Gut Barrier Dysfunction in the *Apc^{Min/+}* Mouse Model of Colon Cancer Cachexia

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Abstract

Background—The *Apc^{Min/+}* mouse, an animal model of colorectal cancer and cachexia, has a heterologous mutation in the *Apc* tumor suppressor gene, predisposing the mouse to intestinal and colon tumor development. This mouse develops intestinal polyps by ~4 weeks of age, and loses body weight gradually between ~14 and ~20 weeks of age. The strengths of this cachexia model derive from several features that mimic human cancer, including a gradual increase in tumor burden, chronic inflammation, and anemia. Little is known about the role of gut barrier dysfunction and endotoxemia in the development of cancer cachexia. We sought to determine how gut permeability and resultant endotoxemia change with the progression of cachexia.

Methods—Intestinal gut barrier integrity was assessed by permeability to FITC-dextran (MW_{av} = 4,000 kDa; FD4). Plasma glucose and triglycerides were measured by enzymatic assays, IL-6 by ELISA, and endotoxin by the limulus amoebocyte assay. Body temperature was measured using a rectal probe.

Results—Progression of cachexia was accompanied by development of gut barrier dysfunction (permeability to FD4), hypertrophy of mesenteric lymph nodes, and an increase in plasma endotoxin concentration. Changes in blood glucose and glucose tolerance, plasma IL-6, triglycerides, and body temperature were characteristic of endotoxemia.

Conclusion—We propose a role for gut barrier dysfunction (GBD) and subsequent endotoxemia in the development of inflammation and progression of cachexia in the *Apc^{Min/+}* mouse.

Keywords

cachexia; colorectal cancer; endotoxin; gut barrier dysfunction; gut permeability; inflammation

1.1 INTRODUCTION

Cachexia, a condition characterized by severe wasting of muscle and adipose tissue, is a common complication of late-stage cancers, especially cancers of the gastrointestinal

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system. It contributes to at least 20% of deaths from cancer [1]. Cachexia is a metabolic syndrome associated with underlying illness causing a loss of muscle mass and fat mass [2]. It is commonly associated with increases in acute phase proteins and pro-inflammatory cytokines in plasma, particularly TNF-α and IL-6 [3–5]. The $Apc^{Min/+}$ mouse is an animal model for colon cancer research [6, 7], which has a mutation in the Apc tumor suppressor gene. This mouse develops intestinal polyps, beginning at ~4 weeks of age and begins to develop a progressive cachexia between 12 and ~20 weeks of age, that culminates in a 20–25% decrease in body weight, The progression of cachexia is associated with an increased concentration of plasma IL-6, and cachexia is inhibited in an IL-6 knockout ($Apc^{Min/+} \times$ IL-6$^{-/-}$) mouse [8] despite the presence of intestinal and colon tumors. The cachectic response can be restored by systemic IL-6 over-expression in the $Apc^{Min/+} \times$ IL-6$^{-/-}$ mouse, while IL-6 over-expression in C57BL/6 mice does not induce cachexia. Thus, IL-6 is necessary, but not sufficient, for induction of cachexia in mice.

Gut barrier dysfunction (GBD), characterized by breakdown and leakage of the gut epithelial barrier, leads to systemic inflammation because of entry of bacterial cell wall components (endotoxin), also known as lipopolysaccharide (LPS), or intact bacteria into the circulation [9]. The resulting inflammation is a common problem in critical care medicine and can lead to multiple organ dysfunction syndrome [10]. Endotoxemia may be caused by a variety of stresses [11], burn injury [12], traumatic brain injury [13] or stroke [14], chronic heart failure [15], pancreatitis [16, 17], and even strenuous exercise [18, 19]. The gut receives a substantial fraction of cardiac output and impairment of the blood supply leads to hypoxia, which may be a common mediator of the development of gut barrier dysfunction and endotoxemia. Indeed, hemorrhagic shock [20] followed by resuscitation (HS/R) induces GBD in mice [21], as measured by increased intestinal permeability to FITC-dextran and translocation of bacteria to mesenteric lymph nodes. Like the ApcMin/+ model, development of cachexia in the HS/R model, GBD is dependent on increased circulating IL-6 concentration; IL-6 knock-out mice do not develop GBD after HS/R [21]. The observation that IL-6 is required, but not sufficient, for development of cachexia in both the $Apc^{Min/+}$ [8] and HS/R models [21], and that GBD is implicated in the pathogenesis of cachexia in the HS/R model led us to investigate the possible role of GBD and endotoxemia in development and progression of cachexia in the $Apc^{Min/+}$ mouse.

1.2 MATERIALS AND METHODS

1.2.1 Animals

C57BL/6 and $Apc^{Min/+}$ mice were originally purchased from Jackson Laboratories (Bar Harbor, ME), and breeding was continued at the University of South Carolina's Animal Resource Facility, as previously described [22]. The room was maintained on a 12:12 light: dark cycle with the light period starting at 0700. Mice were provided standard rodent chow (Harlan Teklad Rodent Diet, #8604, Madison, WI) and water *ad libitum*. Body weights were measured weekly. Male mice (n = 5–10 animals) were used in each group for all experiments. All animal experimentation was approved by the University of South Carolina's Institutional Animal Care and Use Committee.

1.2.2 Tissue sampling and physiological measurements

Mice were anesthetized with a ketamine/xylazine/acepromazine cocktail (1.4 ml/kg body weight), and tissues were removed, weighed and frozen at −80 °C until further analysis. Blood samples were collected in heparinized capillary tubes from the retroorbital sinus under brief isoflurane anesthesia.

Rectal temperature was measured using a rectal probe designed for mice (Thermalert TH-5, Physitemp, Clifton, NJ). Temperature was measured at the same time of day bi-weekly for the duration of the study.

