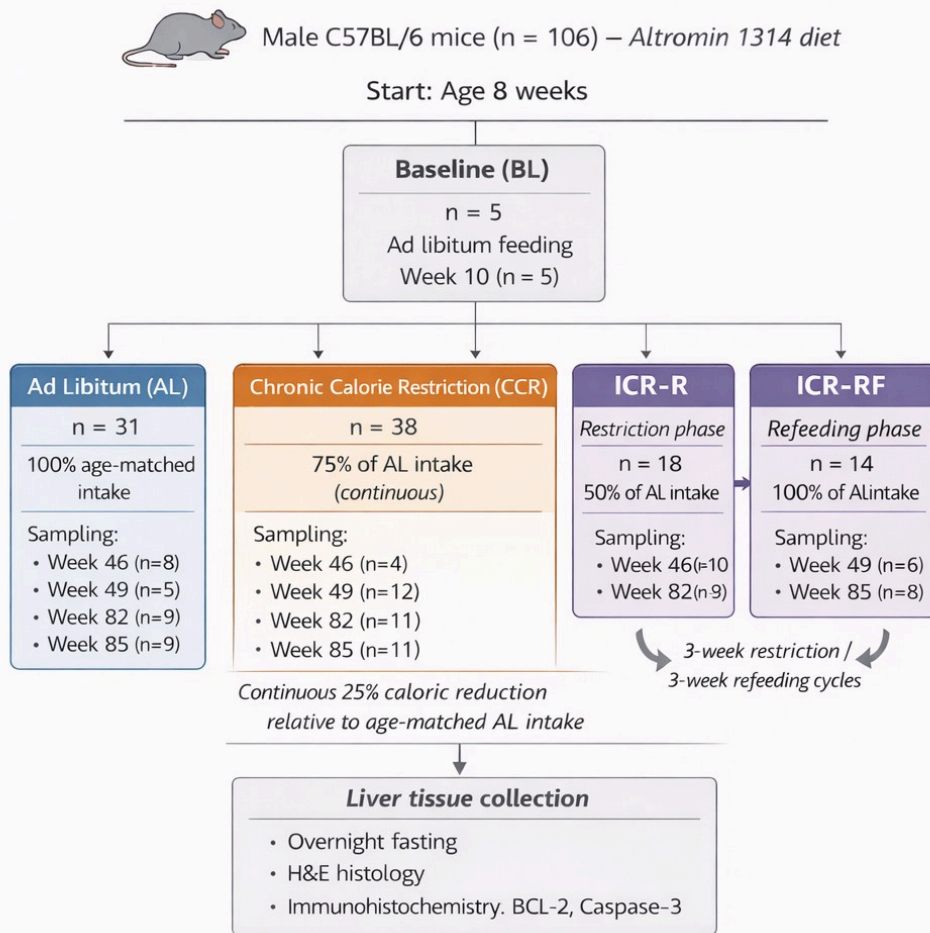


**Figure 1.** Schematic representation of the experimental design and dietary intervention timeline applied in male C57BL/6 mice. Animals were assigned to baseline (BL), ad libitum feeding (AL), chronic calorie restriction (CCR), or intermittent calorie restriction (ICR) groups. The ICR protocol consisted of alternating restriction and refeeding cycles, with tissue collection performed either at the end of the restriction phase (ICR-R) or after the refeeding phase (ICR-RF), CCR indicates a continuous 25% caloric reduction relative to age-matched ad libitum intake.

**Figure 1**

Experimental design and dietary intervention timeline.

Mice were assigned to ad libitum (AL; 100% intake) or continuous caloric restriction (CCR; 75% of AL intake) groups, with separate cohorts sacrificed at the designated time points shown in the experimental timeline. Animals assigned to the intermittent caloric restriction (ICR) protocol originated from the same initial cohort and underwent alternating periods of restriction (ICR-R; 50% of AL intake) and refeeding

(ICR-RF; 100% of AL intake). Within the ICR group, subsets of animals were sacrificed at 46 and 82 weeks during restriction phases, and at 49 and 85 weeks following refeeding. Group sizes (n) for each condition and time point are indicated in the schematic.

