

Establishment and Phenotypic Validation of an Immunocompetent Mouse Model of Neuroblastoma-Associated Cachexia

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Abstract: Neuroblastoma (NB) represents the most prevalent extracranial solid malignancy in children. High-risk NB patients frequently develop cancer cachexia, a life-threatening metabolic disorder characterized by skeletal muscle wasting and fat loss. However, NB-associated cachexia remains understudied. This study established a preclinical model of NB-induced cachexia. Immunocompetent male A/J Gpt mice were subcutaneously inoculated with 1×10^6 murine Neuro-2a cells to establish the NB cachexia model. Longitudinal phenotypic characterization included serial monitoring of body weight, food and water consumption, and grip strength. Histopathological analysis was performed to confirm tissue atrophy. Circulating pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α), and tissue gene transcript levels (Fbxo32, Trim63, Myh4, Pnpla2, Lipe and Plin1) were quantified to characterise the molecular alterations underlying tumor-induced cachexia. The model reproduced core clinical features of cancer cachexia. Tumor-bearing mice entered cachexia progression on Day 12 post-inoculation, exhibiting progressive body weight loss, reduced food intake, impaired motor function, significant bilateral skeletal muscle atrophy, and extensive depletion of white and brown adipose tissues. Histopathological analysis confirmed a reduction in myofiber cross-sectional area and marked adipocyte shrinkage. Elevated circulating pro-inflammatory cytokines and coordinated transcriptional dysregulation of muscle atrophy and adipose lipolysis programmes suggest skeletal muscle and adipose tissue lost. We have established and validated a preclinical model of NB-induced

cachexia that recapitulates key clinical phenotypes. This model provides a platform for investigating disease mechanisms, identifying diagnostic biomarkers, and developing targeted therapies.

Keywords: Neuroblastoma; Cancer Cachexia; Adipose Tissue Loss; Skeletal Muscle Atrophy; Pediatric Oncology

1. Introduction

Neuroblastoma (NB) arises from embryonic neural crest progenitor cells; it is the most prevalent extracranial solid malignancy diagnosed in children, representing 8%–10% of all pediatric cancers. It is the leading malignant tumor in infants under 1 year of age, representing ~50% of childhood cancers in this age group[1,2]. NB among children aged 0–14 years has a reported incidence of 10.1 per million population in Southern and Eastern European (SEE) and 11.7 per million in the US Surveillance, Epidemiology, and End Results (SEER) registry and meanwhile incidence is markedly higher in infancy, with 43.7 and 53.3 per million in SEE and SEER, respectively, and approximately 1/3 of all cases are diagnosed during this period[3]. In China, NB incidence is ~3–4 per million children, consistent with global trends, and has increased slowly in recent years[4]. Prognosis differs dramatically by risk status: 5-year overall survival exceeds 90% for low-risk patients but remains below 50% for high-risk patients, despite multimodal therapy, including radiotherapy, surgery, chemotherapy, and immunotherapy[5-12]. Malnutrition accounts for 20–50% of NB patients[13]. Severe malnutrition may lead to cachexia[14,15]. Cancer cachexia is recognised as a progressive, multifactorial metabolic syndrome driven by

chronic systemic inflammation, cored by persistent skeletal muscle and adipose tissue wasting, metabolic reprogramming, decreased appetite (decreased food and water intake), and gradual functional decline[16,17]. In contrast to the well-established diagnostic criteria for adult cancer cachexia, no unified standardized criteria have been developed for pediatric populations, attributable to four major limitations: children undergo dynamic growth and development with age- and sex-dependent normal ranges of body weight and body composition, making fixed weight loss thresholds inapplicable; pediatric cachexia often presents atypically with growth retardation rather than overt weight loss, and its manifestations overlap with common benign pediatric conditions, causing diagnostic delays[18]; inflammatory and metabolic biomarkers validated in adult cachexia (e.g., CRP, IL-6) have limited diagnostic value in children[19]; and critically, there is a profound lack of well-characterized preclinical models to elucidate the unique pathophysiological features of pediatric cancer cachexia.

In existing NB research, human NB cell lines, including SK-N-BE(2) and SH-SY5Y, have been widely used to establish xenograft mouse models of NB[20-22]. MYCN transgenic mice[23] and MYCN-driven genetically engineered mouse models (MYCN-nGEMM)[24] are also commonly used in NB research. However, the vast majority of these studies have focused primarily on NB tumorigenesis, progression, and therapeutic efficacy. In contrast, dynamic body weight changes and cachexia-associated phenotypes have rarely been incorporated as key observational endpoints, and systematic cachexia-related phenotyping combined with histopathological validation in tumor-bearing mice remains limited. This gap has hindered the development and broader application of NB-induced cachexia models.

In this study, we demonstrate that subcutaneous inoculation of murine Neuro-2a cells into mice generates an NB-induced cachexia model that faithfully recapitulates the core clinical phenotypes of cancer cachexia, including progressive body weight loss, reduced food and water intake, and concomitant skeletal muscle atrophy and systemic adipose tissue wasting. This model provides a preclinical platform for elucidating molecular mechanisms driving initiation and progression of NB-induced cachexia and for evaluating therapeutic strategies

under immunocompetent conditions.

2. Materials and Methods

2.1 Cell Culture

Neuro-2a neuroblastoma cells (RRID: CVCL_0470, male) were sourced from the National Collection of Authenticated Cell Cultures. The cells were cultivated in Gibco DMEM (C11995500BT) containing 10% FBS (A5256701) and 1% penicillin-streptomycin-glutamine (15140-122). Cultivation took place at 37 °C under 5% CO₂. Medium renewal was performed every 48 h. Cells were split at ratios of 1:2–1:5 upon 70–80% confluency. STR authentication was completed for all cell lines before use, and mycoplasma screening was performed semiannually.

2.2 NB Cachexia Model in *Vivo*

All the animals were bred on an A/J Gpt inbred background and housed under pathogen-free conditions at Fudan University (Shanghai, China) with a 12 h light and dark cycle at 24°C, and ad libitum access to water and a standard laboratory chow diet. A/J inbred mice were obtained from GemPharmatech Co., Ltd (Nanjing, China) and bred in-house in our laboratory.

Power Analysis: based on previous study[25], a minimum of six animals per group are required to achieve 80% statistical power at $p < 0.05$ (<https://www.stat.ubc.ca/~rollin/stats/ssize/n2>).