Gut barrier integrity was assessed by permeability to FITC-dextran ($MW_{av} = 4,000$; FD4). The FD4 was administered by gavage (600 mg/kg BW; 125 mg/ml of phosphate-buffered saline) to fasted mice. Plasma was sampled prior to the gavage and 1 hr after the procedure and measured for fluorescence, as described by Yang *et al.*[21]

1.2.3 Biochemical assays

Plasma endotoxin was measured by a chromogenic Limulus Amoebocyte Lysate (LAL) assay (product no. HIT302; Hycult Biotech, Plymouth Meeting, PA). Plasma triglycerides were measured using a colorimetric assay (Thermo Scientific), and IL-6 using a mouse IL-6 ELISA (Invitrogen), performed according to manufacturer's instructions. An intraperitoneal glucose tolerance test was performed after an overnight fast by injecting a 20% glucose solution (2 g glucose/kg body weight). Glucose was measured at 0, 15, 30, 60, and 90 minutes after injection using a Bayer (Mishawaka, IN) Ascencia Contour glucometer.

1.2.4 *IL*-6 over-expression

At 12wk of age mice were electroporated with either an empty vector (vector) or an IL-6 plasmid (IL-6), as previously described [8]. The IL-6 plasmid, driven by the cytomegalovirus (CMV) promoter, was used to increase endogenous IL-6 production in the mice. The mice were anesthetized with a mixture of isoflurane and oxygen during the procedure. While unconscious the right leg was shaved and cleaned with alcohol. A small incision was made over the quadriceps muscle and 50μ of either control vector or IL-6 plasmid was injected into the muscle. To promote uptake of the plasmid into the myofibers a series of eight 100 volt pulses lasting 50 ms each were used on the quadriceps muscle. Each pulse consisted of a 1 second train of square bipolar pulses delivered every other second. Each train consists of 1000 pulses of 200 microseconds in length. The skin was then closed with a wound clip. Mice were then sacrificed at 14wk of age.

1.2.5 Statistical analysis

Repeated measures ANOVA was used to examine changes in body weight and rectal temperature over time in mice categorized by stage of cachexia. One-way ANOVAs or independent t-tests were used to determine significance for all other variables. Post-hoc analyses were performed with Student-Newman-Keuls methods. Significance was set at $p < 0.05$.

1.3 RESULTS

1.3.1 Progression of cachexia Apc$^{Min/+}$ mice

Apc$^{Min/+}$ mice develop tumors by ~4 weeks of age, but continue to grow in parallel with C57BL/6 mice until ~12 weeks, when they begin to lose body weight (Fig. 1A). By 20 weeks of age, *Apc*$^{Min/+}$ mice typically lose 20–25% of body mass, compared to either their maximal weight or the C57BL/6 control mice (Fig. 1A). Polyp number reaches a plateau at ~12 weeks (Fig. 1B), Tumor size continues to increase during the 12th–20th week, as the *Apc*$^{Min/+}$ mice develop cachexia; eventually ~80% of the polyps grow to 2 mm or greater in diameter (Fig. 1C). The weight loss and increase in tumor size is accompanied by a significant increase in plasma IL-6 concentration (Fig. 1D). As previously reported, IL-6, a marker of inflammation, increases with severity of cachexia and age in the *Apc*$^{Min/+}$ mice [23], consistent with a strong inflammatory response during cachexia [8, 24].

1.3.2 Gut Barrier Permeability increases during cachexia

The integrity of the gut barrier can be assessed by measuring its permeability to neutral hydrophilic polymers, such as FD4. Gut barrier permeability was negligible in C57BL/6 controls throughout the 20 weeks of the study, while permeability to FD4 begins to increase in $Apc^{Min/+}$ mouse, starting at 12–14 weeks of age (Fig. 2A), corresponding to the time of onset of cachexia (Fig. 1A). By 20 weeks of age, which corresponds with the severely catechetic condition, there was a significant increase in gut permeability in $Apc^{Min/+}$ mice. There was a strong correlation between plasma IL-6 and intestinal permeability ($r^2 = 0.55$, p <0.001) in untreated $Apc^{Min/+}$ mice at 20 weeks (Fig. 2C) and a significant correlation between the number of large polyps (> 2 mm) and intestinal permeability ($r^2 = 0.38$; p = 0.003). Intestinal permeability also increased 3-fold, compared to vector-injected controls, when IL-6 was over-expressed in $Apc^{Min/+}$ mice for 2 weeks at the onset of cachexia (12 weeks of age) (Fig. 2B), consistent with a role for IL-6 in development of GBD. There was also a measurable increase in FD4 permeability in the vector-injected mice, compared to C57Bl/6 controls, at 14 weeks, which is attributable to the normal increase in intestinal permeability in $Apc^{Min/+}$ at this time.

1.3.3 Endotoxemia develops during cachexia

Trace levels of endotoxin are detectable in plasma of control mice, and similar levels were detected in pre-cachectic $Apc^{Min/+}$ mice at 12 weeks of age. However, endotoxin increased by ~5-fold in severely cachectic animals at 19 weeks of age (Fig. 3), corresponding to the increase in gut permeability to FD4. The bar on the right in this figure represents plasma endotoxin concentration at 12 hours after intraperitoneal injection of 250 µg LPS into a C57BL/6 mouse at 20 weeks of age. Intestinal lymph nodes were also significantly enlarged between 12 and 14 weeks of age and remained enlarged through 20 weeks (Fig. 4), possibly as a consequence of LPS or bacterial penetration into the mesenteric lymphatic system.