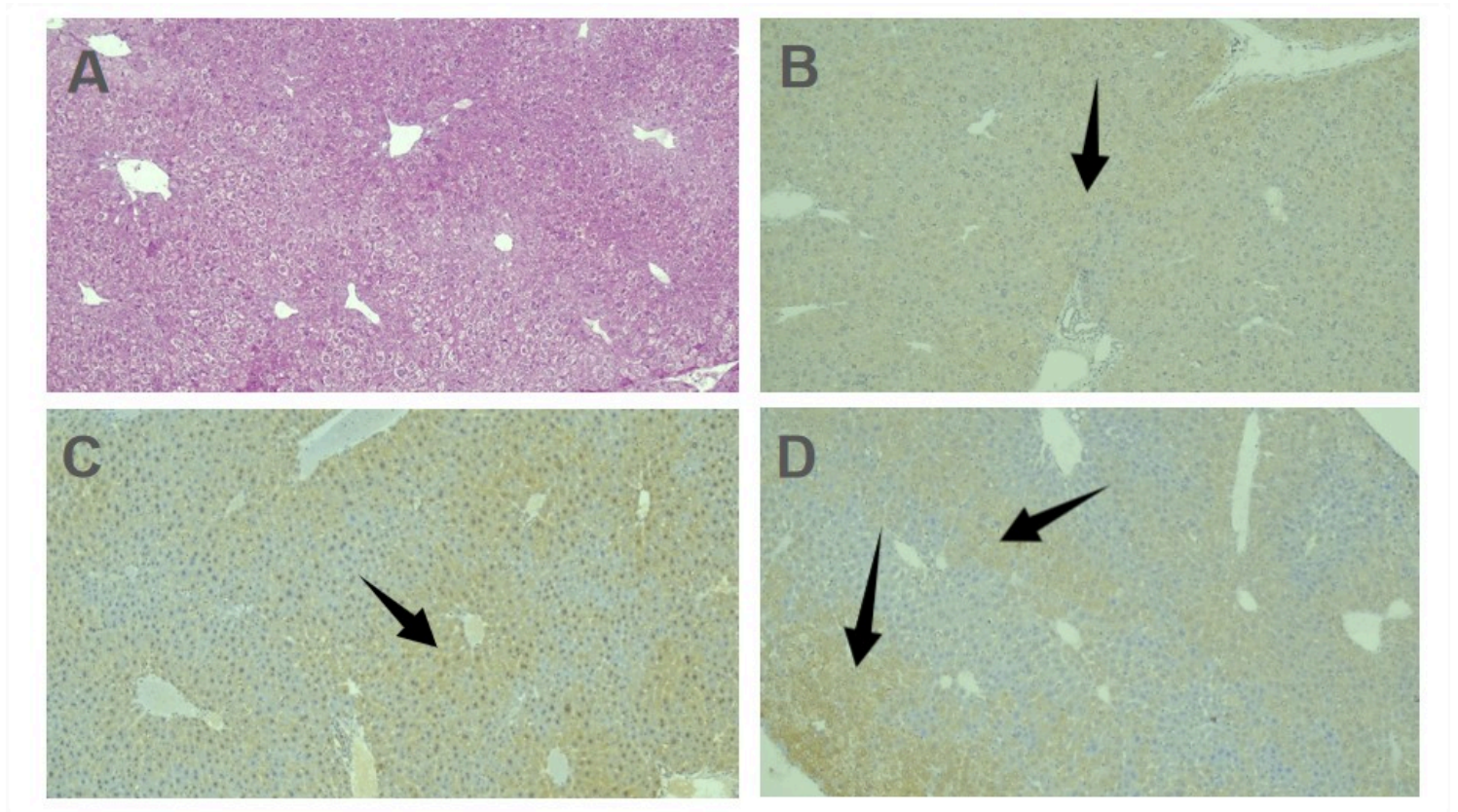


**Figure 2**

Caspase-3 Activity in Hepatocytes Under Caloric Restriction at Week 49.

