HHS Public Access

Author manuscript

Biochim Biophys Acta. Author manuscript; available in PMC 2016 May 01.

Published in final edited form as:

Biochim Biophys Acta. 2015 May ; 1852(5): 816–825. doi:10.1016/j.bbadis.2014.12.015.

Sex Differences in the Relationship of IL-6 Signaling to Cancer Cachexia Progression

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Abstract

A devastating aspect of cancer cachexia is severe loss of muscle and fat mass. Though cachexia occurs in both sexes, it is not well-defined in the female. The *Apc* $^{Min/+}$ mouse is genetically predisposed to develop intestinal tumors; circulating IL-6 is a critical regulator of cancer cachexia in the male *Apc* $^{Min/+}$ mouse. The purpose of this study was to examine the relationship between IL-6 signaling and cachexia progression in the female *Apc* $^{Min/+}$ mouse. Male and female *Apc* $^{Min/+}$ mice were examined during the initiation and progression of cachexia. Another group of females had IL-6 overexpressed between 12-14 weeks or 15-18 weeks of age to determine whether IL-6 could induce cachexia. Cachectic female *Apc* $^{Min/+}$ mice lost body weight, muscle mass, and fat mass; increased muscle IL-6 mRNA expression was associated with these changes, but circulating IL-6 levels were not. Circulating IL-6 levels did not correlate with downstream signaling in muscle in the female. Muscle IL-6r mRNA expression and SOCS3 mRNA expression as well as muscle IL-6r protein and STAT3 phosphorylation increased with severe cachexia in both sexes. Muscle SOCS3 protein increased in cachectic females but decreased in cachectic males. IL-6 overexpression did not affect cachexia progression in female *Apc* $^{Min/+}$ mice. Our results indicate that female *Apc* $^{Min/+}$ mice undergo cachexia progression that is at least initially IL-6-independent. Future studies in the female will need to determine mechanisms underlying regulation of IL-6 response and cachexia induction.

Keywords

IL-6r; STAT3; SOCS3; skeletal muscle; *Apc* $^{Min/+}$

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1. Introduction

Cachexia is a devastating condition that occurs secondary to several chronic diseases, including AIDS, COPD, chronic renal failure, and many forms of cancer (1). There is no FDA-approved treatment for cachexia, though it occurs in 30-50% of cancers (1-3) and has an annual mortality rate of 80% (1); the investigation into its etiology and progression is therefore essential. The most recent definition of cachexia includes an unintentional 5% weight loss over twelve months, comprising the loss of muscle and fat (1). Other abnormalities associated with cachexia include anemia, fatigue, muscle weakness, increased plasma triglycerides and inflammatory markers, and insulin resistance (1). There has been considerable improvement in our understanding of the regulation of cancer cachexia progression due to research employing male mouse models (4-8). However, there is evidence that sex differences exist in the development of cachexia in rodents (9). Additionally, sex differences have been observed in the loss of muscle strength associated with cachexia severity in humans (10). Although there is clear evidence that cachexia occurs in both males and females, the fundamental differences in the pathophysiology due to sex and underlying mechanisms are unknown.

Systemic inflammation related to cancer is thought to be a mediator of cachexia, as several pro-inflammatory pathways are enhanced during the progression of cachexia (11, 12). Sex hormones can modulate the inflammatory response to a variety of stimuli (13-15). Specifically, estrogen is known to inhibit NfκB and tumor necrosis factor α (TNFα) signaling (13, 16), C-reactive protein (CRP)-induced interleukin-6 (IL-6) production (17), and signal transducer and activator of transcription-3 (STAT3) signaling downstream of IL-6 (18). In several rodent models of cachexia and some human cancers, IL-6 is associated with the development of muscle wasting and body weight loss (6, 8, 19-21). Classical IL-6 signaling involves binding of the cytokine to the membrane-bound IL-6 receptor (mIL-6r) on target tissues, which include hepatocytes, immune cells, and skeletal muscle (22-24). mIL-6r is a heterodimer comprising the ligand-specific gp80 unit and the signal-transducing gp130 unit (25). Activation of mIL-6r induces downstream activation of many signaling pathways, including JAK/STAT, p38, and ERK (23, 26-28). Several of these pathways have been implicated in the regulation of muscle mass loss during cancer cachexia (26, 29).

The *Apc* $^{Min/+}$ mouse is genetically predisposed to develop intestinal tumors and becomes cachectic secondary to the tumor burden (4, 30). Our lab initially characterized cachexia in retired female *Apc* $^{Min/+}$ breeders (31); however, the progression and etiology of cachexia in the female *Apc* $^{Min/+}$ mouse has not been described. Development of cachexia in the male *Apc* $^{Min/+}$mouse has an established IL-6 dependence (6-8, 30, 32-34). Administration of an IL-6 receptor antibody to cachectic male *Apc* $^{Min/+}$ mice can attenuate further cachexia progression (7). When IL-6 is systemically overexpressed in the male, more body weight is lost and cachexia is more severe, indicating a causative role (6, 8, 26). However, the response of female *Apc* $^{Min/+}$ mice to IL-6 overexpression has not been examined. Sex differences have been noted in the inflammatory milieu of humans (35, 36) and mice (14, 37) under multiple pathological circumstances. Additionally, estrogen has been shown to inhibit IL-6 transcription and signaling in several tissues (17, 18, 37-39), which may also lead to sex differences in IL-6 response during cancer cachexia progression. Therefore, the

purpose of the present study was to examine the relationship of circulating IL-6 to cancer cachexia progression in the female *Apc* $^{Min/+}$ mouse. Our hypothesis is that cachexia progression in the female *Apc* $^{Min/+}$ mouse would not be associated with increased circulating IL-6 levels as has been reported in the male. This hypothesis was tested through three experiments. The first experiment followed a cohort of female and male *Apc* $^{Min/+}$ mice to 18 weeks of age, at which point the association between cachexia severity and circulating IL-6 level was determined. In the second experiment, 12-week old female *Apc* $^{Min/+}$ mice had IL-6 systemically overexpressed for two weeks (until 14 weeks of age) to determine if supraphysiological IL-6 levels could induce cachexia as we have previously shown in the male (8, 33, 40). In the third experiment, 15-week old female *Apc* $^{Min/+}$ mice had IL-6 systemically overexpressed for three weeks (until 18 weeks of age) to determine if supraphysiological IL-6 levels could accelerate cachexia progression as we have previously shown in the male (30).

