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ADDENDUM

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# Hypogonadism alters cecal and fecal microbiota in male mice

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**ABSTRACT**

Low testosterone levels increase the risk for cardiovascular disease in men and lead to shorter life spans. Our recent study showed that androgen deprivation via castration altered fecal microbiota and exacerbated risk factors for cardiovascular disease, including obesity, impaired fasting glucose, excess hepatic triglyceride accumulation, and thigh muscle weight loss only in high-fat diet (HFD)-fed male mice. However, when mice were administered antibiotics that disrupted the gut microbiota, castration did not increase cardiovascular risks or decrease the ratio of dried feces to food intake. Here, we show that changes in cecal microbiota (*e.g.*, an increased *Firmicutes*/*Bacteroidetes* ratio and number of *Lactobacillus* species) were consistent with changes in feces and that there was a decreased cecal content secondary to castration in HFD mice. Castration increased rectal body temperature and plasma adiponectin, irrespective of diet. Changes in the gut microbiome may provide novel insight into hypogonadism-induced cardiovascular diseases.

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## Association of testosterone levels with cardiovascular disease and mortality

Testosterone is produced in the testis and is the major androgen (male sex hormone) circulating in the blood. Testosterone production temporarily increases during the prenatal and neonatal periods, is highly elevated at puberty, and then steadily declines with age (~2% per year) in men.[1,2] Low blood testosterone is a criterion for the diagnosis of late-onset hypogonadism (LOH), which impairs general physical and mental health status in men.[3] A meta-analysis showed that blood testosterone levels are negatively associated with longevity in men, with an increase in cardiovascular disease risk accounting for this shortened lifespan.[4] In addition, androgen deprivation therapy for prostate cancer patients increases cardiovascular events,[5] and testosterone replacement therapy in men with LOH extends longevity.[6] These results suggest the causality between testosterone deficiency and increased mortality. Androgen deprivation therapy is a widely accepted therapeutic approach for the treatment of prostate cancer, the most diagnosed cancer in Western countries.[5] In addition, LOH is diagnosed in ~2% of elderly men.[3] Therefore, it is important to clarify how hypogonadism affects cardiovascular disease and overall survival in males.

## Sex hormones and gut microbiota

The gut microbiota consists of $> 10^{14}$ microorganisms that contain more than 100-fold the number of genes than in humans,[7] and is considered a hidden metabolic organ because of its profound effect on the host, such as its role in promoting inflammatory and metabolic diseases such as obesity and type 2 diabetes mellitus (T2DM).[8-11] The gut microbiota is estimated to be composed of ~1000 bacterial species,[7] 80–90% of which belong to the *Firmicutes* or *Bacteroidetes* phyla.[9] Alterations in the composition and/or function of gut microbiota, which can occur in pathological states, is called dysbiosis. For example, lower bacterial diversity

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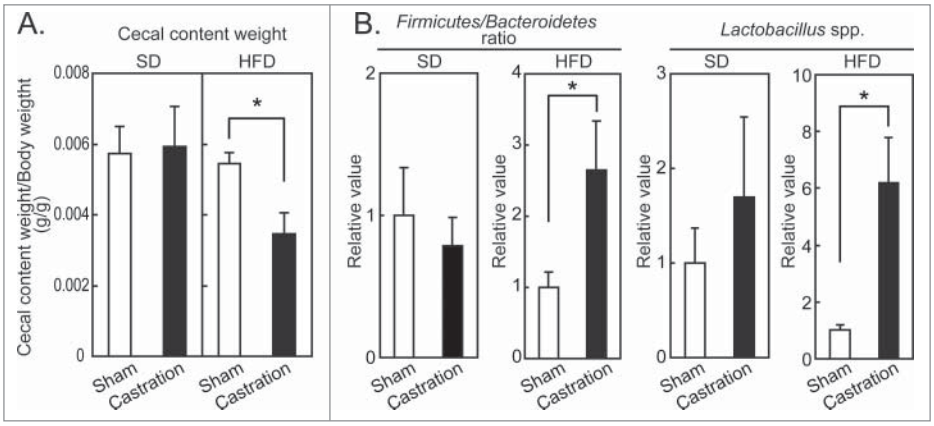


**Figure 1.** Effects of castration and diet on cecal weight and cecal microbiota. Mice were castrated or sham operated at 8-weeks-old and grown to 13 weeks with either a standard diet (SD) or high-fat diet (HFD), as described previously [16] (A) Cecal content was weighed. (B) DNA was extracted from cecal microbiota and analyzed by real-time PCR using specific primers.[16] Data were analyzed by Student's *t*-test using JMP statistical software version 8.0.1 (SAS Institute, Cary, NC, USA). Data were expressed as means ± SEM, and the threshold for a statistically significant difference between groups was set at $p < 0.05$ and was denoted by an asterisk (SD sham, n = 8; SD castration, n = 7; HFD sham, n = 8; HFD castration, n = 6).

in the gut has been associated with obesity.[12] An increased *Firmicutes*/*Bacteroidetes* phyla ratio had also been linked to obesity in mice, although this relationship is still controversial in humans.[9,13]

Sex differences in the gut microbiota are associated with sex-biased disorders such as female-biased autoimmune disease.[14] Although sex differences are also observed in the prevalence of metabolic diseases,[15] the involvement of sex hormones in the gut microbiota in sex-biased metabolic changes remains poorly understood. Our recent study showed that androgen deprivation by castration changes the composition of fecal microbiota in a high-fat diet (HFD)-dependent manner.[16] In addition, castrated male mice fed an HFD also exhibited obesity, impaired fasting glucose, excess accumulation of liver triglyceride, and thigh muscle loss. Notably, when mice were administered antibiotics, which disrupt the gut microbiota, these undesirable effects were not induced by castration in HFD-fed mice.