A: Hematoxylin and Eosin X100, B (AL), C: (CCR), D (ICR-RF): Representative images illustrate caspase-3 immunostaining in hepatocytes across dietary groups; no specific caspase-3 staining was observed in Kupffer cells.

\*Arrows indicate staining in hepatocytes.

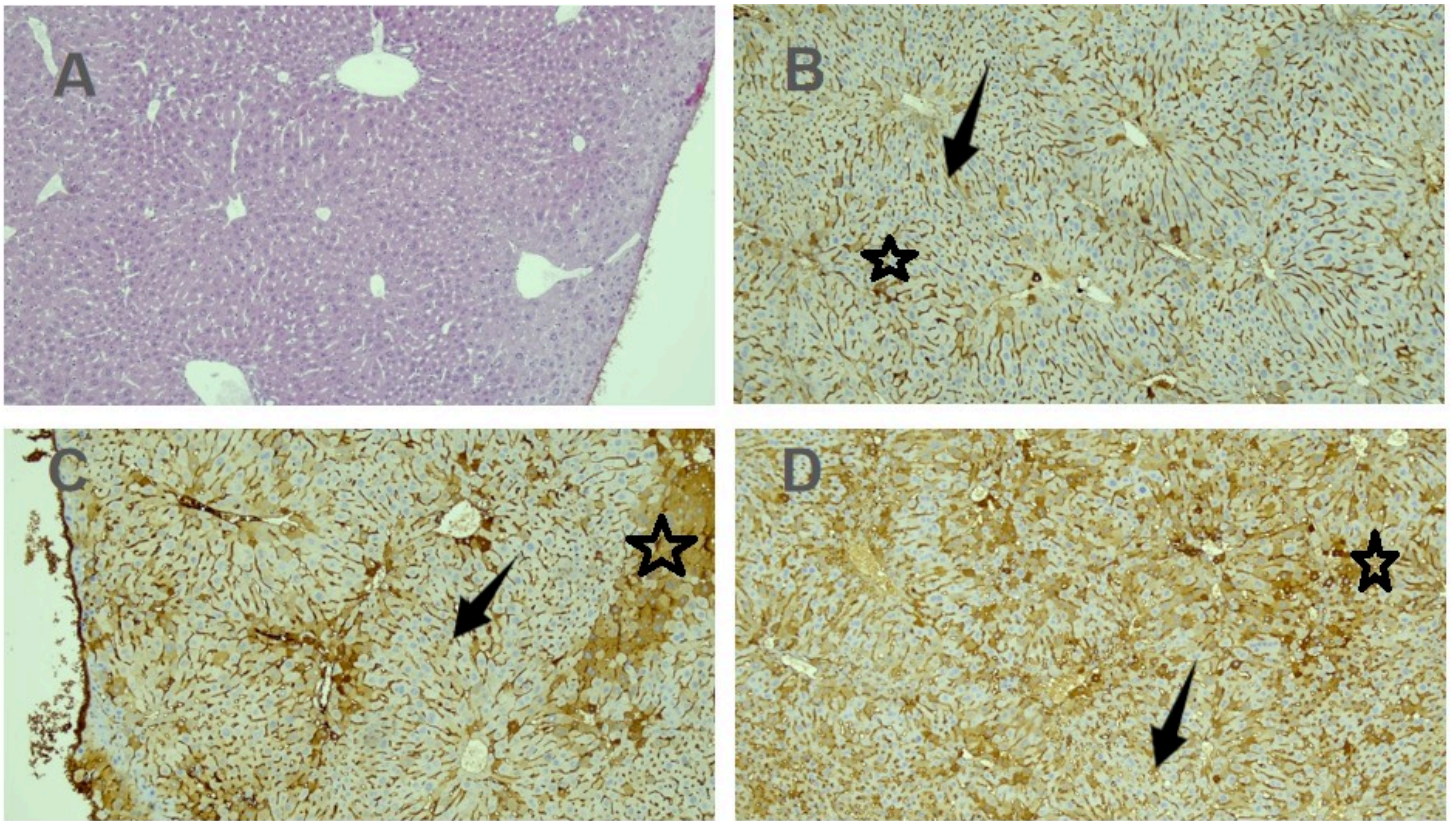


**Figure 3**

Impact of Caloric Restriction Regimens on BCL-2 Levels in Liver Cells at Week 85. A: Hematoxylin and Eosin, B (AL), C: (CCR), D (ICR-RF) X100. The strongest BCL-2 immunoreactivity was observed in the CCR group, followed by the ICR-RF group, whereas AL showed the lowest expression.