2. Methods

2.1 Animals

Female *Apc* $^{Min/+}$mice (N=32), male *Apc* $^{Min/+}$ mice (N=12), female C57BL/6 mice (N=6), and male C57BL/6 mice (N=6) were bred and maintained at the University of South Carolina Animal Resource Facility. *Apc*$^{Min/+}$ mice used were offspring from breeders originally purchased from Jackson Labs (Bar Harbor, ME, USA). Male and female mice were taken during the three-month period from a standing inbred *Apc*$^{Min/+}$ breeding colony. *Apc*$^{Min/+}$ mice used were on a C57BL/6 background. Mice were kept on a 12:12h light/dark cycle beginning at 7:00 AM and were given unrestricted access to standard rodent chow (Harlan Teklad Rodent Diet, #8604). Mice were weighed weekly. Blood was collected by retro-orbital eye bleed at 12, 14, 16, 18, and 20 weeks for IL-6 analysis. All experiments were approved by the University of South Carolina's Institutional Animal Care and Use Committee.

2.2 Procedures

Three experiments were performed. *Experiment 1* examined the progression of cachexia. Female *Apc* $^{Min/+}$mice (n=18) were sacrificed at 18 weeks of age. Male *Apc* $^{Min/+}$ mice were sacrificed at 18-20 weeks of age. Female (n=6) and male (n=6) C57BL/6 (B6) were sacrificed at 18 weeks of age as non-cancer controls. Prior to sacrifice, blood was taken for analysis of IL-6 levels. *Experiment 2* examined 2 weeks of IL-6 over-expression in 12 week-old, weight stable female *Apc* $^{Min/+}$ mice as we have previously completed in the male (40). Female *Apc* $^{Min/+}$ mice were electroporated with a control plasmid or with an IL-6 overexpression plasmid (n=4 per group) at 12 weeks and were sacrificed at 14 weeks. Blood was taken at sacrifice (post-treatment) to determine plasma IL-6 levels. Mice from this experiment were not used for any other analysis. *Experiment 3* examined IL-6 over-expression in 15-week-old female *Apc* $^{Min/+}$ mice corresponding with the initiation of cachexia, a time course we have previously examined in the male (30). At 15 weeks of age mice were randomly separated into IL-6 over-expression (n=6), control plasmid (N=3), or non-electroporated control (n=3) treatment groups. All female *Apc* $^{Min/+}$ mice were sacrificed at 18 weeks of age. Blood was taken at sacrifice (post-treatment) for

determination of plasma IL-6 levels. No differences were found in body weight (p=0.78) or muscle mass (p=0.36) between control plasmid and non-electroporated mice. Therefore, the control plasmid and non-electroporated female *Apc* $^{Min/+}$ mice were then pooled for analysis. Additionally, these 6 mice are in the cohort of 18 female *Apc* $^{Min/+}$ mice related to Experiment 1. In all experiments, blood, inguinal fat, hindlimb muscles, and spleen were removed at the time of sacrifice. Hindlimb muscle mass measurements comprise the sum of left soleus, plantaris, gastrocnemius, tibialis anterior, extensor digitalis longus, and quadriceps masses.

2.3 IL-6 overexpression by electroporation

In vivo intramuscular electroporation of an IL-6 plasmid was used to increase circulating IL-6 levels in mice as previously described (41). The right quadriceps muscle was used to synthesize and secrete exogenous IL-6 into circulation from the injected expression plasmid, and was not used for any analyses in the study. The gastrocnemius muscle used in the study was not subjected to electroporation. Briefly, mice were injected with 50 μg of IL-6 plasmid driven by the CMV promoter, or control plasmid (pV1J), into the right quadriceps muscle. Mice were anesthetized with a 2% mixture of isoflurane (IsoSol, VEDCO, St. Joseph, MO, USA) and oxygen (1 L/min). The leg was shaved, and a small incision was made over the quadriceps muscle. Fat was dissected away from the muscle, and the plasmid was injected in a 50 μl volume of sterile phosphate-buffered saline (PBS). A series of eight 50 ms, 100 V pulses was used to promote uptake of the plasmid into myonuclei, and the incision was closed with a wound clip. In Experiment 2, electroporation was performed at 12 weeks and mice were sacrificed at 14 weeks of age. In Experiment 3, electroporation was performed at 15 weeks and mice were sacrificed at 18 weeks of age.

2.4 RNA isolation and RT-PCR

RNA isolation, cDNA synthesis, and real-time PCR were performed as previously described (42). Reagents from Applied Biosystems (Foster City, CA, USA) were used. Briefly, proximal gastrocnemius muscle was homogenized in TRIzol (Life Technologies, Grand Island, NY, USA) and mixed with chloroform, then centrifuged. The RNA phase was removed and washed several times with ethanol in DEPC-treated water. cDNA was synthesized using 1 μg of RNA. RT-PCR was performed on an Applied Biosystems 7300 thermocycler. A Taqman gene expression assay was used to determine IL-6 mRNA expression (Life Technologies). IL-6 receptor (forward, 5'-CTGCCAGTATTCTCAGCAGCTG-3', and reverse, 5'-CCTGTGTGGGGTTCCAGAGGAT-3'); SOCS3 (forward, 5'-TGCAGGAGAGCTGATTCTAC-3', and reverse, 5'-TGACGCTCAACGTGAAGAAG-3'); gp130 and GAPDH primer sequences have been published elsewhere (43).

2.5 Western blotting

Western blots were performed as described previously (7, 44). Briefly, gastrocnemius muscle samples were run on 6-8% acrylamide gels and transferred overnight to PVDF membrane (Thermo Scientific, Waltham, MA, USA). After transfer, Ponceau stains were

imaged to verify equal loading. The membrane was blocked for 1 hour in 5% milk-TBS-Tween 20, and incubated with primary antibodies at 1:2000 dilution overnight at 4°C. After several washes, membranes were incubated with secondary antibodies at 1:2000 dilution for 1 hour. Blots were visualized with WesternBright ECL (BioExpress, Kaysville, UT, USA). SOCS3 antibody was obtained from Abcam (Cambridge, MA, USA). STAT3, phopho-STAT3 (Y705), anti-mouse IgG, and anti-rabbit IgG antibodies were obtained from Cell Signaling (Danvers, MA, USA).

2.6 Grip strength measurement

Grip strength measurements were determined as previously described (8). Mice underwent 2 sets of 5 grip strength trials. The first mouse was removed from its cage, held firmly by the base of the tail, and allowed to grasp the top of the grate attached to the force gauge (Chatillon, Largo, FL, USA) with its paws. The mouse was firmly pulled down the grate, and grip strength was recorded in Newtons. The mouse completed the 5 trials of the first set and was returned to its cage. Each mouse tested went through the same procedure for its first trial, and mice were cycled through in the same order for the second trial.

2.7 Fasting glucose

Mice were fasted for five hours prior to blood glucose measurements. Blood glucose measurements were performed using a handheld glucometer (Bayer CONTOUR®, Whippany, NJ, USA) according to the manufacture's instructions.