1.3.4 Hypothermia develops during cachexia

Acute injection of LPS into normal mice induces hypothermia [25], possibly as a protective mechanism to limit tissue damage from the inflammatory response [26]. As shown in Fig. 5, $Apc^{Min/+}$ mice also experience a gradual decline in body temperature during development of cachexia. Intraperitoneal injection of a bolus of 250 µg endotoxin into control C57BL/6 mice led to a similar decrease in body temperature to 29.9 ± 0.2 °C at 12 hours (n = 2). Although their temperatures were comparable, the LPS-injected control animals were more lethargic than $Apc^{Min/+}$ mice at 16–20 weeks of age, consistent with lower-level, chronic exposure to endotoxin in the cachectic animals (Fig. 3).

1.3.5 Changes in lipemia and insulin resistance during development of cachexia

$Apc^{Min/+}$ mice developed severe hypertriglyceridemia, which is maintained during the progression of cachexia (Fig. 6). Although fasting blood glucose was not significantly altered, glucose tolerance tests were abnormal in cachectic animals, based on delayed clearance of glucose at 90 minutes and the 29% increase (p = 0.05) in Area-Under-the Curve (AUC) for the glucose tolerance test (Fig. 7). Alterations in lipemia and insulin resistance are commonly seen in cachexia [27]. These changes are indicative of major shifts in energy metabolism in response to inflammation in cachectic animals and, as discussed below, are also common sequellae of endotoxemia.

1.4 DISCUSSION

1.4.1 Role of endotoxemia in cachexia

Cachexia is a challenging complication of end-stage cancer, affecting a patient's overall health and vitality, ability to withstand infection, and to respond to chemotherapy or other interventions. Dealing aggressively with cachexia is critical for increasing patient survival. While there has been considerable focus on the distal consequences of cachexia, *i.e.* the mechanisms involved in adipose and muscle wasting, it is equally, if not more, important from a clinical perspective to elucidate the primary, proximal mechanisms initiating cachexia. The $Apc^{Min/+}$ mouse is an accepted model of colorectal cancer cachexia. In this model the mice grow at a normal rate until approximately 12 weeks when intestinal polyps reach a plateau in numbers. After this the polyps grow in size without increasing in number and the animals begin to lose weight gradually until 16 weeks of age when there is a more severe decrease in body weight. These data suggest that it is not just the presence of the polyps that leads to cachexia, but the size of the polyps contributes to the degree of weight loss.

1.4.2

In the present study, we have developed both direct and indirect evidence supporting a role for endotoxemia in development of cachexia in the $Apc^{Min/+}$ mouse model of colon cancer. The direct evidence is the detection of GBD (increased FD4 permeability) and the appearance of endotoxin in the circulation during the progression of cachexia. The plasma endotoxin concentration, while only modestly (~5-fold) increased, represents a steady state balance between its rate of entry into the circulation through the mesenteric lymph ducts and its rate of clearance from plasma in reticuloendothelial and other organs. The enlargement of mesenteric lymph nodes may be consistent with entry of endotoxin (or intact bacteria) through the intestinal lymphatic system [28, 29]. Acute intravenous administration of endotoxin also increases intestinal permeability in rodents [30, 31] and humans [32], suggesting a possible amplification loop in which gastrointestinal damage promotes endotoxin leakage, which then promotes an escalating cycle of endotoxin exposure and intestinal leakage. Indirect evidence for endotoxemia in cachectic mice includes a number of pathological changes that are characteristic of endotoxemia, including hypothermia [25, 26, 33], hypertriglyceridemia [34, 35], and insulin resistance [36, 37], as well as the chronic inflammatory state characterized by increased plasma concentrations of IL-6 [8]. However, the increase in the inflammatory biomarker IL-6 alone is not sufficient to cause these changes since IL-6 injection does not induce cachexia in control C57Bl/6 mice [8] or GBD (increased FD4 permeability) in $Apc^{Min/+}$ mice (Fig. 2B).

1.4.3 Source of GBD

While we have documented increases in GBD and endotoxemia in the $Apc^{Min/+}$ mouse, we do not have sufficient information at this stage to identify the primary cause of GBD. Tumor growth or macrophage infiltration and inflammation in the intestinal wall may affect gastrointestinal permeability, either locally or throughout the intestine through alterations in epithelial tight junctions. Soler *et al.* showed that in humans and rats tight junction permeability is increased in the region surrounding intestinal tumors [38]. Tight junction protein such as ZO-1 and occludin are also decreased in tumor rich regions of the intestines and colon in humans [39]. Decreases in tight junction proteins would increase permeability and allow passage of large molecules such as LPS into the lymphatic circulation. Changes in mucin secretion and mucin profiles in gastrointestinal carcinomas [40, 41] may also contribute to increased gut permeability. It is also possible that, as observed in HS/R [21] and acute pancreatitis [42], decreased splanchnic blood flow and resultant hypoxia might contribute to GBD. Hypoxia may also develop as a result of severe anemia, which may also

cause hypoxia. We have reported previously [43] that blood hemoglobin concentration and red cell hemoglobin content fall by ~50% during the rapid phase of weight loss (16–20 weeks) in the $Apc^{Min/+}$ mouse, suggesting that decreases in oxygen transport to the intestine may underlie the development of hypoxia. All of these mechanisms may be linked through hypoxia-induced mucosal damage and subsequent lifting of the intestinal epithelia. This lifting of the epithelia disrupts junctional proteins and leads to increased gut permeability [44].

1.4.4 Role of inflammation in GBD

Increases in circulating IL-6 are necessary for development of cachexia in the $Apc^{Min/+}$ mouse [8]. We show here that plasma IL-6 concentration also increases in concert with changes in intestinal permeability, and that tumor size also correlates with increased intestinal permeability. The strong correlations between permeability to FD4, tumor size, and plasma IL-6 in the $Apc^{Min/+}$ mouse, as well as the increase in permeability following IL-6 overexpression, suggest that GBD and endotoxemia are associated with, and perhaps the immediate cause of, both inflammation and cachexia. While more work is required to define the mechanisms underlying the increase in gut permeability, the general observations regarding compromised barrier function in this model of colon cancer may also apply to other cancers with a high frequency of cachexia, such as pancreatic or lung cancer.