Twelve A/J Gpt inbred male mice (6 weeks old) were randomly allocated into a control group ($n = 6$) and a tumor-bearing group ($n = 6$). Control group (CTRL) received a subcutaneous injection of 50% (v/v) phosphate-buffered saline (PBS) and 50% (v/v) Matrigel on the left posterior side of the back. A dedicated sham injection group was not incorporated into the original experimental design. Throughout the entire experimental period, we performed daily close visual inspection of all injection sites to monitor local inflammatory reactions. In contrast, the tumor-bearing group (CAC) received an equivalent subcutaneous injection of 0.1 mL cell of a cell suspension containing 1 million Neuro-2a cells in 50% (v/v) PBS and 50% (v/v) Matrigel (Corning, Cat. No.356234) on the left posterior side of the back. After 3 days of tumor cell inoculation, the weight and tumor length and width of the CTRL and CAC group mice were measured every 3 days, with daily measurements starting from the 4th time point. Tumor length

(longest diameter, y , mm) and width (shortest diameter, x , mm) were recorded with digital callipers. Tumor volume was calculated as V (mm^3)

$$= \frac{x^2 \times y}{2}.$$

Each mouse was kept individually. Weekly food and water consumption were measured on a fixed schedule. A pre-weighed amount of pelleted chow was placed in the cage food hopper at the beginning of each 7-day measurement cycle. At the end of the cycle, all remaining food in the hopper and any spilled chow collected from the cage bedding were carefully retrieved and weighed using an analytical balance. Weekly food intake per mouse was calculated as the difference between initial and final (residual + spilled) chow weight. Water intake was determined gravimetrically. Each filled water bottle was weighed to mounting on the cage, and reweighed after a 7 day measurement period. Weekly water intake per mouse was computed by subtracting the final bottle weight from the initial weight obtained from the control bottles. Final values were converted to millilitres (mL) assuming a pure water density of 1 g/mL for data presentation. All measurements were performed by a single trained investigator to minimize systematic bias.

At endpoint, adipose tissue, liver, tumor specimens and serum samples were promptly harvested, weighed, snap-frozen in liquid nitrogen, and stored at -80°C . The remaining Tissues were fixed in 4% paraformaldehyde (Biosharp, BL539A) overnight, then processed for paraffin embedding. One mouse in the CAC group exhibited body weight loss exceeding 20% at the experimental endpoint; this animal was excluded from all subsequent data analyses, yielding a final sample size of $n = 5$ for this group. Animal procedures were approved by the Institutional Laboratory Animal Welfare and Ethics Committee (No.: Fudan No. A20250038). Mice were kept under proper housing conditions; all experimental manipulations followed standard guidelines, and maximum efforts were exerted to reduce animal suffering throughout the research.

2.3 Optical Coherence Tomography (OCT) Measurement

Subcutaneous fat thickness was quantified using optical coherence tomography (OCT). Briefly, the actual physical thickness corresponding to each pixel in the OCT images was pre-calibrated and denoted as u . For each acquired image, the

number of vertical pixels between the pre-defined blue and green lines (representing the superior and inferior boundaries of the subcutaneous fat layer, respectively) was counted and denoted as y . The subcutaneous fat thickness at a single anatomical site (l) was calculated using the formula: ($l = y \times u$). The average subcutaneous fat thickness ($l_{\text{aver}} = \frac{l_1 + l_2 + \dots + l_n}{N}$) for each mouse was derived by averaging the thickness values measured from multiple independent anatomical locations. OCT image acquisition was conducted on mice from both the CTRL group and CAC group at 3, 17, and 21 days post-tumor inoculation, with Day 21 designated as the experimental endpoint. At the final OCT measurement on Day 21, two mice from the CAC group were excluded from analysis as their body weight loss $>20\%$; accordingly, the CAC group had a final sample size of $n = 4$ for OCT.

2.4 Hematoxylin and Eosin Staining (H&E)

Tissue were fixed in 4% paraformaldehyde (Biosharp, BL539A; adipose tissue in special fixative, Servicebio Cat No.G1119) at 4°C for 24–48 h, then processed through a standard histological workflow: after sequential ethanol dehydration, xylene clearing and paraffin infiltration, specimens were embedded in paraffin blocks. Four-micrometer tissue sections were deparaffinized and rehydrated. Nuclear staining was performed with Harris hematoxylin (8 min), followed by differentiation in 1% acid alcohol and bluing under tap water. Eosin Y was applied for 2 min as the cytoplasmic counterstain. Finally, sections were dehydrated, cleared and mounted using neutral balsam. Representative images were captured at $400\times$ magnification using an inverted light microscope; representative adipocyte, muscle, and skin tissue images were quantitatively analyzed by ImageJ software.

2.5 Morphometric Analysis of H&E-Stained Muscle and Adipose Sections

Per the experimental design, three mouse gonadal adipose tissue samples ($n = 3$) were randomly selected, processed into paraffin-embedded blocks, and subjected to downstream analysis. Five representative fields of view were selected from each digitally scanned H&E-stained muscle or adipose tissue section, each data each dot indicates the average quantification of 5 fields. Cellular segmentation was performed using the Cellpose platform (<http://www.cellpose.org>), and

the resulting segmentation mask files were downloaded for subsequent morphometric quantification. The area (μm^2) and diameter (μm) of all segmented cells were measured with ImageJ software. For each representative field, the mean cell area and mean cell diameter were calculated by averaging the values of all individual cells within the field.

2.6 Serum Cytokine Quantification

Serum samples collected from CTRL and CAC mice and stored at -80°C . Concentrations of circulating pro-inflammatory mediators, including IL-1 β (Servicebio; No. GEM0002), IL-6 (Servicebio; No. GEM0001), TNF- α (Servicebio; No. GEM0004), were quantified by ELISA.

2.7 Quantitative Real-Time PCR (RT-qPCR)

Total RNA (TIANGEN, DP419) was extracted from skeletal muscle and gonadal white adipose tissue and reverse-transcribed into cDNA (Takara, RR047A). RT-qPCR was performed using SYBR Green detection reagent (Takara, CN830) with custom gene-specific primer pairs. In skeletal muscle, we quantified transcripts associated with muscle atrophy and fibre-type specification: Fbxo32, Trim63 and Myh4. In gonadal white adipose tissue, we profiled core genes governing lipolytic activation: Pnpla2, Lipe and Plin1. All transcript levels were normalized to the endogenous housekeeping gene Actb. Three replicates per sample, take the average for calculation. Relative gene expression was computed using $2^{-\Delta\Delta\text{Ct}}$ method. Full primer sequences are provided in Source Data.