2.8 IL-6 Enzyme-linked immunosorbent assay

Plasma IL-6 concentrations were determined as previously described (8). A commercial IL-6 ELISA kit was obtained from Invitrogen (Fredrick, MD, USA) and the manufacturer's protocol was followed. Briefly, blood was centrifuged after sacrifice; plasma was removed and stored at −80°C until analysis. A Costar clear 96-well plate (Corning, NY, USA) was coated with IL-6 capture antibody and allowed to incubate overnight. The next morning, the plate was blocked with assay diluent buffer. The plate was washed; plasma samples and IL-6 standards were diluted with assay diluent buffer and added in duplicate to the plate. The plate was again washed and sAV-HRP reagent was added to wells. After several washes, TMB substrate was added to wells and color was allowed to develop. The reaction was stopped with sulfuric acid and absorbance was read in a BioRad iMark plate reader (Hercules, CA, USA) at 450 nm.

2.9 IL-6R Enzyme-linked immunosorbent assay

Muscle IL-6R protein levels were determined using a DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA) as previously described (45). Briefly, gastrocnemius muscle tissue was homogenized in buffer containing 50mM HEPES, 4mM EGTA, 10mM EDTA, 15mM $Na_4P_2O_7$, 100mM β-glycerophosphate, 25mM NaF, 5mM $NaVO_4$, 0.1% Triton-X, and 0.1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). A Costar 96-well plate (Corning) was coated with IL-6r capture antibody and allowed to incubate overnight. The next morning, the plate was blocked with assay diluent. After washing, IL-6r standards and 50-500 μg protein of samples were added to wells in duplicate. The plate was again washed

and detection antibody was added. Another wash was performed and streptavidin-HRP was added to the plate. After a final wash, substrate solution was added to the plate and color was allowed to develop before the addition of stop solution. Absorbance was read at 450 and 570 nm in a BioRad iMark plate reader (BioRad). Standard and sample concentration was determined using a third-order polynomial curve.

2.10 Intestinal polyp quantification

Quantification of intestinal polyps was determined as previously described (46). Briefly, intestinal sections were fixed in formalin at time of sacrifice. At time of analysis, sections were rinsed in deionized water and dipped briefly in 0.1% methylene blue. Polyps from segment 4 were counted under a dissecting microscope; it has been determined that tumor number in segment 4 is representative of total tumor number (47).

2.11 Statistical analysis

All results are reported as means ± SEM. Differences between degrees of cachexia severity were analyzed by one-way ANOVA using Tukey *post hoc* test where appropriate. Differences between sexes and genotypes were determined by two-way ANOVA with Student-Newman-Keuls *post hoc* test where appropriate. Correlations were determined by Pearson's test for correlation. Differences between 12-14 week and 15-18 week IL-6 treatment groups were determined by Student's t-test. Level of significance was set at 0.05.

3. Results

3.1 Differential cachexia progression in male and female Apc $^{Min/+}$ *mice (Experiment 1)*

Both male and female *Apc* $^{Min/+}$ mice undergo varying degrees of cachexia; however, efforts to characterize progression have focused overwhelmingly on the male. Our lab has previously described the male (7, 8, 19), but limited data has been presented related to the female response (48). Both male (n=12) and female (n=18) *Apc* $^{Min/+}$ mice lost a significant amount of body weight from their peak weight (Figure 1A); however, males lost a greater percentage of body weight than females ($p<0.0001$). *Apc* $^{Min/+}$ mice generally have the greatest density of tumors in segment 4 of the small intestine, and the number of tumors in this segment correlates with total tumor number (47). The number of tumors in male and female *Apc* $^{Min/+}$ mice, though significantly higher than male and female B6 mice ($p<0.0001$), did not differ (Figure 1B), indicating that the differences in body weight loss were not due to tumor burden. Regardless of sex, *Apc* $^{Min/+}$ mice had significantly higher circulating IL-6 levels than B6 mice ($p<0.0001$). Despite the consistency in tumor burden, male *Apc* $^{Min/+}$ mice had higher circulating IL-6 levels than females ($p<0.0001$) (Figure 1C). Both male and female *Apc* $^{Min/+}$ mice had significantly larger spleens than B6 mice of the same sex ($p<0.0001$), indicating a higher level of systemic inflammation (Figure 1D); however, male *Apc* $^{Min/+}$ had larger spleens than females ($p=0.03$).

3.2 Progression of cachexia in female Apc $^{Min/+}$ *mice (Experiment 1)*

To determine the changes seen with progression of cachexia in the female *Apc* $^{Min/+}$ mouse, a cohort of 18 week old female *Apc* $^{Min/+}$ mice (n=18) were stratified based on body weight change from peak (Table 1). The cohort was divided based on body weight loss; "Weight

Stable" comprises mice with less than 2% body weight loss from peak weight (n=6), "Initial Cachexia" comprises mice with 2-9% body weight loss (n=6), and "Cachexia" comprises mice with greater than 10% body weight loss (n=6). Body weight loss ranged from 0% to −15.8%; ~30% of the mice were classified as "cachectic," with severe body weight loss, fat and muscle mass loss, and decreased muscle strength (Table 1). As expected, body weight change from peak weight was significantly different between all groups (Table 1). Spleen weight did not differ between weight stable and the initiation of cachexia. Cachectic female *Apc* $^{Min/+}$ mice had significantly larger spleens than either of the other groups (p=0.01) (Table 1). Segment 4 tumor count did not differ between weight stable and the initiation of cachexia. Cachectic *Apc* $^{Min/+}$ mice had significantly more tumors than either of the other groups (p=0.002) (Table 1). This indicates a relationship between tumor burden and cachexia development in the female that has also been reported in the male (4). Hindlimb muscle mass and inguinal fat mass did not differ between weight stable and initiation of cachexia. Cachectic *Apc* $^{Min/+}$ females had significantly less hindlimb muscle mass and inguinal fat mass than weight stable or the initial cachexia groups (Table 1). Importantly, there were no differences in circulating IL-6 levels between any of the groups (Table 1). Cachectic *Apc* $^{Min/+}$ females had significantly lower voluntary forelimb grip strength than either of the other groups, though this difference was eliminated when normalized to body weight (Table 1).

All female *Apc*$^{Min/+}$ mice continued to gain weight until 16 weeks of age; differences in body weight between groups are only seen after this point (Figure 2A; p=0.016). Across all female *Apc* $^{Min/+}$ mice, there was a significant relationship between hindlimb muscle mass and body weight loss (Figure 2B; p<0.0001, R^2=0.64). Inguinal fat mass demonstrated a curvilinear relationship (p=0.0002, R^2=0.58) with body weight loss, with fat mass having the most direct relationship with body loss during the initiation of cachexia (Figure 2C). Taken together, this indicates that during the progression of cachexia in the female *Apc* $^{Min/+}$ mouse the loss of inguinal fat mass occurs early, while muscle mass loss demonstrates a consistent decline over time.