\*Arrows indicate Kupffer cells, and stars indicate staining in hepatocytes.

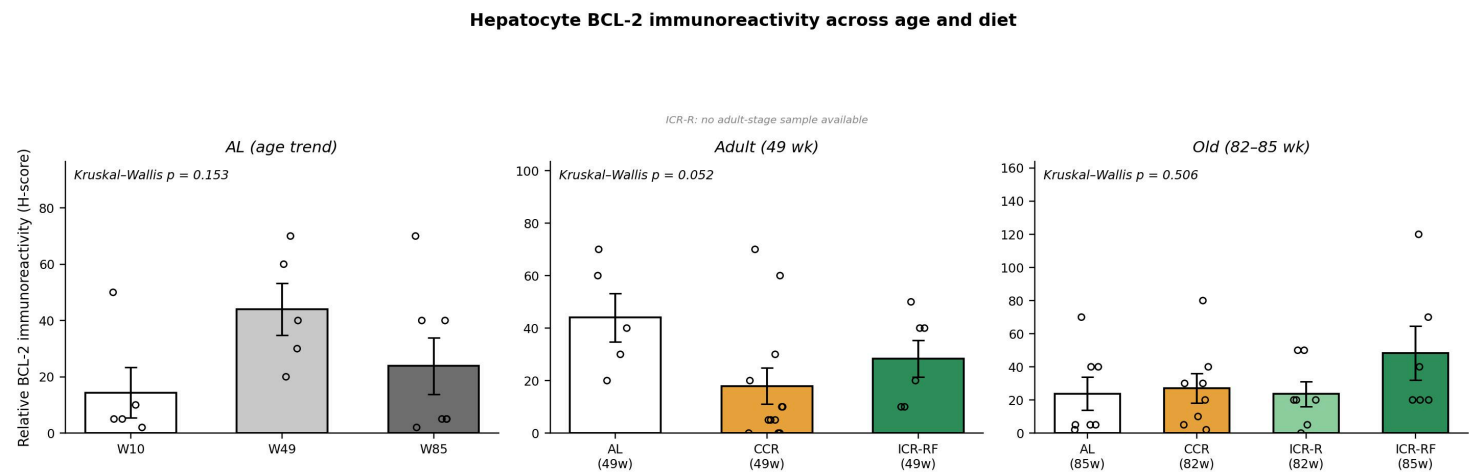


**Figure 4**

Hepatocyte BCL-2 immunoreactivity across age and diet.

(A) Hepatocyte BCL-2 immunoreactivity across the baseline and available AL cohorts (BL at 10 weeks; AL at 49 and 85 weeks). (B) Diet comparison at 49 weeks (Set 1: AL, CCR, ICR-RF; no adult-stage sample was available for ICR-R). (C) Exploratory old-age comparison (Set 5, 82–85 weeks: AL and ICR-RF at 85 weeks, CCR and ICR-R at 82 weeks).