1.5 CONCLUSION

Effective treatment of cachexia would have a significant impact on survival of patients with colorectal and other cancers. We show data to support that gut barrier dysfunction and endotoxemia develops concurrently with a surge in IL-6 and tumor growth present during cachexia. While our studies on cachexia are limited thus far to the $Apc^{Min/+}$ mouse model, our observations suggest that endotoxemia should be evaluated in clinical studies of cachectic patients, especially those with gastrointestinal cancers. If endotoxemia or GBD is a common feature of cachexia, then efforts to limit gastrointestinal toxicity and/or preserve the integrity of the gut epithelial barrier during therapy may have a significant impact on cancer morbidity and mortality.

Highlights

The $Apc^{Min/+}$ model of colon cancer cachexia develops endotoxemia and gut barrier dysfunction

IL-6 over-expression increases intestinal permeability in $Apc^{Min/+}$ mice

Intestinal permeability correlates with increases in tumor size and Il-6 levels

Acknowledgments

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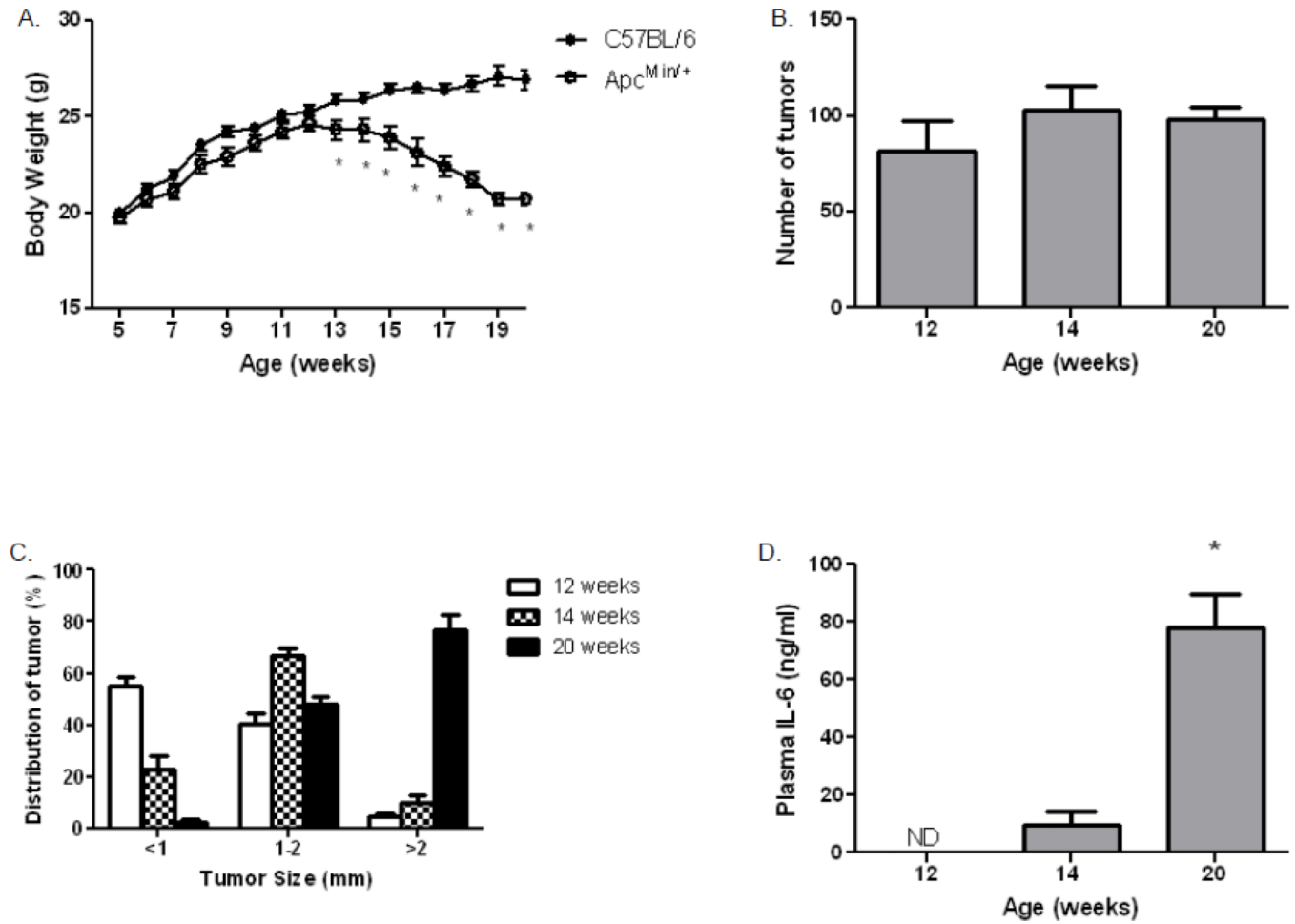
Figure 1. Changes in body weight and tumor distribution in *Apc^Min/+* mice