3.3 Muscle IL-6 signaling-associated mRNA expression in male and female Apc $^{Min/+}$ *mice (Experiment 1)*

The levels of circulating IL-6 in female *Apc* $^{Min/+}$ mice were not associated with body weight loss, hindlimb muscle mass, or inguinal fat mass (Table 2). However, muscle mRNA expression of IL-6 was significantly correlated with both body weight loss and hindlimb muscle mass (Table 2). There was a main effect of cachexia independent of sex to increase muscle IL-6 receptor mRNA expression (p=0.043; Figure 3A). In female *Apc* $^{Min/+}$ mice, muscle IL-6 receptor mRNA expression was significantly correlated with muscle gp130 mRNA and SOCS3 mRNA expression (Table 2). Neither sex nor cachexia had a significant effect on muscle gp130 mRNA expression (Figure 3B). Although gp130 mRNA expression was not associated with body weight loss in female *Apc* $^{Min/+}$ mice, it was significantly associated with muscle SOCS3 mRNA expression (Table 2). Cachexia increased muscle SOCS3 mRNA expression independent of sex (p=0.01; Figure 3C). However, muscle SOCS3 mRNA expression was not correlated with body weight loss in female *Apc* $^{Min/+}$ mice (Table 2).

3.4 Muscle IL-6 signaling-associated protein expression in male and female Apc $^{Min/+}$ *mice (Experiment 1)*

Muscle IL-6 receptor protein expression was examined in gastrocnemius muscle of male and female b6 and *Apc* $^{Min/+}$ mice. Interestingly, male B6 mice have significantly higher levels of muscle IL-6r than female B6 mice (Figure 4A; p=0.006). In female *Apc* $^{Min/+}$ mice, muscle IL-6r increases with cachexia severity (Figure 4B; p=0.002); however, cachectic male Apc $^{Min/+}$ mice have significantly higher expression than cachectic females (Figure 4B; p=0.02).

Muscle STAT3 phosphorylation was examined during the progression of cachexia in female *Apc* $^{Min/+}$ mice (Figure 4C). Muscle STAT3 phosphorylation (Y705) was normalized to female B6 levels of phosphorylation. Total STAT3 muscle expression did not change with the progression of cachexia (B6: 1 ± 0.05; Weight stable: 1.03 ± 0.03; Cachexia: 1.02 ± 0.07). Muscle STAT3 phosphorylation was significantly different across groups (p=0.010). Muscle STAT3 phosphorylation significantly increased in cachectic muscle when compared to the initially cachectic female *Apc* $^{Min/+}$ mice (p=0.049). The induction of STAT3 phosphorylation had a strong trend towards correlation with body weight loss in female *Apc* $^{Min/+}$ mice, but there was no association with circulating IL-6 levels (Table 2). There was no difference in muscle STAT3 phosphorylation between female and male cachectic muscle (Figure 4C). These results demonstrate that muscle STAT3 phosphorylation is not related to circulating IL-6 level during the progression of cachexia in female *Apc Min/+* $_{\text{mice.}}$

SOCS3 protein expression was examined in muscle of male and female B6 and cachectic *Apc* $^{Min/+}$ mice. As with the IL-6 receptor, male B6 mice had significantly higher levels of SOCS3 protein than female B6 mice (Figure 4D; p=0.015). However, cachexia had differential effects on SOCS3 protein expression between the sexes. SOCS3 protein expression was significantly higher in female *Apc* $^{Min/+}$ mice than female B6 (Figure 4D; p=0.03); however, there was a strong trend towards a decrease in SOCS3 protein with cachexia in the male versus the B6 male (Figure 4D; p=0.053).

3.4 Effect of IL-6 overexpression on cachexia initiation in Apc $^{Min/+}$ *females (Experiment 2)*

We have previously shown that IL-6 overexpression in male *Apc* $^{Min/+}$ mice is sufficient to initiate or accelerate cachexia progression (8, 30). To determine the effect of IL-6 on the initiation of cachexia in the female *Apc* $^{Min/+}$ mouse, we overexpressed IL-6 from 12-14 weeks of age, when tumor burden is fully present but cachexia has not been initiated. Plasma IL-6 levels were significantly higher in mice treated with the IL-6 overexpression vector (Table 3). There were no differences in body weight between treatments groups at the initiation of IL-6 overexpression, and IL-6 treatment did not alter body weights after two weeks (Table 3). In spite of IL-6 overexpression, 12-14 week-old female *Apc* $^{Min/+}$ mice continued to grow (Table 3). Inguinal fat mass and hindlimb muscle mass did not significantly change in response to IL-6 overexpression (Table 3). Tumor number was quantified at the end of the study. Although tumor numbers were variable, there were no significant differences between treatment groups (not shown).

3.5 Effect of IL-6 overexpression on cachexia progression in Apc $^{Min/+}$ *females (Experiment 3)*

To determine the effect of IL-6 on cachexia progression in the female *Apc* $^{Min/+}$ mouse, we overexpressed IL-6 between 15 and 18 weeks of age, which generally corresponds with the development of cachexia. Plasma IL-6 levels were significantly higher in mice overexpressing IL-6 ($p=0.002$, Figure 5A), though it is important to note that the control mice had significantly higher levels of IL-6 than B6 females ($p=0.01$, data not shown). IL-6 overexpression above this cancer-induced increase did not induce an acceleration of body weight loss in female *Apc* $^{Min/+}$ mice that had initiated cachexia (Table 3, Figure 5B). It is unlikely that tumor counts would have been affected by IL-6 overexpression, as polyp number stabilizes at approximately 12 weeks of age (32). Tumor number was highly variable, but there were no differences between treatment groups in the number of intestine segment 4 tumors (not shown). Spleen weight was not further increased with IL-6 overexpression (Table 3). Hindlimb muscle mass and inguinal fat mass loss were not accelerated by IL-6 overexpression in 18 week female *Apc* $^{Min/+}$ mice (Figure 5B, Table 3). Importantly, muscle STAT3 phosphorylation was not increased by systemic IL-6 overexpression (Figure 5D). These results are in direct contrast to previous work by our lab with the male *Apc* $^{Min/+}$ mouse, which showed decreases in body weight and muscle mass, and an increase in spleen weight as a result of IL-6 overexpression in addition to the cancer-induced levels between 16 and 18 weeks (30).