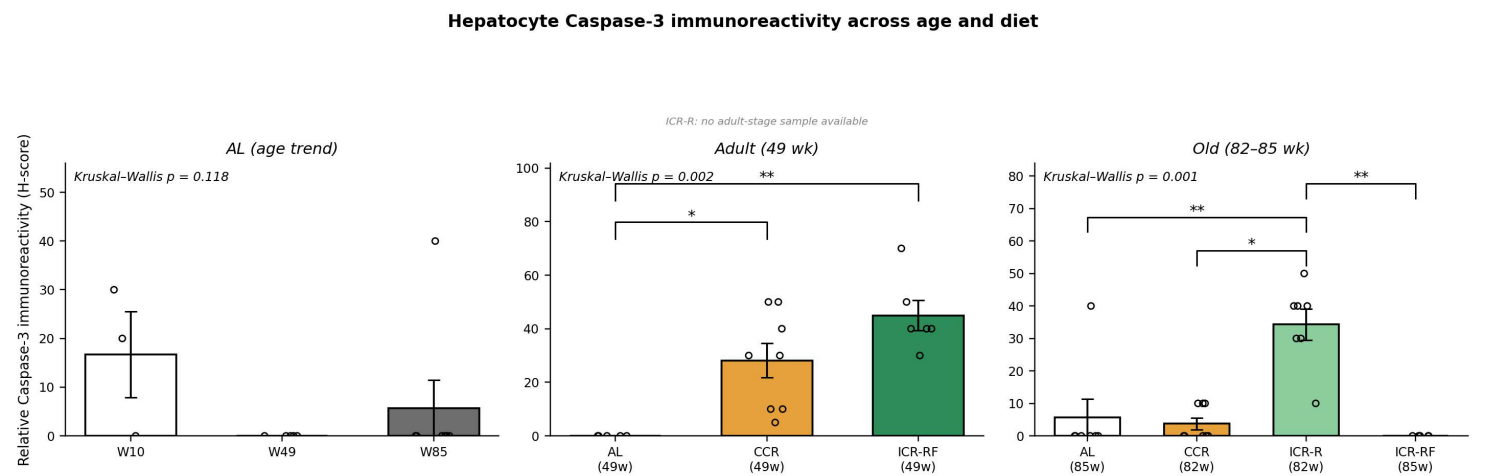


**Figure 5**

Hepatocyte Caspase-3 immunoreactivity across age and diet.

Panel layout as in Figure 4. In panel C (Set 5, old age), the ICR-R group shows significantly higher hepatocyte caspase-3 immunoreactivity than AL, CCR, and ICR-RF (Dunn’s post-hoc, Bonferroni-corrected; \*  $p < 0.05$ , \*\*  $p < 0.01$ ), consistent with higher apoptotic signaling in the ICR-R group in this exploratory late-life comparison.