2.8 Statistics

All data presented in figures are from a single in vivo cohort with $n = 5-6$ animals per group; and representative results are presented. Normality was verified for all datasets prior to statistical analysis. For two-group comparisons at a single time point, Student's t-test was used for normally distributed data with equal variance, whereas Welch's t-test was used. For two-group comparisons across multi-timepoints, two-way ANOVA was employed. All associated p-values are labeled directly within the figures. Data are presented as $\bar{x} \pm \text{SEM}$. Image processing was performed in ImageJ software (version 2.14.0), and statistical analysis and graphing were conducted using GraphPad Prism (version 9.5.0).

3. Results

3.1 Establishment of NB-Associated Cachexia Model

Six-week-old male A/J Gpt mice were used as experimental animals. Twelve mice were randomized into cancer cachexia group (CAC group) and blank control group (CTRL group), and ectopic subcutaneous injections were performed in the left posterior back of each mouse. CAC group mice received an injection of a mixture containing 1×10^6 mouse-derived NB Neuro-2a cells and Matrigel. In contrast, mice in the CTRL group were subcutaneously injected with a PBS-Matrigel mixture without tumor cells. After injection, tumor formation and the physiological status of the mice were monitored over time. The results showed a slightly elevated tumor mass with good overall mobility at the injection site on Day 3 post-injection in the CAC group, indicating subcutaneous tumor inoculation. In the CTRL group receiving PBS-Matrigel injection only, a mild local bulge was detectable at the injection site during the first three days post-inoculation. This prominence progressively subsided and the injection site became flattened over time; by Day 12, no visible Matrigel-associated bulge remained at the injection site. During the first 12 days, body weight as well as tumor size were measured every 3 days. From Day 12 post-injection, when body weight divergence and rapid tumour growth became apparent, monitoring frequency was increased to daily to ensure accurate identification optimal humane endpoint. Meanwhile, to comprehensively evaluate the effects of tumor burden in tumor-bearing mice, food and water intake, grip strength, and body composition were measured in each group. The timeline schematic of model establishment and data monitoring is shown in Figure 1.

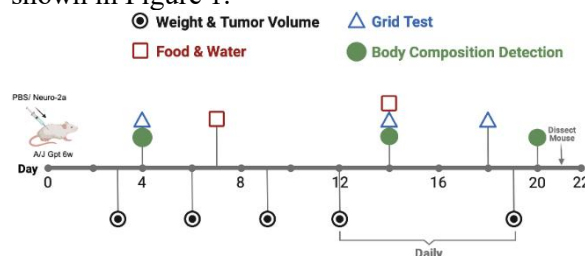


Figure 1. NB-Induced Cachexia Mouse Model Timeline Schematic

Note: Six-week-old immunocompetent A/J Gpt mice were randomly assigned to receive subcutaneous injection of either PBS (vehicle

control) or 1×10^6 murine Neuro-2a NB cells. Serial measurements of body weight, tumor volume, weekly food intake, and weekly water intake were conducted at the intervals shown in the figure. Grip strength assessment (grid test) and whole-body composition analysis were performed at the specified time points during the experimental period. Created with BioRender.com.

3.2 Metabolic Derangements in Mice with NB-Induced Cachexia

Adult cachexia is well defined: patients who have not progressed to the refractory stage are classified as cachectic if they meet any one of following: a stable body weight loss $>5\%$ over six months; BMI <20 kg/m² with ongoing weight loss $>2\%$; or sarcopenia accompanied by sustained weight loss of $>2\%$ [26]. By contrast, no formal consensus definition for pediatric cachexia has been established to date. Referring

to the above definition of adult cachexia[26], we defined the presence of a cachectic phenotype in our tumor-bearing mouse model as somatic wasting, decreased food and water intake coupled with a sustained, stable body weight reduction of greater than 5%. For body weight changes (Figure 2a), no significant intergroup difference was observed between the CAC and CTRL groups in the first three measurements (Day 3, 6, 9), indicating that tumor growth at this stage had not yet caused significant interference with systemic metabolism in mice. From Day 12 post-inoculation, the body weight of CAC mice declined rapidly, with an average reduction of 11% relative to baseline at the experimental endpoint. In contrast, the body weight of CTRL mice increased steadily or remained stable with feeding time. The intergroup difference in body weight was statistically significant from Day 12 through to the endpoint (Figure 2a, $p = 0.0012$ at the final measurement).