4. Discussion

Circulating IL-6 has been demonstrated to be a regulator of cancer cachexia progression in male *Apc* $^{Min/+}$ mice. Circulating IL-6 is a known cachectic factor in human cancer populations as well (21, 49-51). Treatments for cancer cachexia that target IL-6 signaling are currently being examined (52, 53). However, it is known that sex differences exist in inflammatory responses (13, 14, 54, 55), and sex differences have been reported in cancer cachexia progression in both rodents and humans (9, 10, 27). We therefore sought to determine the relationship between circulating IL-6 and cachexia progression the female *Apc* $^{Min/+}$ mouse. We have demonstrated for the first time that circulating IL-6 level is not associated with the degree of cachexia in the female *Apc* $^{Min/+}$ mouse, as IL-6 levels were similar between weight stable and cachectic mice. We also report that, unlike the male (8, 30), IL-6 overexpression above cancer-induced levels does not induce or accelerate cachexia progression in female *Apc* $^{Min/+}$ mice. Additionally, we report that sex alters muscle IL-6 transcription during cachexia. Female muscle IL-6 mRNA expression increased with cachexia severity, which contrasts with male cachexia progression (7, 30). We found that the female muscle may become more sensitive to circulating IL-6 levels during the progression of cachexia, since muscle IL-6r protein and phosphorylated STAT3 increased with severe cachexia. Though the female *Apc* $^{Min/+}$ mouse loses both muscle and fat with the progression of cachexia, the development of this loss occurs differently from that reported in the male (7). The male *Apc* $^{Min/+}$ mouse initiates cachexia with the rapid loss of both muscle and fat (7). We provide evidence that the initiation of cachexia in the female *Apc* $^{Min/+}$ mouse involves a rapid loss of fat mass, while the loss of muscle mass occurs later in the

development of cachexia. These data clearly show that sex influences the regulation of cachexia progression in the *Apc* $^{Min/+}$ mouse.

As therapies targeting IL-6 for the treatment of cancer cachexia are gaining traction (52, 53), it is of utmost importance that basic research support this modality. The majority of investigations into human cancer cachexia do not separately analyze men and women (49-51, 56), which does not allow for the determination of sex differences in IL-6 levels. Recent investigation into single nucleotide polymorphisms (SNPs) in the IL-6 and IL-6 receptor promoter sequences has shown that certain SNPs affect levels of circulating IL-6 and soluble IL-6r; however, there are no differences between the sexes in allele frequency, indicating that sex differences do not likely occur at the transcriptional level (57). While we have consistently found that cachexia development in the male *Apc* $^{Min/+}$ mouse is directly related to tumor burden and the level of circulating IL-6 (6, 8, 19, 30, 33, 40, 58), this relationship has not been firmly established in the female. Though we found that male and female *Apc* $^{Min/+}$ mice had similar tumor burdens, females had significantly lower levels of circulating IL-6 than males, even when weight loss was comparable. However, the female also appears to have a differential cachectic response to IL-6 that is independent of circulating level. Specifically, plasma IL-6 did not increase as cachexia progressed, while muscle IL-6 mRNA expression and STAT3 phosphorylation were increased. Additionally, systemic overexpression of circulating IL-6 did not accelerate the progression of cachexia, indicating that higher circulating IL-6 levels are not sufficient to induce cachexia in the female *Apc* $^{Min/+}$ mouse. We also found that circulating IL-6 levels were not correlated with increased muscle IL-6r mRNA expression. Though the C26 and *Apc* $^{Min/+}$ models of cancer cachexia are IL-6 dependent in the male (26, 29, 58), sex differences have been found in the C26 model of cachexia, providing evidence that this is not a singular phenomenon related to the *Apc* $^{Min/+}$ mouse (9). Importantly, estrogen inhibits IL-6 transcription and signaling in many tissues (17, 18, 37-39); however, IL-6 is a known mediator of diseases in females including polycystic ovarian syndrome, ovarian cancer, and osteoporosis (38, 59, 60), indicating that females are susceptible to the pathophysiological effects of IL-6 under multiple circumstances regardless of estrogen status. The overall inflammatory environment related to the underlying disease may also alter IL-6 action. The circulating levels of IL-10, IL-4, and interferon-α (IFNα) have been shown to influence IL-6 action (50, 61). Interestingly, sex differences in inflammation have been reported in IL-10 knockout mice (12). Further investigation is required to determine if the systemic inflammatory environment, particularly the presence of estrogen, is differentially regulating IL-6 function in female *Apc* $^{Min/+}$ mice.

A key factor that determines tissue response to the inflammatory environment is receptor expression. IL-6 initiates intracellular signaling through binding with mIL-6r, which interacts with the signal-transducing gp130 (62). The importance of muscle IL-6 signaling through mIL-6r and gp130 in the development of cachexia has been clearly demonstrated in many tumor models (27, 33, 60, 63). Signaling through gp130 activates many signaling pathways, including JAK/STAT, p38, and ERK (23, 26-28). We have previously demonstrated that LLC-induced cachexia in males suppresses muscle gp130 expression (11). However, alterations in the expression level of gp130 or IL-6r during cachexia progression

in *Apc* $^{Min/+}$ mice have not been previously reported. Interestingly, cachectic muscle from both male and female *Apc* $^{Min/+}$ mice demonstrated an induction of muscle IL-6r mRNA expression and protein expression, without a significant change in muscle gp130 mRNA expression. The novel observation that male C57BL/6 mice have significantly higher muscle IL-6r protein level than females may explain why males have a more robust response to IL-6 than females. As IL-6 signaling has been linked to the induction of muscle protein degradation (7, 26), it remains to be determined why this signal is being amplified in cachectic muscle of both sexes at both the mRNA and protein levels. It also appears unlikely that differential muscle gp130 and IL-6r expression can account for differential sex responses to circulating IL-6 in *Apc* $^{Min/+}$ mice.

In addition to IL-6 interaction with its receptor complex, downstream regulators have the potential to alter IL-6 signaling in muscle (64-66). STAT3 is activated by phosphorylation at Y705 by Janus kinases downstream of the IL-6r/ gp130 complex (26). Activation of JAK/ STAT signaling in cachectic muscle is associated with ubiquitin/proteasome and autophagy-mediated protein degradation (7, 26). SOCS3 has the capacity to directly bind to gp130, inhibiting IL-6 signaling (66). Cachexia increased muscle SOCS3 mRNA expression in *Apc* $^{Min/+}$ mice of both sexes. However, the observation that SOCS3 protein has a differential sex response both in B6 and cachectic *Apc* $^{Min/+}$ mice may have implications for differential control of STAT3 expression and downstream signaling between the sexes. The pattern of increased SOCS mRNA and decreased SOCS3 protein that we report in the male *Apc*$^{Min/+}$ has been previously noted with cachexia in the C26 tumor-bearing model; it has been shown that phosphorylation of SOCS3 by Jak can destabilize SOCS3 and lead to its proteasome-mediated degradation (29). Further, there is evidence that a second chronological activation of STAT3 signaling may occur even in the presence of SOCS3 binding; this activation is induced by binding of the epidermal growth factor receptor (EGFR) to IL-6r in the continued presence of IL-6, though it is unknown if sex differences exist (67). We demonstrate a strong trend towards increased STAT3 phosphorylation during cachexia progression in female *Apc* $^{Min/+}$ mice, as previously seen in the male (7). This also corresponds with female muscle becoming more sensitive to inflammatory signaling with the progression of cachexia, and may represent the loss of a protective mechanism present in female muscle that suppresses inflammatory signaling. However, IL-6 signaling is only one of many inflammatory pathways involved in the progression of cachexia, and other inflammatory factors may regulate cachexia development in the female *Apc* $^{Min/+}$ mouse. Further work is needed to determine the regulation of JAK/STAT signaling during the progression of cachexia in female muscle and its relationship to wasting.