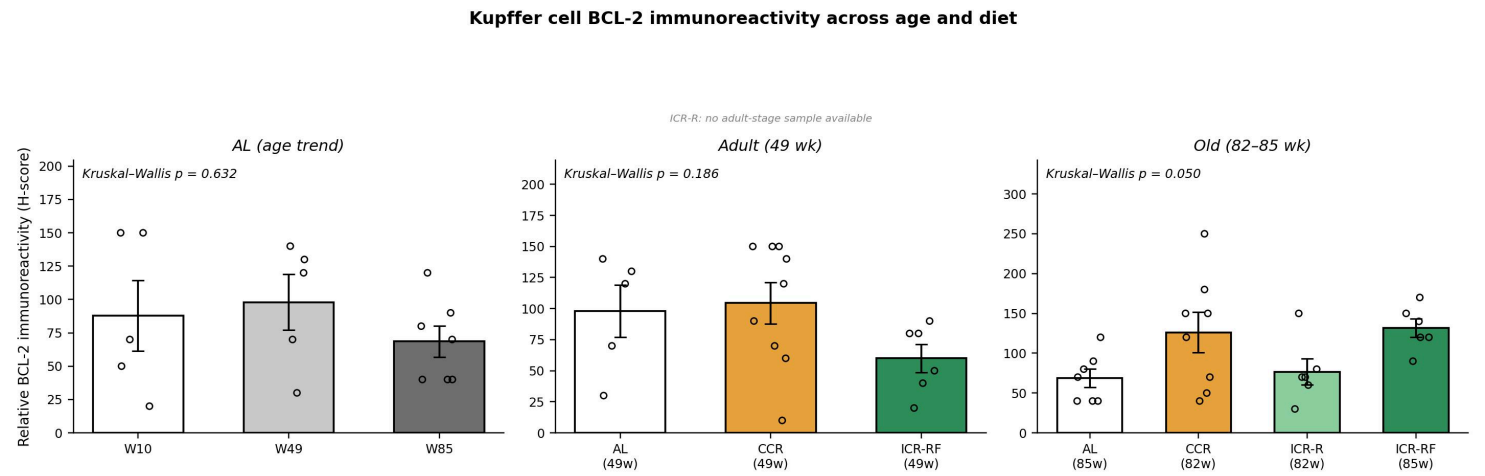


**Figure 6**

Kupffer-cell BCL-2 immunoreactivity across age and diet.

Panel layout as in Figure 4. The old-age (Set 5) omnibus comparison was borderline (Kruskal–Wallis,  $p = 0.050$ ); no individual pairwise comparison remained significant after Bonferroni correction.

# Kupffer cell Caspase-3 immunoreactivity across age and diet (near/below detection threshold)

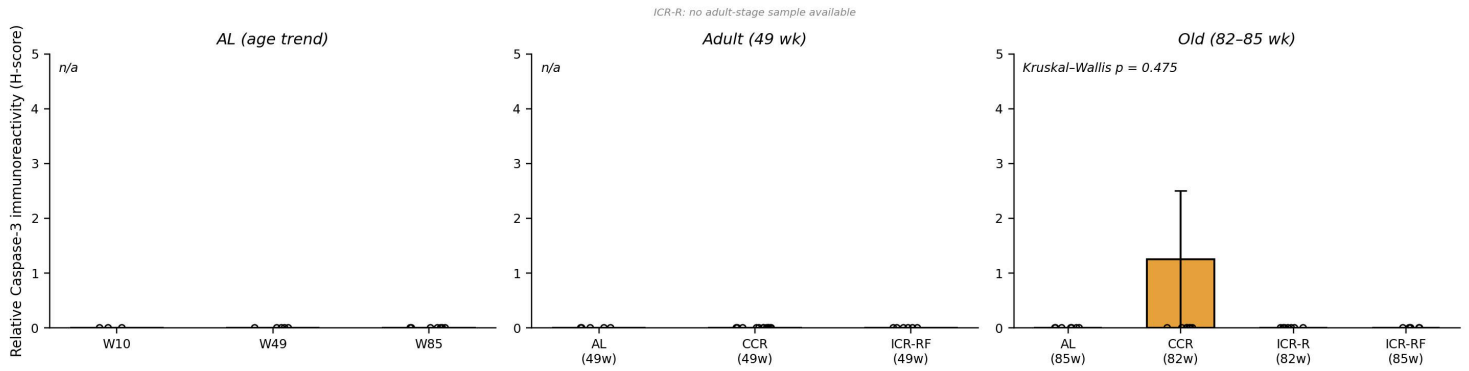


**Figure 7**

Kupffer-cell Caspase-3 immunoreactivity across age and diet.

Panel layout as in Figure 4. Caspase-3 immunoreactivity in Kupffer cells was at or near the detection threshold across all groups and ages, consistent with the pattern reported in Table 3.