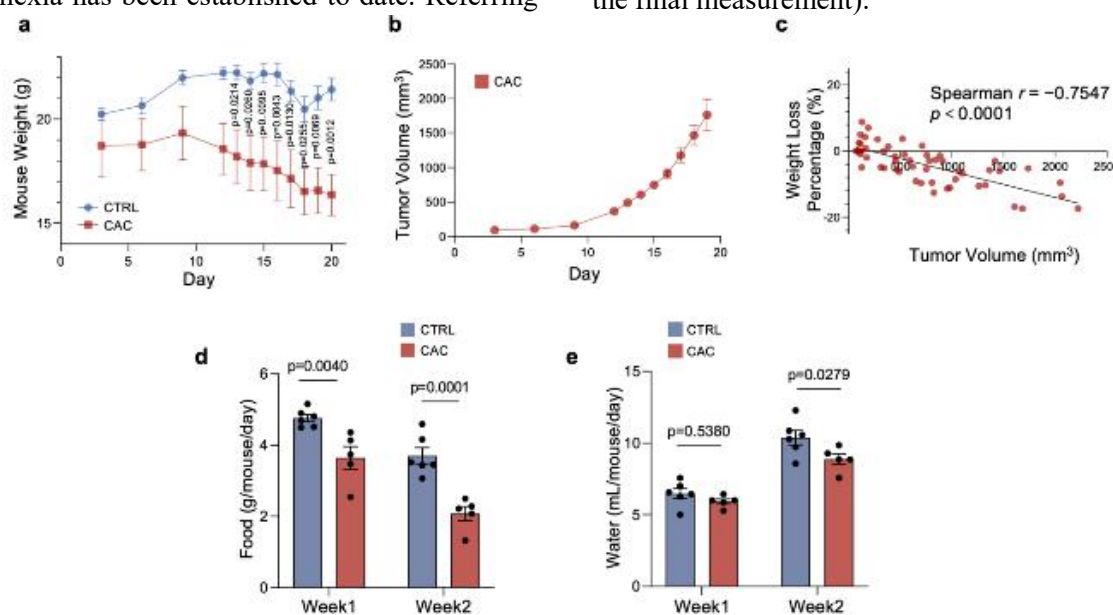


Figure 2. Systemic Metabolic Derangements in NB-Induced Cachexia Mice

Note: (a) Body weight change dynamics of mice in the CTRL and CAC groups (CTRL, $n = 6$; CAC, $n = 5$). (b) Tumor volume growth curve of mice in the CAC group ($n = 5$). (c) Correlation between body weight loss and tumor volume in CAC mice (analyzed by Spearman's rank correlation test, $n = 5$, each mouse has 10 body weights corresponding to the tumor volume). (d) Comparison of average food intake of mice in the CTRL and CAC groups during the 1st and 2nd weeks (CTRL, $n = 6$; CAC, $n = 5$). (e) Comparison of average water intake of mice in the CTRL and CAC groups during the 1st and 2nd weeks (CTRL, $n = 6$; CAC, $n = 5$). Abbreviations: CAC, cancer cachexia (NB tumor-bearing cancer cachexia model); CTRL, control (healthy control group). Mean $\pm$ SEM, Two-way ANOVA was performed, with exact p -values labeled in the figure.

Regarding tumor growth kinetics in the CAC group (Figure 2b), tumors with a volume of approximately 50–75 mm³ were measurable on Day 3 post-inoculation, and growth was slow during the first three measurements. From Day 12

post-inoculation, the tumor entered a rapid growth phase, with an average daily tumor growth rate of approximately 50–150 mm³. The average tumor volume reached 1766.28 mm³ at the experimental endpoint, which was below the

limit specified in the ethical review guidelines. Further correlation analysis revealed a significant negative correlation between percentage body weight loss and tumour volume in the CAC group (Figure 2c, $r = -0.7547$, $p < 0.0001$), suggesting that rapid tumor growth may be the primary driver of body weight loss in mice.

Food and water intake were measured on Day 7 and Day 14 post-injection to calculate the average daily food and water intake per mouse for each week (Figure 2d, e). In the first week, the average daily food intake was 4.76 g/mouse in CTRL group and 3.64 g /mouse in CAC group, representing a significant intergroup difference (Figure 2d, $p = 0.0040$). In the second week, the food intake of the CAC group decreased further to 2.08 g/mouse/day, a reduction of approximately 44% compared with 3.70 g/mouse/day in the CTRL group (Figure 2d, $p = 0.0001$). Similarly, no significant difference in water intake was observed between two groups in the first week (Figure 2e, $p = 0.5380$). In contrast, a significant 14.4% reduction in water intake was detected in the CAC group in the second week (Figure 2e, $p = 0.0279$).

Taken together, the measurement and analysis of body weight, tumor volume, food intake, and water intake in the two groups during model establishment revealed that NB tumor-bearing mice developed progressive weight loss and reduced feeding as tumor volume increased. These changes may further aggravate the negative energy balance in tumor-bearing mice and promote systemic metabolic derangements. The phenotypes of persistent weight loss and reduced feeding are consistent with the clinical manifestations of cancer cachexia.

3.3 Impaired Motor Function and Progressive Fat Loss in Mice with NB-Induced Cachexia

To further investigate the effects of NB tumor burden on body function and tissue morphology in mice, we measured and analyzed grip strength and body composition in the two groups. Grip strength is a key indicator of muscle and physical activity capacity in mice and can indirectly reflect the degree of motor impairment in tumor-bearing mice. Dynamic monitoring of grip strength was performed at three time points (Day 4, Day 14, and Day 18 post-injection) in both groups (Figure 3a). No significant intergroup difference in grip strength was observed on Day 4 post-injection (Figure 3a, $p = 0.3210$). The grip strength of CTRL mice increased moderately with age, while

CAC mice showed only minimal improvement in the two tests on Day 14 and Day 18, with values significantly lower than CTRL mice (Figure 3a, $p = 0.0003$). With tumor progression, the grip strength of CAC mice did not increase with age. We therefore hypothesized that the NB tumor burden can impair muscle function, leading to reduced motor capacity in mice, which is also one of the classic clinical features of cancer cachexia. Another core hallmark of cachexia is adipose tissue loss. Body composition was measured during model establishment, with a particular focus on total fat mass and total fat percentage (Figure 3b). Consistent with the grip strength results, no significant changes in total fat mass and percentage were observed between two groups (Figure 3b, $p < 0.0001$). Body composition analysis at different time points showed that, on Day 13, total fat mass and percentage in the CAC group had not yet decreased significantly compared with the CTRL group but continued to trend downward over time. This suggests that the degree of fat loss gradually intensifies with tumor progression, further confirming the long-term negative impact of NB tumor burden on adipose metabolism in mice.

In vivo subcutaneous fat thickness was also measured using optical coherence tomography (OCT) (Figure 3c). As shown in the representative light microscopic images, the area bounded by the red and blue lines denotes the epidermal and dermal layers. In contrast, the green and blue lines demarcate the subcutaneous fat layer. Notably, the subcutaneous adipose tissue layer was substantially thinner in CAC mice compared with control mice. Meanwhile, H&E staining of mouse skin cross-sections (Figure 3d) showed that the epidermal and dermal layers were structurally intact, with a uniform, continuous subcutaneous adipose layer in CTRL mice. In CAC mice, although the epidermal and dermal layers showed no obvious damage, the subcutaneous adipose layer was significantly thinner, with sparsely distributed and discontinuous adipocytes. This result is consistent with the aforementioned OCT finding of a thinner subcutaneous adipose layer in CAC mice, further confirming progressive subcutaneous fat loss induced by NB tumor burden from a histopathological perspective.

Subcutaneous fat measured by OCT showed that the subcutaneous adipose layer was significantly

thinner in CAC mice (Figure 3e, $p < 0.0001$ on Day 21), directly visualizing subcutaneous fat loss in tumor-bearing mice. Meanwhile, to further quantify the association between subcutaneous fat changes and body weight, Spearman's rank correlation analysis was performed on Day 21 in CAC mice. Subcutaneous fat thickness was positively correlated with mouse weight (Figure 3f, Spearman $r = 0.7660$, $p = 0.0130$), indicating

that subcutaneous adipose tissue mass decreased in synchrony with body weight loss in CAC mice. This is consistent with the aforementioned result of significant body weight reduction with tumor growth in CAC mice, indicating that tumor growth may disrupt systemic adipose metabolism, leading to whole-body fat loss and exacerbation of cachexia progression.