A) Body weight was measured weekly, C57BL/6 (●, N = 10) and Apc^Min/+ (○, N = 9) mice. (B and C) Intestines were evaluated in mice that were sacrificed at 12, 14 and 20 weeks of age. Polyps were counted and categorized by diameter. D) Changes in IL-6 concentration during progression of cachexia. Data are expressed as means ± SE. *p<0.05 indicates significant difference from controls.
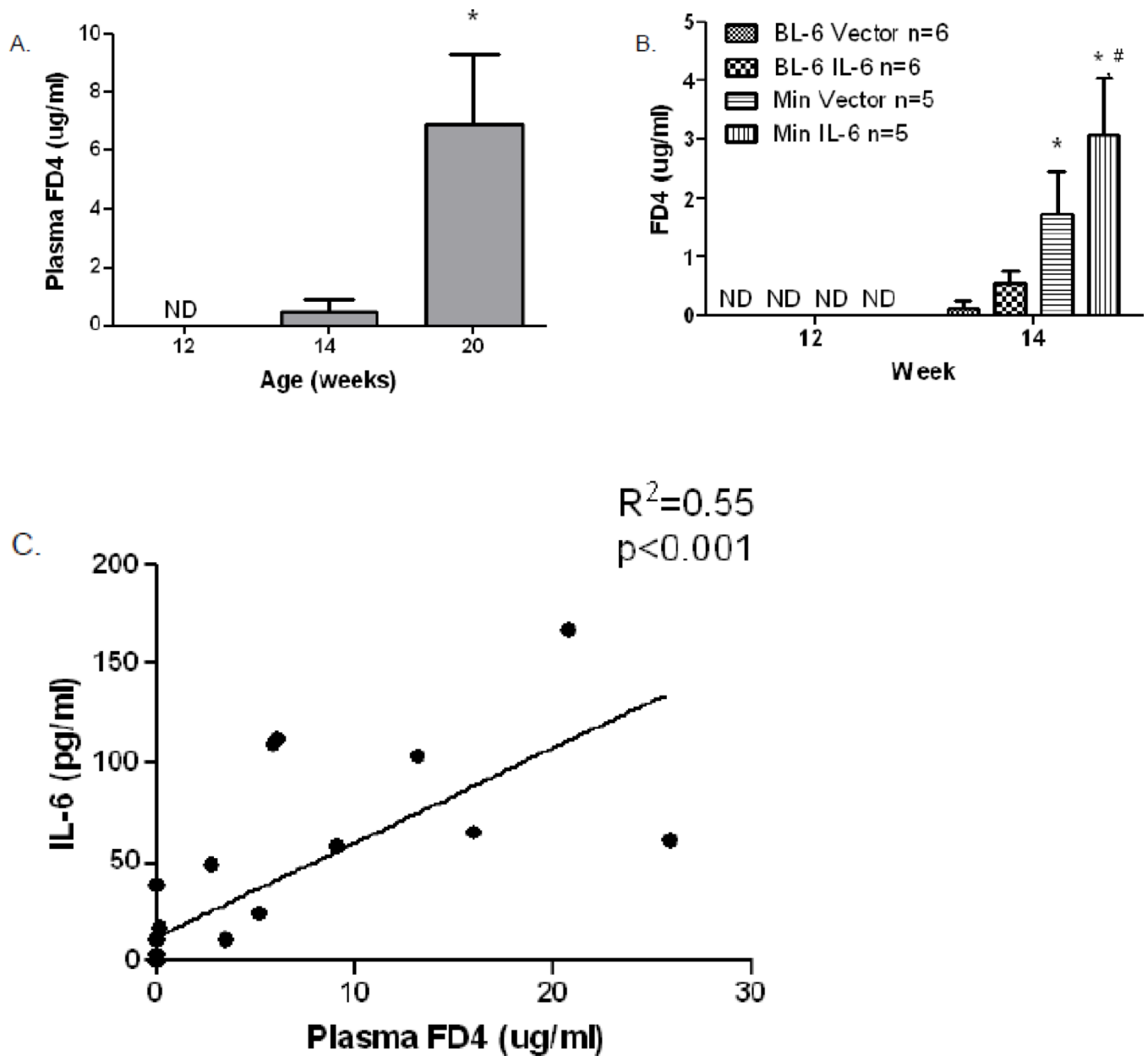
Figure 2. Gut permeability (barrier dysfunction) increases during development of cachexia in *Apc^Min/+* mice