We have shown that female *Apc* $^{Min/+}$ mice undergo cachexia that is, at least initially, independent of IL-6 regulation. This finding has important ramifications, particularly as IL-6 has garnered interest as a potential therapeutic target for cancer cachexia. We report for the first time levels of IL-6r, gp130, and SOCS3 mRNA and protein expression in the muscle of male and female *Apc* $^{Min/+}$ mice. We also report the novel finding that there is differential expression of muscle IL-6r protein in male and female mice. Further, we have reported a difference in the timing of muscle and fat loss between the sexes that may have important ramifications for metabolism and cytokine signaling during the progression of cachexia. It

appears that factors involved in the regulation of cancer cachexia progression are subject to differential sex regulation, and more work is needed to mechanistically understand these differences. In addition, future work will need to determine the mechanisms in skeletal muscle during the progression of cachexia related to protein turnover, oxidative metabolism, and apoptosis that undergo differential regulation in the female.

5. Acknowledgements

This work was supported by National Institutes of Health grant # NCI-R01CA121249A501 (JAC), National Institutes of Health Grant P20 RR-017698 from the National Center for Research, and by the USC Behavioral-Biomedical Interface Program, which is a NIGMS/NIH-T32 supported program. Contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the NIGMS or NIH. The authors would like to acknowledge Tia Davis and Dr. M. Marjorie Peña for assistance with mouse breeding and IL-6 overexpression experiments. The authors declare no competing interests.

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Highlights

- Cachexia in female *Apc* $^{Min/+}$ mice is unrelated to plasma IL-6 level, unlike the male.
- Muscle IL-6r and downstream targets increase with cachexia in the female.
- IL-6 overexpression has no effect on cachexia in the female *Apc* $^{Min/+}$, unlike the male.
- IL-6 expression does not correlate with STAT3 phosphorylation in the female *Apc*$^{Min/+}$.
- Muscle IL-6r and SOCS3 protein levels are higher in males than females.

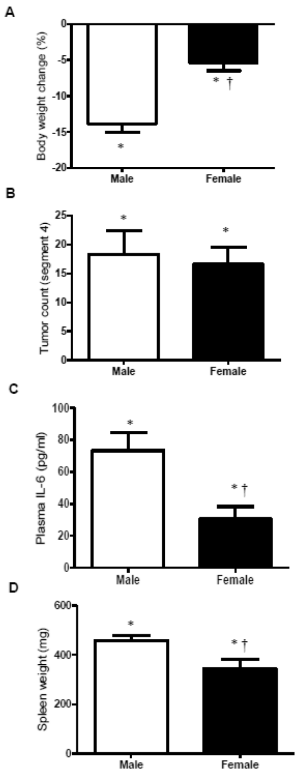

Figure 1. Characteristics of male and female *Apc* $^{Min/+}$ mice (Experiment 1)

A) Average body weight change from peak weight in male and female *Apc*$^{Min/+}$ mice (n=12 males, n=18 females). Male *Apc* $^{Min/+}$ mice had more body weight loss than female *Apc* $^{Min/+}$ mice. B) There is no difference in the average number of tumors in segment 4 of the small intestine between male and female *Apc* $^{Min/+}$ mice. C) Average level of plasma IL-6 (pg/ml). D) Average spleen weight in male and female *Apc* $^{Min/+}$ mice. Male *Apc* $^{Min/+}$ mice had larger spleens than female *Apc* $^{Min/+}$ mice. * indicates significantly different from B6 mice of the same sex ($p<0.05$); † indicates significant difference from male *Apc* $^{Min/+}$ ($p<0.05$).

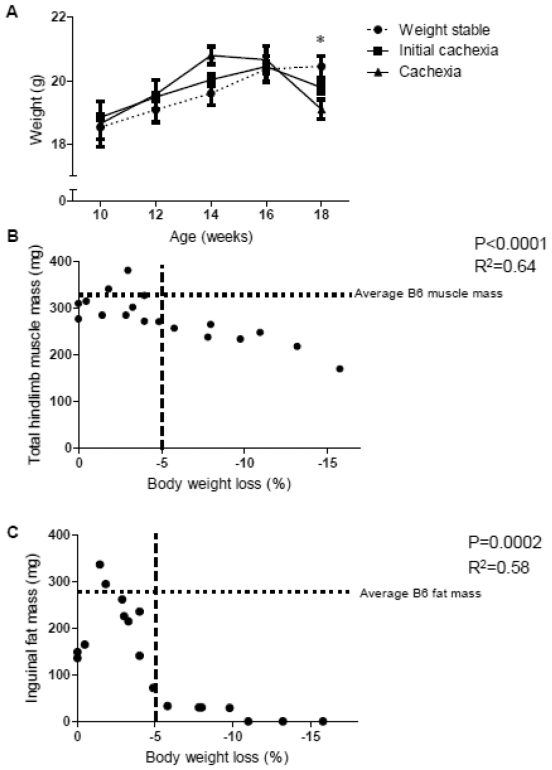

Figure 2. Cachexia progression in female *Apc* $^{Min/+}$ mice (Experiment 1)

A) Average body weight from 10-18 weeks for weight stable, initial cachexia, and cachexia groups (n=6 per group). Groups are significantly different at 18 weeks of age (p=0.016, repeated measures ANOVA). B) Hindlimb muscle mass has a negative correlation with body weight loss during cachexia in the female *Apc* $^{Min/+}$ mouse (Pearson's correlation, p<0.0001). C) Inguinal fat mass has a negative correlation with body weight loss during cachexia in the female *Apc* $^{Min/+}$ mouse (Pearson's correlation, p=0.0002). Horizontal dotted lines in B and C show average B6 muscle and fat masses. Dotted line at -5% body weight loss intended to show the contrast between fat and muscle loss before and after 5% body weight loss. * indicates significant differences between groups (p<0.05).