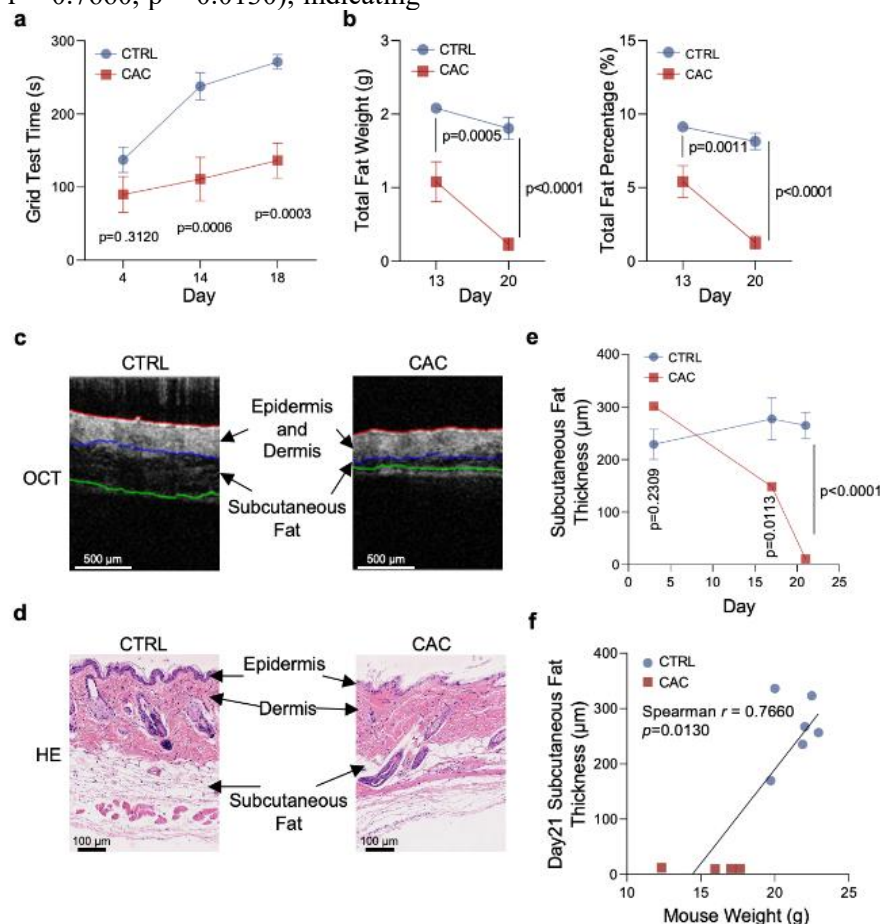


Figure 3. NB-Induced Cachexia Causes Impaired Motor Function and Progressive Systemic Adipose Tissue Loss

(a) Dynamic changes in grip strength measured by the grid test in two group mice (CTRL, $n = 6$; CAC, $n = 5$). (b) Change trends of total fat mass and total fat percentage from body composition analysis in mice of the two groups (CTRL, $n = 6$; CAC, $n = 5$, error bars are not visible for some groups due to their small magnitude). (c) Representative OCT images of subcutaneous adipose tissue (the subcutaneous adipose layer is located between the blue and green lines; the representative figure shows the measurement of Day 17). Scale bar = 500 μm . (d) Representative H&E staining images of cross-sections of skin tissue harvested and fixed upon dissection at Day 21. Scale bar = 100 μm . (e) Changes in subcutaneous adipose tissue thickness of mice in the two groups at each time point (CTRL group: $n = 6$; CAC group: $n = 4$, two mice were excluded due to weight loss $> 20\%$ at the time of analysis). (f) Correlation analysis between subcutaneous adipose tissue thickness and corresponding body weight of mice on day 21 (analyzed by Spearman rank correlation test; CTRL group: $n = 6$; CAC group: $n = 4$, two mice were excluded due to weight loss $> 20\%$ at the time of analysis). Abbreviations: CAC, cancer cachexia (NB tumor-bearing cancer cachexia model); CTRL, control (healthy control group). Mean $\pm$ SEM, Two-way ANOVA was performed, with p -values labeled in the figure.

In summary, with tumor progression in the NB tumor-bearing mouse model, CAC mice developed characteristic changes, including reduced grip strength and progressive loss of

systemic adipose tissue, and fat loss was closely correlated with changes in body weight. These phenotypes are consistent with the clinical manifestations of persistent fat loss and motor function impairment in patients with cachexia.

3.4 Macroscopic Loss of Adipose and Skeletal Muscle Tissues in Mice with NB-Induced Cachexia

At the experimental endpoint, gross morphological observation revealed that CAC mice had a smaller overall body size and shorter body length than CTRL mice (Figure 4a, left panel), indicating that tumor burden significantly affected overall growth and systemic status. The major white adipose tissue (WAT) and brown adipose tissue (BAT) depots of mice were identified, dissected, and harvested. The mesenteric (M), perirenal (P), subcutaneous (S), and gonadal (G) adipose tissue depots (marked

with black wireframes) in CTRL mice were evenly distributed and plump. In contrast, these WAT depots in CAC mice showed varying degrees of fat loss, with significantly reduced tissue volume and thinner texture (Figure 4a, right panel).

Each adipose tissue depot was weighed for quantitative analysis. The results showed that the mass of perirenal, subcutaneous, gonadal, and BAT was significantly reduced in CAC mice, with the most significant change in gonadal adipose tissue mass (Figure 4b, upper panel, $p = 0.0003$). In addition, the bilateral gastrocnemius and soleus muscles of the mice were completely dissected and weighed. The results showed that the mass of the left and right gastrocnemius and soleus muscles was significantly lower in CAC mice (Figure 4b, lower panel, left side $p = 0.0104$, right side $p = 0.0004$).