A) Measurements were taken at 12 (n = 8), 14 (n = 7) and 20 (n = 10) weeks of age. B) Measurements were taken before and after two weeks of IL-6 over-expression (n=5–6 per group). C) IL-6 correlates with increases in gut permeability in mice measured at 12, 14, and 20 weeks of age. Serum fluorescein dextran concentrations (λ_{ex} = 485; λ_{em} = 535 nm) were measured at one hour after gavage. Data are expressed as means ± SE. **$p<0.05$, indicates significant difference from other groups; *$p<0.05$ compared with 14 week BL-6 Vector; # $p<0.05$ compared with 14 week BL-6 IL-6; ND = not detected.
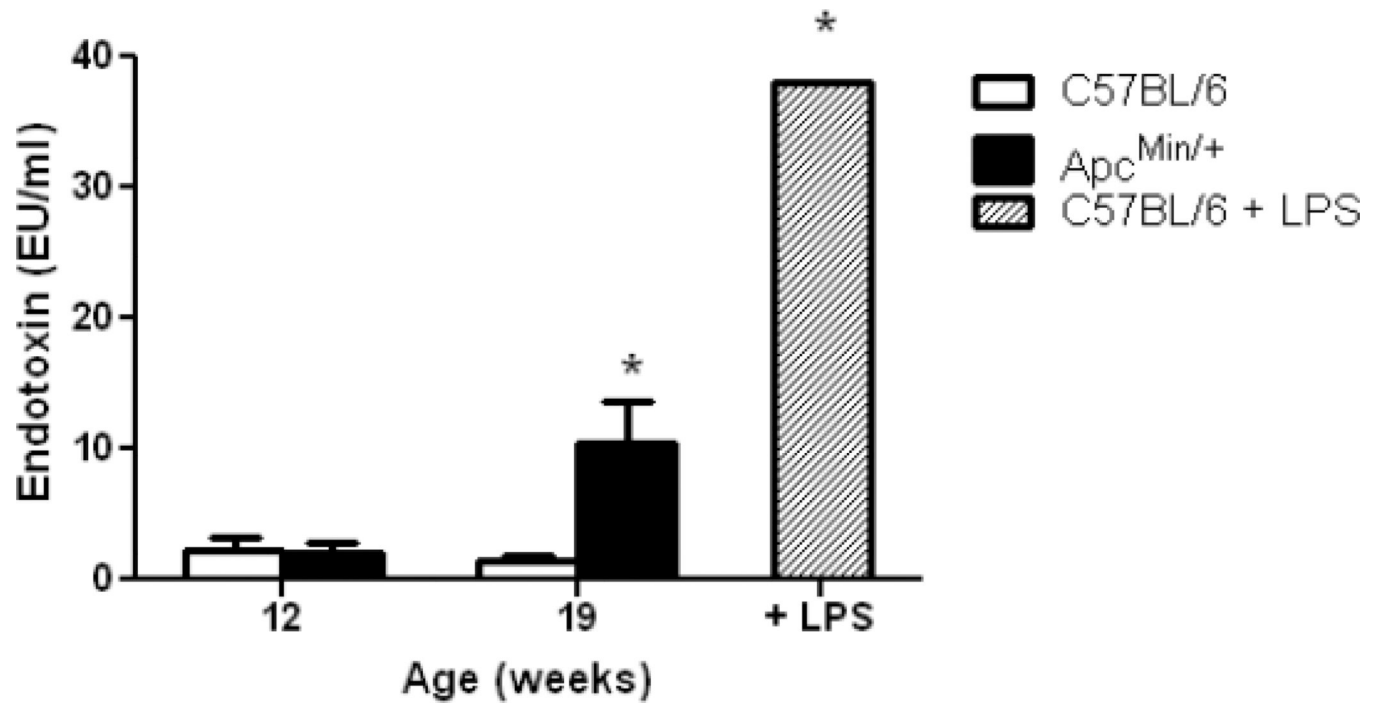
Figure 3. Plasma endotoxin concentration increases during development of cachexia in *Apc^Min/+*
mice
N = 7–9 per group. Right hand bar represents plasma endotoxin concentration in C57BL/6
mice at 12 hr after intraperitoneal injection of 250µg of endotoxin (N = 2). Data are means ±
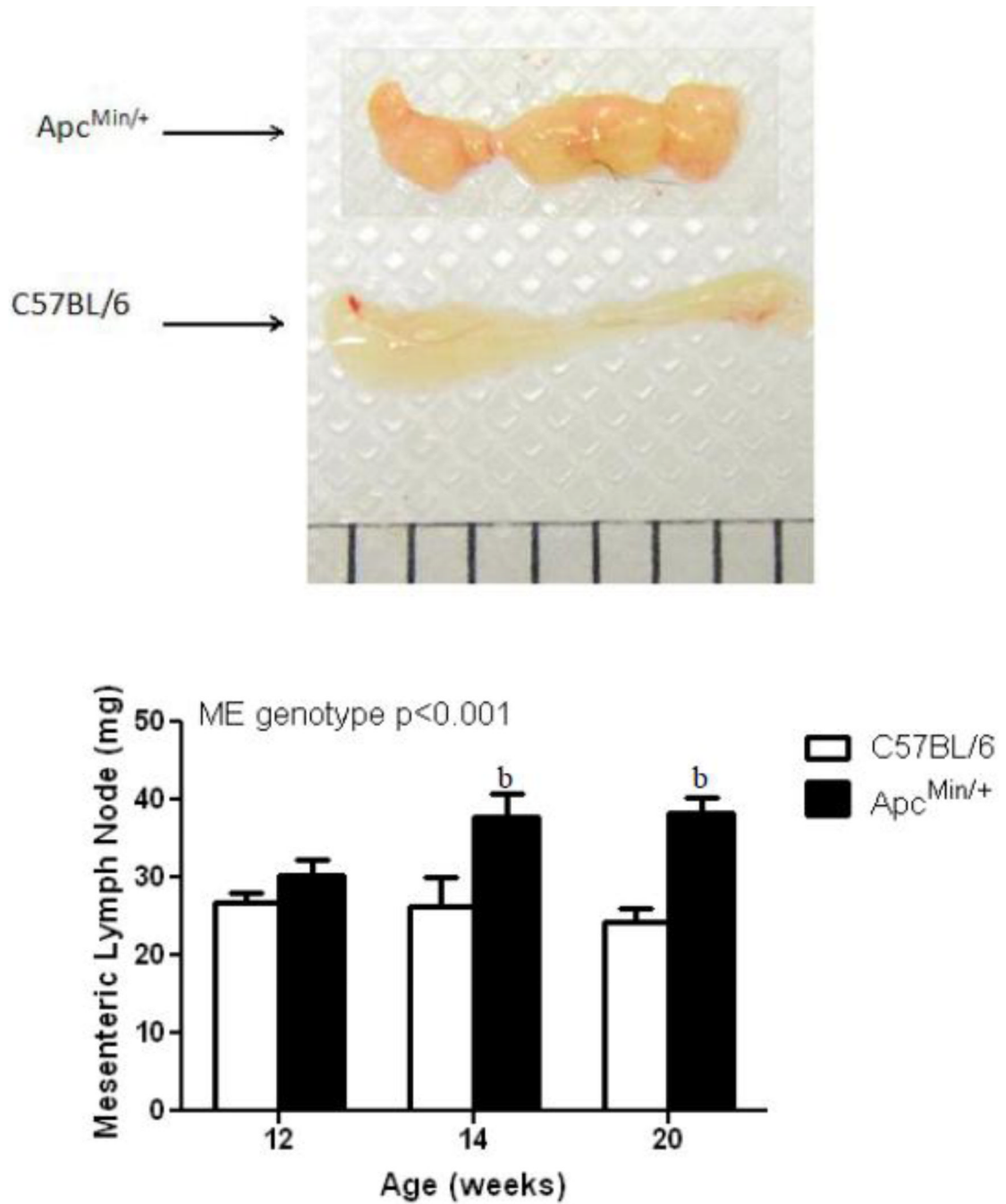SE. * p<0.05. vs. all other groups.