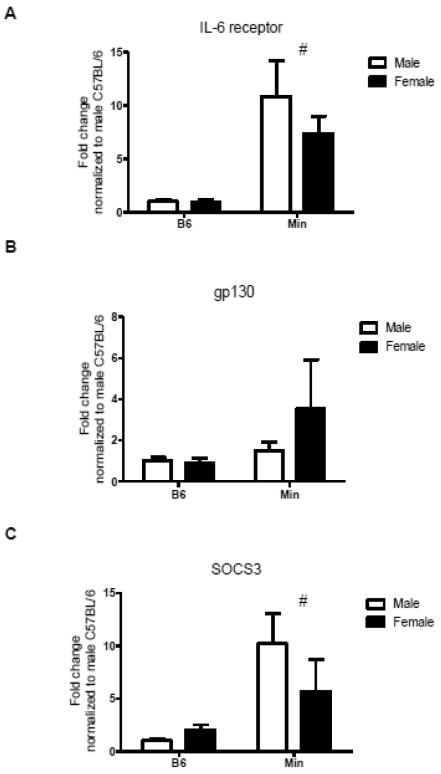

Figure 3. Muscle IL-6 signaling-associated mRNA levels (Experiment 1)

A) IL-6 receptor mRNA expression is significantly higher in male and female *Apc* $^{Min/+}$ mice than B6 mice. B) There is no difference in gp130 mRNA expression between male or female *Apc* $^{Min/+}$ mice or B6 mice. C) SOCS3 mRNA expression is higher in male and female *Apc* $^{Min/+}$ mice than B6 mice. All differences were analyzed by two-way ANOVA. # indicates main effect of *Apc* $^{Min/+}$ ($p<0.05$).

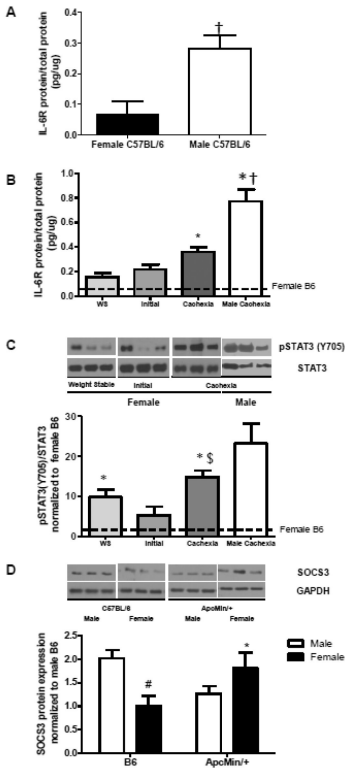

Figure 4. Muscle IL-6 signaling-associated protein levels (Experiment 1)

A) Muscle IL-6 receptor levels (pg/ug total protein) are significantly higher in male B6 than in female B6 mice (0.28 ± 0.04 vs 0.07 ± 0.04, p=0.01, Student's t-test) B) Muscle IL-6 receptor levels (pg/ug total protein) increases with cachexia severity in female *Apc* $^{Min/+}$ mice (one-way ANOVA, p<0.0001). Cachectic levels were different from all other groups (p<0.05, Tukey *post hoc*). IL-6 receptor levels are significantly higher in male cachexia than female cachexia ± 0.11 vs. 0.42 ± 0.09, p=0.02, Student's t-test). C) Upper: Representative blots shown. All samples per group were run on a gel with all weight stable samples, and then normalized during analysis. Lower: Quantification of pSTAT3/STAT3 blots. The ratio of phosphorylated STAT3 (Y705)/STAT3 increases with cachexia severity in the female *Apc* $^{Min/+}$ mouse (one-way ANOVA, p=0.01). There is no difference in phosphorylation of STAT3 (Y705) between cachectic females and cachectic males (p=0.154, Student's t-test). No differences were seen between groups in total STAT3. D) Upper: Representative blots shown. All *Apc*$^{Min/+}$ samples were run on a gel with male B6 samples for normalization. Lower: Quantification of SOCS3 blots. There is a significant interaction of sex by genotype in muscle SOCS3 protein expression (p=0.006, two-way ANOVA). Female B6 mice had significantly lower SOCS3 expression than male B6 mice (p=0.015, Student-Newman-Keuls *post hoc*). Female *Apc*$^{Min/+}$ mice had significantly higher SOCS3 expression than female B6 (p=0.029, Student-Newman-Keuls *post hoc*), while male *Apc*$^{Min/+}$ mice showed a strong trend towards lower SOCS3 expression than male B6 (p=0.053, Student-Newman-Keuls *post hoc*). $ indicates significant difference from initial cachexia (p=0.005, Tukey *post hoc*); * indicates significantly different from same-sex B6; # indicates significant difference from male B6; † indicates significantly different from female. Dotted lines on graphs indicate B6 levels.

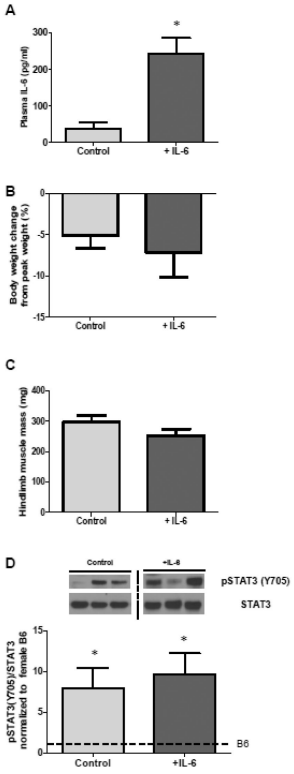

Figure 5. Effect of IL-6 overexpression in 18 week-old female *Apc* $^{Min/+}$ mice (Experiment 3)

A) Female *Apc* $^{Min/+}$ mice that had IL-6 systemically overexpressed from 15-18 weeks of age had higher circulating plasma IL-6 than control mice (p=0.002). B) There is no difference in body weight change from peak body weight (%) between female *Apc* $^{Min/+}$ mice that have had IL-6 systemically overexpressed from 15-18 weeks of age and control female *Apc* $^{Min/+}$ mice at 18 weeks of age. C) There is no difference in hindlimb muscle mass between female *Apc* $^{Min/+}$ mice that have had IL-6 systemically overexpressed from 15-18 weeks of age and control female *Apc* $^{Min/+}$ mice at 18 weeks of age. D) There is no difference in the ratio of phosphorylation of STAT3/STAT3 between female *Apc* $^{Min/+}$ mice that have had IL-6 systemically overexpressed from 15-18 weeks of age and control female *Apc* $^{Min/+}$ mice at 18 weeks of age (p=0.662, Student's t-test). Upper: Representative blots showing pSTAT3 and STAT3 expression. Dashed line indicates that samples were run on same gel, but separated by other samples. Lower: Quantification of pSTAT3/STAT3 expression. Dotted line on graph indicates female B6 level of phosphorylation/total STAT3. * indicates pSTAT3/STAT3 levels were significantly higher than B6 (p=0.008).