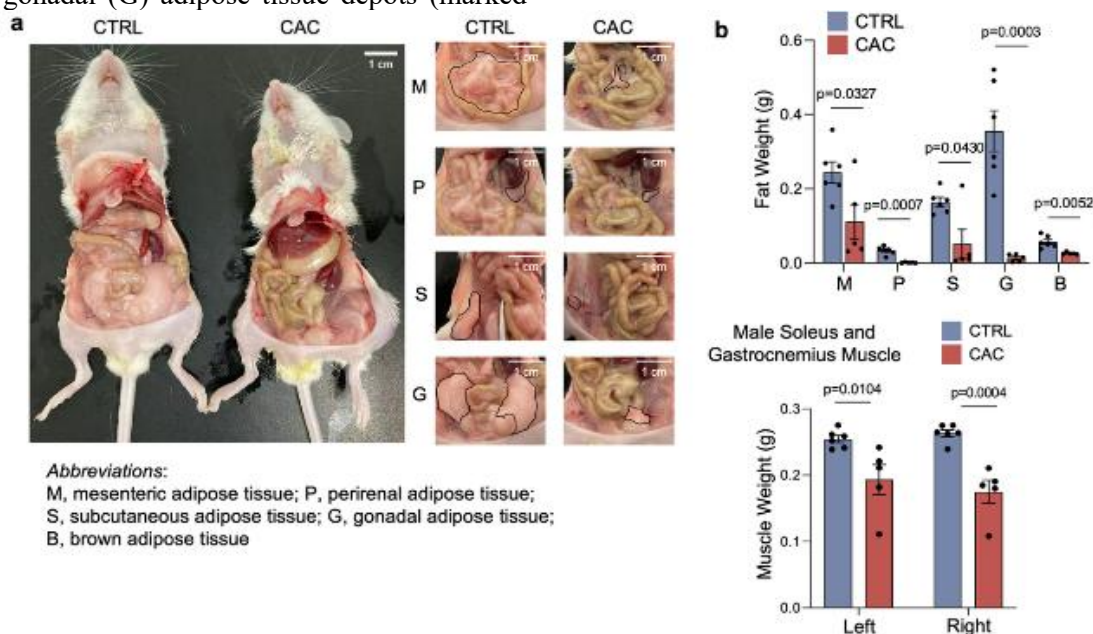


Figure 4. NB-Induced Cachexia Causes Extensive Macroscopic Loss of Adipose Tissues and Skeletal Muscles

(a) Representative image of gross morphology of mice (left panel) and the distribution of adipose tissue in the thoracic and abdominal cavities (right panel, adipose tissue regions outlined with black wireframes) in the CTRL and CAC groups. Of note, representative images of brown adipose tissue are not included in panel 4a; only tissue mass measurements were obtained upon dissection. (b) Quantification of adipose tissue mass at different anatomical sites (upper panel) and the weight of bilateral gastrocnemius and soleus muscles (lower panel) in mice of the two groups (CTRL, $n = 6$; CAC, $n = 5$). Abbreviations: CAC, cancer cachexia (NB tumor-bearing cancer cachexia model); CTRL, control (healthy control group). Student's t-test. P-values labeled in the figure.

Taken together, NB tumor-bearing mice developed extensive loss of both systemic WAT and BAT, accompanied by significant skeletal muscle wasting at the experimental endpoint. This characteristic, "concurrent fat and muscle

loss," is consistent with the clinical cachexia state in cancer patients, further verifying that this model recapitulates the core pathological manifestations of clinical cancer cachexia.

3.5 Pathomorphological Atrophy of Muscle and Adipose Tissues in Mice with NB-Induced Cachexia

H&E staining of lower leg muscle tissue (Figure 5a) revealed that muscle fibers in the CTRL mice were tightly arranged with intact morphology, uniform cross-sectional diameter, and even sarcoplasm distribution. In contrast, the cross-sectional diameter of muscle fibers in CAC mice was significantly reduced, with widened intermyofibrillar spaces. Quantitative analysis showed that the mean diameter (Figure 5a, upper panel, $p = 0.0022$) and mean area (Figure 5a, lower panel, $p = 0.0014$) of lower leg muscle fibers were significantly decreased in CAC mice.

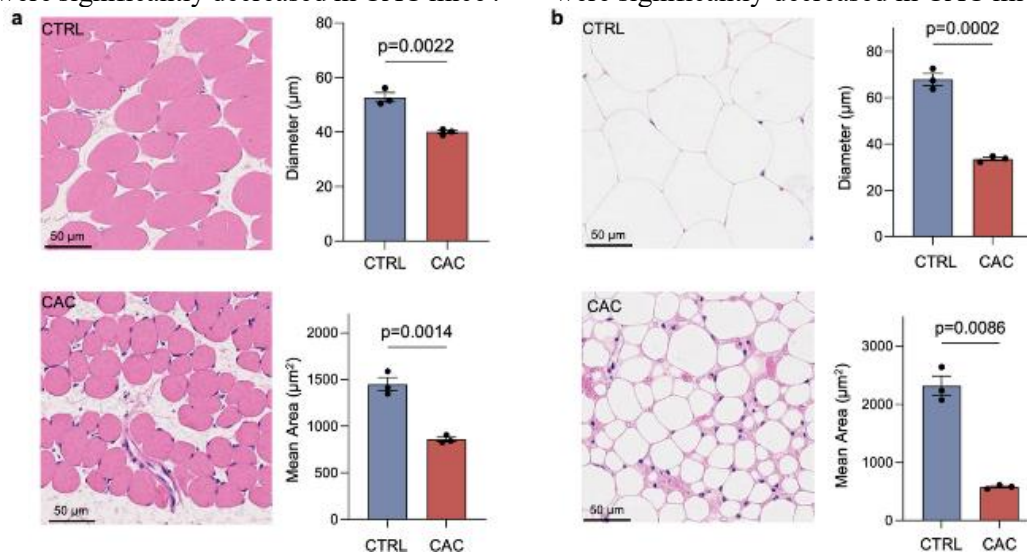


Figure 5. NB-Induced Cachexia Causes Extensive Histopathological Loss of Skeletal Muscles and Adipose tissues

(a-b) Representative H&E staining images of lower leg skeletal muscle tissue(a) and gonadal adipose tissue(b) harvested and fixed upon dissection at Day 21 (left), and quantitative analysis of the mean cross-sectional diameter and mean cross-sectional area (right). Scale bar = 50 μm (n = 3, with 5 representative fields of view taken from each mouse, each dot indicates the average quantification of 5 fields). Abbreviations: CAC, cancer cachexia (NB tumor-bearing cancer cachexia model); CTRL, control (healthy control group). Mean ± SEM. Student's t-test. P-values labeled in the figure.