Figure 4. Mesenteric lymph nodes are enlarged in *Apc^Min/+* compared to C57BL/6 mice at 12, 14, and 20 weeks of age

Mesenteric lymph nodes were taken in C57BL/6 and ApcMin/+ mice at 12 (n = 8/8), 14 (n = 8/8) and 20 (n = 9/6) weeks of age. Data are expressed as means ± SE. ^{b}p<.05 compared to 12 week *Apc^Min/+*.
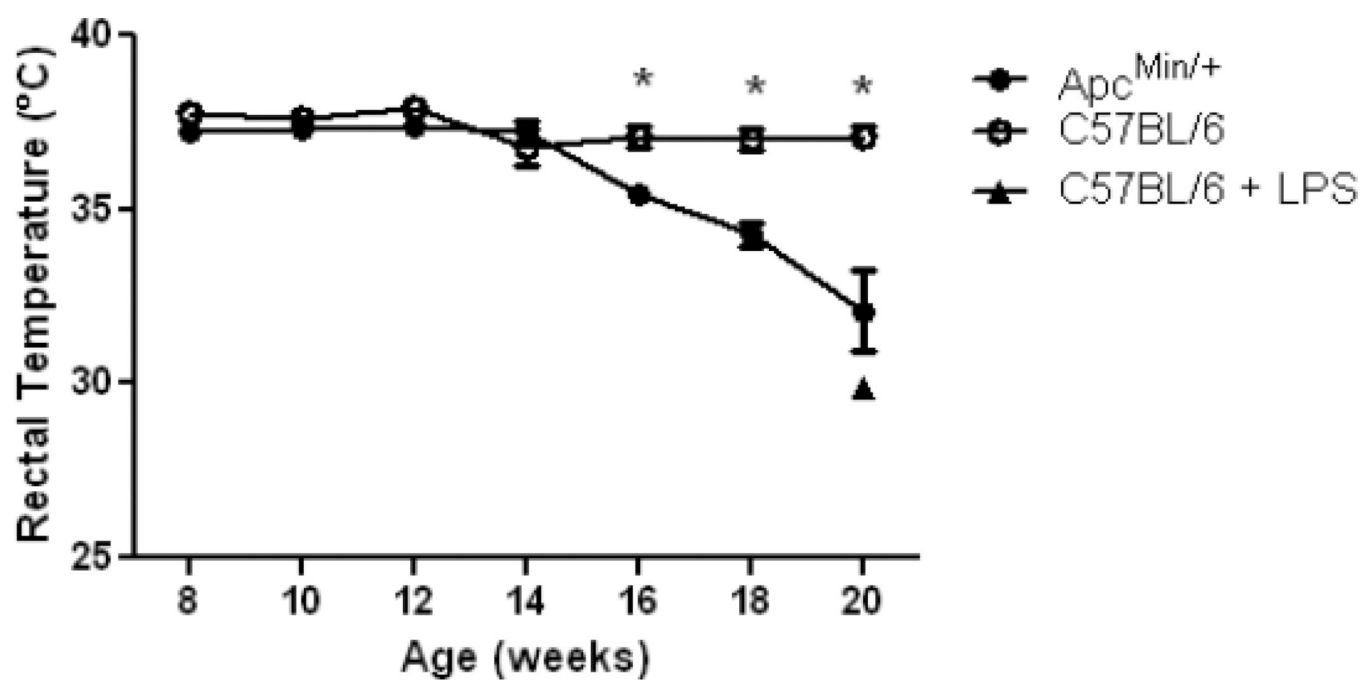
Figure 5. Decrease in body temperature with development of cachexia in Apc^Min/+ mice
Mean rectal temperature is shown for C57BL/6 (●, N = 10) and Apc^Min/+ (○, N = 9) mice,
C57BL/6 mice at 12 hr after intraperitoneal injection of 250μg of endotoxin (▲, N = 2).
Data are expressed as means ± SE. *p<.05 indicates significant difference from controls.
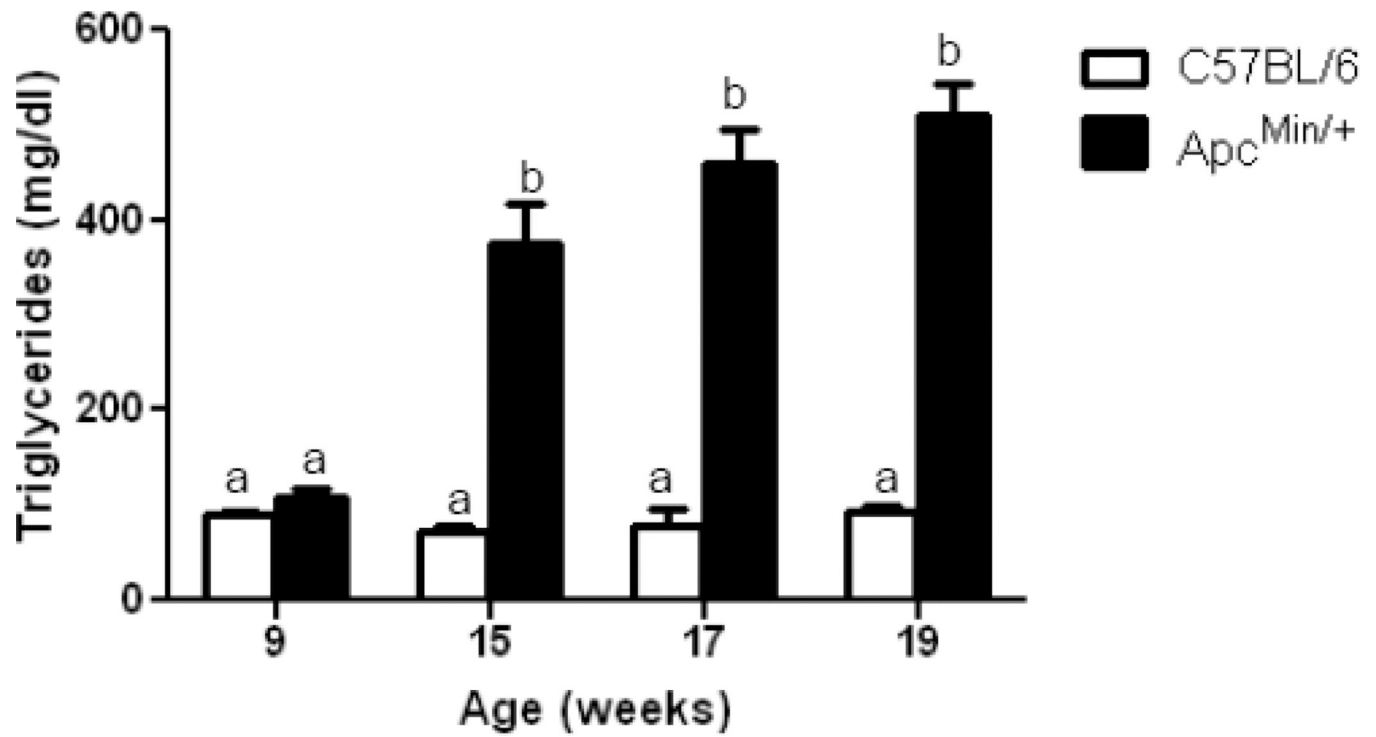
Figure 6. Plasma triglyceride concentration increases during development of cachexia in the *Apc^Min/+* mouse