In the present paper, we assessed the effects of castration on cecal microbiota because few studies have examined the consistency of bacterial components between the feces and cecum. In accordance with our recent paper,[16] C57BL/6J mice were castrated at 8-weeks-old and fed the AIN93G-based standard-diet (SD, containing 7% corn oil), or the HFD (containing 14% beef tallow, 14% lard, and 2% corn oil substituted for cornstarch). Castrated mice (sacrificed at 13-weeks-old) exhibited smaller cecal content weight than sham-operated mice in the HFD-dependent manner (Fig. 1A). In addition, castration increased the ratio of the *Firmicutes*/*Bacteroidetes* phyla and *Lactobacillus* species when mice were fed with the HFD (Fig. 1B). These results were consistent with the changes in fecal microbiota.[16]

## Obesity

Obesity is strongly associated with an increased risk of cardiovascular disease.[17] Unlike in humans, dogs, cats, or pigs, androgen deprivation (*e.g.*, castration or luteinizing hormone-releasing hormone analog) had not been reported to induce obesity in mice or rats.[18-20] However, our recent study showed that castration-induced obesity in C57BL/6J mice occurred in a diet-dependent manner, despite decreased food intake.[16] In general, decreased body temperature secondary to lower heat production is considered a risk for the development of obesity because of the decreased energy expenditure. However, the body temperature of obese castrated mice was higher than that in sham-operated mice (Fig. 2A). Therefore, a castration-induced alteration of body temperature counters the induction of obesity, and thus is not a suitable explanation of castration-induced obesity.

The ratio of total dried fecal weight to food intake was significantly decreased in castrated mice fed the HFD.[16] The amount of food consumption was used to normalize dried fecal weight because it affects fecal weight.[21] Feces are composed of ~75% water and residual solid materials, the majority of which are

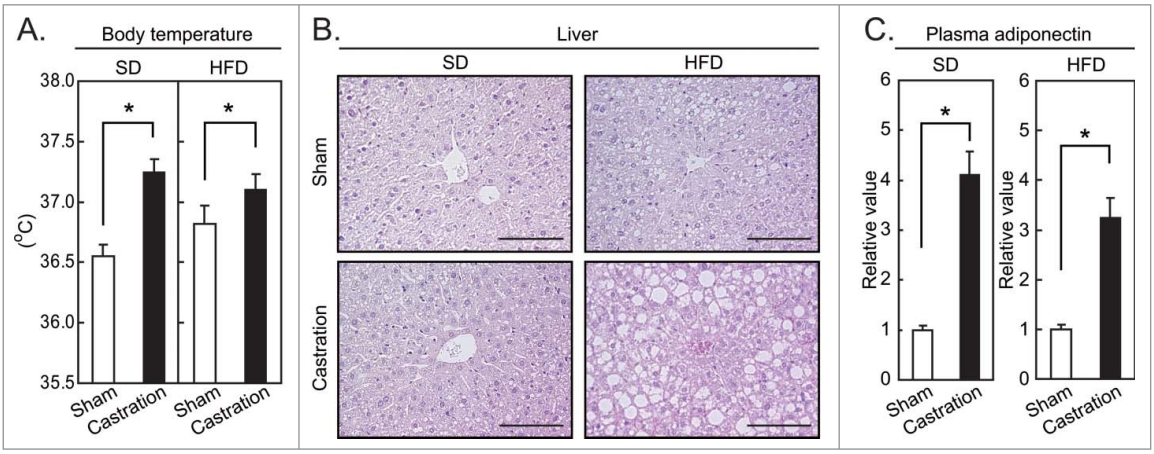


**Figure 2.** Effects of castration and diet on the development of fatty liver, plasma adiponectin levels, and rectal body temperature. Mice were castrated or sham operated at 8-weeks-old and grown to 24 weeks with either a standard diet (SD) or high-fat diet (HFD).[16] (A) Rectal body temperature was measured with a digital thermometer (KN-91, Natsume Seisakusho, Tokyo, Japan) at 17 weeks of age (SD sham, n = 6; SD castration, n = 6; HFD sham, n = 7; HFD castration, n = 7). (B) The liver sections were stained with hematoxylin and eosin. Representative images of each group are shown (scale bar = 100 $\mu$m). (C) Plasma adiponectin levels were determined by western-blotting using anti-adiponectin rabbit polyclonal antibody (GTX23455, GeneTex, San Antonio, TX, USA), and the immunoreactive bands were developed as described previously.[60] The band intensity was quantified using Image J software (ver. 1.48, National Institutes of Health, Bethesda, MD, USA) (SD sham, n = 5; SD castration, n = 5; HFD sham, n = 7; HFD castration, n = 7). Data were analyzed by Student's *t*-test using JMP statistical software version 8.0.1. Data were expressed means ± SEM, and the threshold for a statistically significant difference between groups was set at $p < 0.05$ and was denoted by an asterisk.