Summary, the histopathological results demonstrate that NB tumor burden induces characteristic atrophic changes in adipocytes and skeletal muscle fibers. These microstructural damages are consistent with macroscopic tissue mass loss and functional impairment, providing direct histopathological evidence that NB tumor burden drives the development of cancer cachexia in mice.

3.6 Elevated Circulating Inflammatory Cytokines, Dysregulation Atrophy-Related and Lipolysis-Related Gene Expression in NB-Induced Cachexia.

We next examined systemic inflammatory status

Consistent with the findings in lower leg muscle tissue, H&E staining of gonadal adipose tissue sections (Figure 5b) showed that adipocytes in the CTRL group had regular morphology, plump lipid droplets with uniform diameter, and narrow intercellular spaces, with adipose tissue as the main component in the visual field. In contrast, gonadal adipocytes in CAC mice exhibited significant atrophy, with markedly reduced lipid droplet volume, irregular cell contours, and widened intercellular spaces. Quantitative analysis of the mean diameter (Figure 5b, $p = 0.0002$) and mean area (Figure 5b, $p = 0.0086$) of lipid droplets confirmed that both parameters were significantly decreased in CAC mice.

and tissue-specific transcriptional changes to define the molecular features of NB-induced cachexia.

Circulating concentrations of pro-inflammatory cytokines tightly linked to cancer cachexia — IL-6, IL-1β and TNF-α — were all significantly elevated in CAC mice compared with CTRL controls (Figure 6a, IL-6, $p = 0.0018$; IL-1β, $p = 0.0012$; TNF-α, $p = 0.0093$), reflecting a systemic inflammatory response driven by tumor growth.

In skeletal muscle, we assessed expression of genes governing protein turnover and fibre type specification. Transcript levels of the muscle-specific E3 ubiquitin ligases Fbxo32 and Trim63, two well-established markers of muscle atrophy,

were markedly upregulated in CAC mice (Figure 6b, upper panel, *Fbxo32*, $p = 0.0104$ and *Trim63*, $p = 0.0043$), in line with enhanced proteasomal protein degradation. By contrast, *Myh4*, which encodes the myosin heavy chain isoform of fast-

twitch glycolytic type IIb fibres, was significantly downregulated in the CAC group (Figure 6b, upper panel, $p = 0.0186$), consistent with the preferential atrophy of glycolytic fibres widely reported in cachexia.

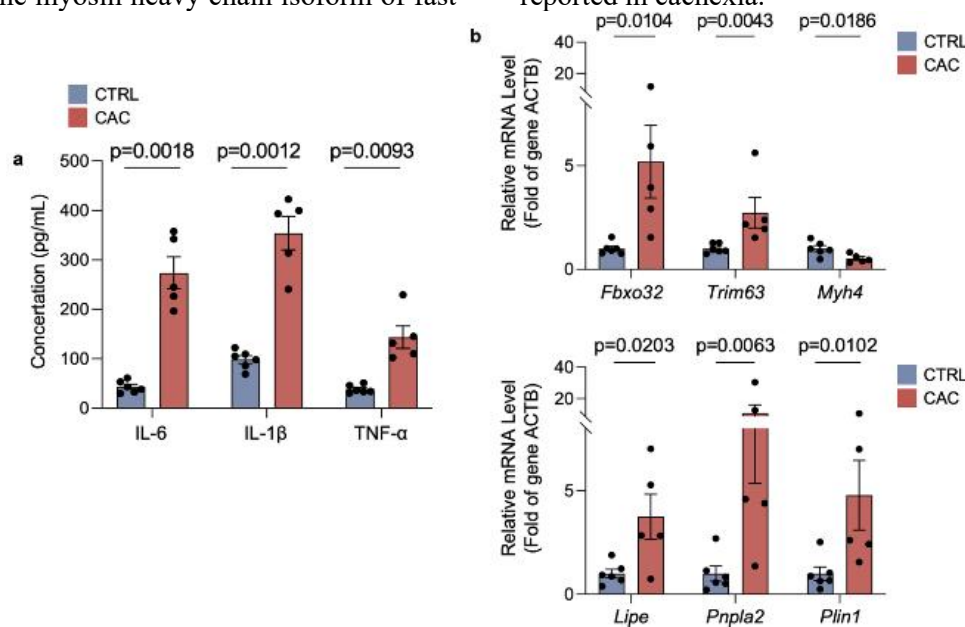


Figure 6. NB-Induced Cachexia Causes Elevated Circulating Inflammatory Cytokines and Dysregulated Expression of Muscle Atrophy- and Lipolysis-Related Genes

(a) Serum concentrations of IL-6, IL-1 β and TNF- α in CTRL and CAC group (CTRL group, $n = 6$; CAC group, $n = 5$), measured by ELISA. (b) Relative mRNA expression levels of skeletal muscle atrophy-associated genes (*Fbxo32*, *Trim63*, *Myh4*) in leg skeletal muscle (upper panel) and adipose lipolysis-related genes (*Pnpla2*, *Lipe*, *Plin1*) in gonadal adipose tissue (lower panel) from CTRL and CAC mice (CTRL group, $n = 6$; CAC group, $n = 5$). Abbreviations: CAC, cancer cachexia (NB tumor-bearing cancer cachexia model); CTRL, control (healthy control group). Mean $\pm$ SEM, Student's t-test. P-values labeled in the figure.

In gonadal white adipose tissue, we observed significant upregulation of key lipolytic genes in CAC mice, including *Lipe*, *Pnpla2* and *Plin1* (*Lipe*, $p = 0.0203$; *Pnpla2*, $p = 0.0063$; *Plin1*, $p = 0.0102$; Figure 6b, lower panel). This expression profile points to increased lipolytic activity in white adipose tissue, which probably underlies the progressive fat mass depletion in this model. Taken together, these data show that NB-driven cachexia triggers systemic inflammation and coordinates the transcriptional activation of muscle atrophy and adipose lipolysis programmes, which collectively drive the widespread reduced muscle and adipose mass.

4. Discussion

Here, we established an NB-induced cancer cachexia model in immunocompetent A/J mice via subcutaneous inoculation of Neuro-2a cells. This model recapitulates clinical cachexia characteristics defined by authoritative clinical

guidelines, including body weight loss, muscle and fat wasting, decreased appetite (decreased food and water intake), and motor dysfunction, and closely simulates the clinical progression of pediatric NB-induced cachexia. Tumor growth synchronizes well with cachexia onset, and tumor burden is significantly correlated with body weight decline, which is consistent with clinical manifestations. The model also reproduces typical negative energy balance and reduced food intake seen in patients, making it a reliable and standardized preclinical tool for exploring mechanisms and developing targeted interventions against NB-induced cachexia.