Plasma triglyceride concentration was measured in the same animals over time, N = 7–9. Data are expressed as means ± SE. [b]p<.05 indicates significant difference from controls and Apc^Min/+ at 9 weeks.
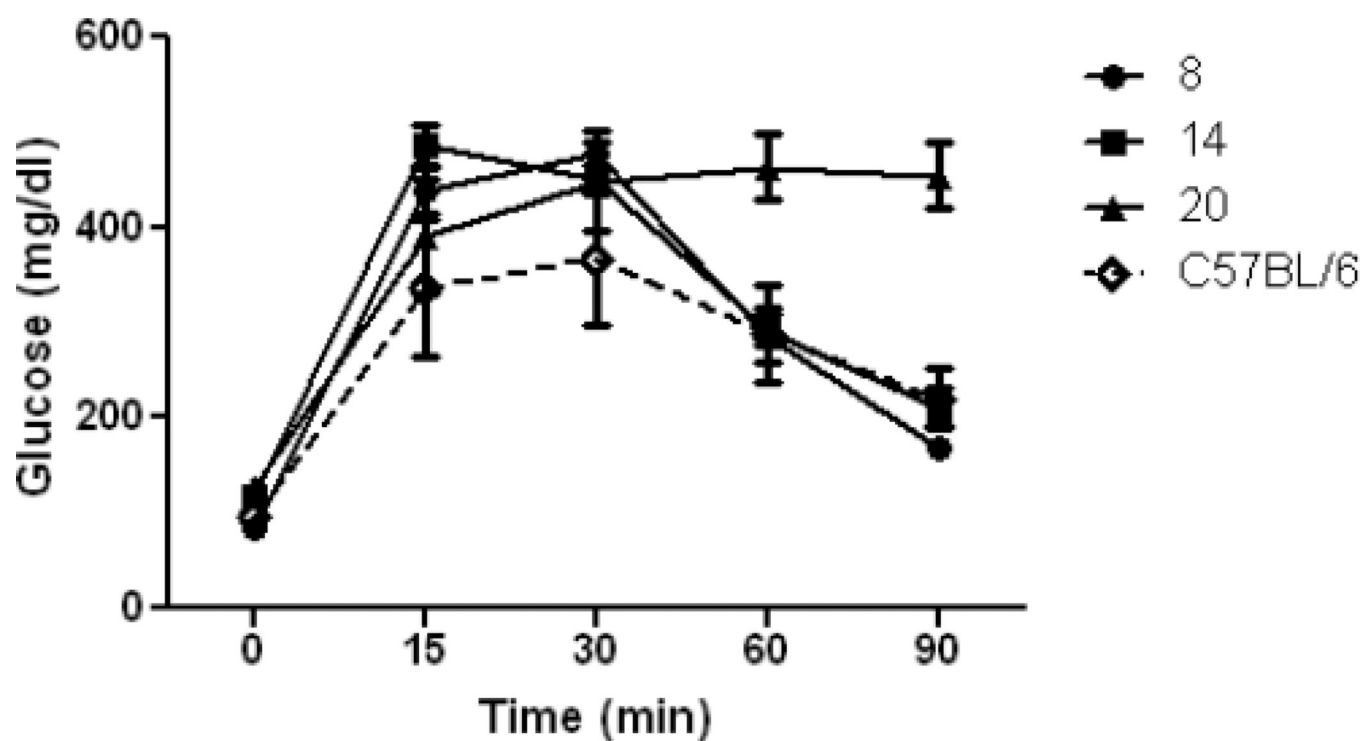
Figure 7. Insulin resistance increases during development of cachexia in *Apc*$^{Min/+}$ mice
Glucose (2.0 g / kg of body weight) was injected intraperitoneally. Glucose was measured at
0, 15, 30, 60 and 90 min. Results are means ± SE, N = 6.