Table 1

Characteristics of female *Apc* $^{Min/+}$ mice (Experiment 1).

	Female *Apc* $^{Min/+}$ mice Weight stable	Initial Cachexia	Cachexia
n	6	6	6
Weight change (%)	−1.1 ± 0.5%	−4.2 ± 0.4%*	−11.0 ± 1.3%*†
Spleen weight (mg)	250.5 ± 50.2	310.5 ± 64.4	485.7 ± 37.7*†
Tumor count (seg 4)	7.8 ± 2.2	6.8 ± 3.0	26.3 ± 1.0*†
Hindlimb muscle mass (mg)	300.2 ± 9.9	299.7 ± 18.9	226.8 ± 13.4*†
Inguinal fat mass (mg)	224.0 ± 34.7	153.8 ± 35.2	14.8 ± 6.6*†
Plasma IL-6 (pg/ml)	17.6 ± 6.7	34.4 ± 18.9	39.3 ± 13.1
Grip strength (N)	1.9 ± 0.2	1.9 ± 0.1	1.6 ± 0.1*†
Grip strength/Body weight (N/g)	0.092 ± 0.008	0.098 ± 0.006	0.084 ± 0.003

Weight change (%): Percent weight change from peak body weight to weight at sacrifice [(weight at sacrifice-peak weight)/peak weight]. Hindlimb muscle mass comprises left soleus, plantaris, gastrocnemius, tibialis anterior, extensor digitalis longus, and quadriceps mass. n, number. seg 4, segment 4 of the small intestine. mg, milligrams. pg, picograms. ml, milliliters. N, Newtons.

*indicates significantly different from weight stable ($p<0.05$).

†indicates significantly different from initial cachexia ($p<0.05$).

Table 2

Relationships between circulating IL-6 and skeletal muscle signaling during cachexia development (Experiment 1).

Muscle mRNA expression correlations

Factor 1	Factor 2	R^2	p
Circulating plasma IL-6 (pg/ml)	Body weight loss	0.004	0.802
	Hindlimb muscle mass	0.070	0.289
	Inguinal fat mass	0.004	0.797
	IL-6 mRNA	0.041	0.436
	IL-6 receptor mRNA	0.038	0.468
	gp130 mRNA	0.014	0.701
	SOCS3 mRNA	0.045	0.428
Muscle IL-6 mRNA expression	Body weight loss	0.380	0.008*
	Hindlimb muscle mass	0.315	0.019*
	Inguinal fat mass	0.151	0.124
	IL-6 receptor mRNA	0.183	0.112
	gp130 mRNA	0.000	0.981
	SOCS3 mRNA	0.224	0.075
Muscle IL-6 receptor mRNA expression	Body weight loss	0.189	0.092
	Hindlimb muscle mass	0.142	0.150
	Inguinal fat mass	0.218	0.068
	IL-6 receptor protein	0.246	0.060
	gp130 mRNA	0.769	0.000*
	SOCS3 mRNA	0.913	0.000*
Muscle gp130 mRNA expression	Body weight loss	0.200	0.125
	Hindlimb muscle mass	0.130	0.226
	Inguinal fat mass	0.151	0.190
	SOCS3 mRNA	0.757	0.000*
Muscle SOCS3 mRNA expression	Body weight loss	0.212	0.110
	Hindlimb muscle mass	0.124	0.181
	Inguinal fat mass	0.045	0.428

Muscle protein expression correlations

Factor 1	Factor 2	R^2	p
	IL-6 receptor protein STAT3	0.098	0.221

Factor 1	Factor 2	R^2	p
Circulating plasma IL-6 (pg/ml)	phosphorylation/STAT3	0.008	0.728
	Body weight loss	0.005	0.795
Muscle	Hindlimb muscle mass	0.007	0.745
IL-6 receptor protein (pg/ug)	Inguinal fat mass	0.001	0.913
	STAT3 phosphorylation/STAT3	0.001	0.912
Muscle	Body weight loss	0.205	0.059
pSTAT3/STAT3	Hindlimb muscle mass	0.385	0.006*
	Inguinal fat mass	0.034	0.465

Correlations calculated in 18-week female *Apc* $^{Min/+}$ mice of varying degrees of cachexia (n=18). R^2, coefficient of determination; p, p-value. Correlations determined by Pearson's correlation. mRNA levels are expressed as fold change from female C57BL/6 mice.

* indicates significant correlation between factors ($p<0.05$).

Table 3

Characteristics of 14-week and 18-week female *Apc* $^{Min/+}$ mice with IL-6 overexpression (Experiments 2 and 3).

	Female *Apc* $^{Min/+}$ mice		
IL-6 overexpression	**−**	**+**	
12-14 week treatment			p-value
n	4	4	
12-week body weight (g)	20.0 ± 0.7	19.0 ± 0.7	0.38
14-week body weight (g)	20.3 ± 0.6	19.5 ± 0.7	0.49
Spleen weight (mg)	312.3 ± 35.0	336.5 ± 22.3	0.59
Fasting Glucose (mg/dl)	140.7 ± 1.3	171.0 ± 28.0	0.34
Plasma IL-6 (pg/ml)	1.5 ± 1.5	65.0 ± 18.1*	0.02
Grip strength (N)	2.1 ± 0.1	2.0 ± 0.1	0.67
15-18 week treatment			
n	6	6	
Peak body weight (g)	20.4 ± 0.3	19.4 ± 0.6	0.21
15-week body weight (g)	19.6 ± 0.3	18.8 ± 0.7	0.41
18-week body weight (g)	19.3 ± 0.3	17.9 ± 0.6	0.06
Inguinal fat mass (mg)	130.3 ± 39.9	65.5 ± 36.3	0.27
Spleen weight (mg)	318.7 ± 77.6	367.3 ± 75.7	0.68
Fasting Glucose (mg/dl)	119.3 ± 6.8	111.3 ± 4.7	0.37
Grip strength (N)	1.9 ± 0.1	1.9 ± 0.1	0.89

Female *Apc* $^{Min/+}$ mice had the right quadriceps muscle electroporated with control plasmid or IL-6 overexpression plasmid. G, grams; mg, milligrams; dl, deciliter; pg, picograms; ml, milliliters; seg 4, segment 4 of the small intestine; N, Newtons.

* denotes significant difference from control group ($p<0.05$).