Our study provides functional and histopathological validation of the two defining pathological cancer cachexia hallmarks — skeletal muscle and adipose tissue wasting—in a syngeneic immunocompetent NB model. Tumor-bearing mice exhibited progressive declines in grip strength, accompanied by significant

symmetrical reductions in gastrocnemius and soleus muscle mass, as well as marked decreases in myofiber cross-sectional diameter and area. Concurrently, these mice displayed progressive systemic depletion of both WAT and BAT depots, with pronounced subcutaneous fat thinning that correlated significantly with total body weight loss. Histopathological analyses confirmed gonadal adipocyte atrophy and reduced lipid droplet volume, providing direct structural evidence of adipose wasting.

Consistent with the tissue atrophy observed above, NB tumor growth induces a systemic inflammatory response and drives transcriptional activation of atrophy programmes in skeletal muscle and lipolytic pathways in white adipose tissue, which together underpin the extensive tissue wasting phenotype observed in this model. Most preclinical NB studies focus on tumor growth and therapeutic efficacy, with minimal or no systematic phenotyping of cachexia. Even studies that report body weight changes rarely provide detailed functional or histopathological validation of muscle and adipose tissue alterations. For instance, although Biosacetalin has been shown to improve survival in an SH-SY5Y-induced murine cachexia model, this work documented no marked body weight loss during cachexia progression and did not assess changes in adipose or skeletal muscle mass[27].

Preclinical cachexia models have been described for other pediatric cancers, such as osteosarcoma and pediatric acute myeloid leukemia. However, these models have largely focused on skeletal muscle atrophy as the primary phenotype, with limited characterization of adipose tissue loss[28,29]. This preclinical gap aligns with observations from clinical research: available studies of pediatric cancer cachexia universally employ body weight dynamics as a foundational clinical readout, and substantial work has delineated the prevalence and prognostic significance of skeletal muscle wasting in this population. However, systematic quantitative characterization of adipose tissue loss remains comparatively scarce in clinical settings as well, and is typically confined to secondary descriptive observations rather than dedicated mechanistic investigation[30-33].

Against this backdrop, our NB cachexia model fills a critical preclinical research gap, particularly given that cachexia affects approximately 50% of high-risk NB patients. By recapitulating concurrent wasting of skeletal

muscle and adipose, our model provides a phenotypic foundation for cross-model comparisons. Given the divergent metabolic pathways driving cachexia across distinct pediatric malignancies, such comparisons informed by our complete tissue phenotypic datasets will help delineate the unique signaling cascades underlying NB-associated wasting.

In children, adipose tissue functions not only as an energy reserve but also as a key regulator of growth, immunity, and metabolic homeostasis. Thus, early adipose wasting may have unique long-term developmental consequences for pediatric survivors that are absent in adult patients. Furthermore, our model recapitulates concurrent WAT and BAT loss—a feature rarely characterized in preclinical pediatric cancer models. BAT dysfunction is recognized as a key driver of the metabolic reprogramming underlying cachexia progression, yet remains largely unexplored in NB research. The comprehensive characterization of both fat depots in NB-induced NB cachexia model provides a platform for researching tissue-specific mechanisms of adipose depletion and developing targeted interventions to preserve adipose function in pediatric cancer patients.

This study also has several limitations. Our phenotypic characterisation of NB-associated cachexia was restricted exclusively to male mice, a limitation we acknowledge in light of sex disparities in paediatric NB epidemiology[34]. While the male-only experimental design presented here aligns with the skewed male predominance seen in older paediatric NB patients, we also analysed a group of female tumor-bearing mice, whose skeletal muscle and adipocyte atrophic phenotypes closely mirror those measured in male mice. These datasets generated from this female mice group have been compiled into a standalone companion manuscript to prevent duplicate data reporting across related publications. We utilized subcutaneous tumor inoculation rather than orthotopic implantation, despite the superior physiological relevance of orthotopic models for replicating native tumor microenvironments and metastatic progression. Subcutaneous delivery was chosen for consistent tumor growth, straightforward non-invasive longitudinal monitoring and uniform humane endpoint assessment. The present analysis prioritized full phenotypic validation of late-stage cachexia, and longitudinal profiling dissecting stage-specific

wasting signatures across pre-cachectic, established and refractory phases will be further investigated.

5. Conclusions

Cancer cachexia is a major contributor to poor prognosis in high-risk pediatric neuroblastoma (NB), yet disease-specific preclinical models remain limited. In this study, we established and phenotypically validated an NB-induced cancer cachexia model in immunocompetent male A/J Gpt mice through subcutaneous inoculation of syngeneic Neuro-2a cells. This model recapitulates the key clinical and pathological hallmarks of cancer cachexia. It provides a robust preclinical platform for elucidating the mechanisms underlying NB-associated cachexia, identifying diagnostic biomarkers, and evaluating therapeutic interventions for NB.

Availability of Data

Datasets acquired in this research are available via the corresponding author when reasonably requested.

Author Contributions

Conception, Y.C.K., B.Q.D. and L.T.; Methodology, Y.C.K., K.Y.L, J.Y.W., X.R.L.; Validation, Y.C.K., K.Y.L, J.Y.W., X.R.L., Y.A, W.Q.L., L.Z., Y.Z. and L.T.; Formal Analysis, Y.C.K., J.Y.W., X.R.L.; Investigation, Y.C.K., J.Y.W., X.R.L., Y.A, W.Q.L., L.Z. and Y.Z.; Resources, B.Q.D. and L.T.; Data Curation, Y.C.K. and J.Y.W.; Visualization, Y.C.K., J.Y.W. and L.T.; Writing – Original Draft, Y.C.K. and L.T.; Writing – Review & Editing, Y.C.K. and L.T.; Supervision, L.T.

Ethics Approval

This study received ethical approval from the Ethics Committee of Fudan University (Approval ID: A20250038). All mice were raised under suitable husbandry conditions. Standard protocols were adopted for all experimental manipulations, and all feasible measures were implemented to reduce animal distress during the research period. Written informed consent was not required for this investigation, given that only laboratory animals were used in the experiments.

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Conflicts of Interest

None of the authors have any potential conflicts of interest to state regarding this study.

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