organic materials.[21] The organic matter consists of unabsorbed food, cellular debris, and bacteria, with the bacterial biomass making up to half of these contents.[21] On the other hand, a large part of dried cecal content is attributed to indigestible food such as fiber,[22] and thus, cecal content is greatly increased in germ-free or antibiotic-treated mice. Turnbaugh *et al.*[9] indicate that the increased ratio of the *Firmicutes*/*Bacteroidetes* phyla decreases the bomb calorimetry of the fecal gross energy content, and thus results in an enhancement of the energy harvest from food. The increase in the *Firmicutes*/*Bacteroidetes* phyla ratio observed in the gut microbiota of the HFD-fed castrated mice may enhance the energy harvest from the HFD, which is associated with obesity or decreased cecal contents. Although dried fecal weight is highly associated with the fiber content in the diet,[21] the change in dried fecal weight may partly reflect the energy harvest from food whenever the same diet is fed. In addition, our results suggest that the calculation of dried fecal weight per food intake is valuable for the speculation of changes in the gut microbiota.[16]

Unlike our recent study,[16] previous models in which castrated C57BL/6 mice were fed with an HFD did not develop obesity.[18,20] These inconsistent results suggest that feeding an HFD is insufficient for castrated mice to exhibit the obese phenotype secondary to androgen deficiency. We propose that diet contents such as the fat source and/or fiber may be important for castration-induced obesity because these dietary factors strongly affect the gut microbiota.[23,24] The gut microbiota is generally considered to be involved in the response of the host to dietary components that affect metabolisms; the underlying mechanisms of this response have been discussed in a recent review.[24]

## Non-alcoholic fatty liver disease (NAFLD)

The liver plays a central role in glucose and lipid metabolism, and NAFLD is associated with an increased risk of cardiovascular disease.[25] NAFLD develops in ~20% of adults and progresses to non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma.[26] NAFLD is increasing with the prevalence of obesity and T2DM, and in fact, 50–90% of obese individuals are diagnosed with NAFLD.[25,27] In our previous study, the castrated mice accumulated excessive triglycerides in the liver in the HFD-dependent manner.[16] Histopathological analysis demonstrated the development of hepatic steatosis in HFD-fed castrated mice (Fig. 2B). Our results are consistent with previous studies that showed that

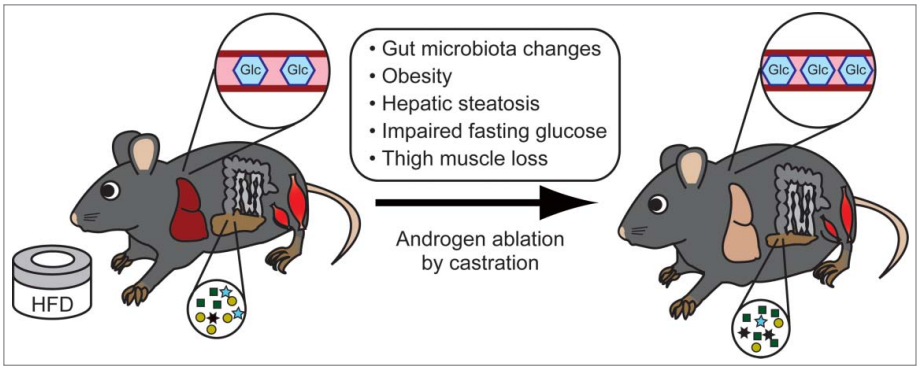


**Figure 3.** Schematic presentation of the effects of interactive effects between castration and high-fat diet intake in male mice. Castration influenced the gut microbiota and caused obesity, hepatic steatosis, thigh muscle loss, and impaired fasting glucose in male mice in the high-fat diet (HFD)-dependent manner.[16] These observations were not induced by castration when antibiotics were provided.[16]

androgen deprivation enhances (and androgen replacement suppresses) the development of hepatic steatosis and NAFLD in HFD-fed rodents.[28,29]

The gut microbiome and mesenteric fat both strongly influence the development of NAFLD.[27,30,31] The gut-visceral fat (*i.e.*, mesenteric and omental)-liver axis is central to this association.[32] The liver receives ~70% of its blood supply from the intestine,[33] and the venous blood stream from the mesenteric and omental fat directly flows into the liver.[32] Our castrated mice with NAFLD also exhibited excess mesenteric fat deposition.[16] Therefore, the development of NAFLD by castration could be affected by changes in the gut microbiome and the accumulation of mesenteric fat.

## Type 2 diabetes mellitus (T2DM)

T2DM is strongly associated with NAFLD and obesity,[34,35] as well as increases the risk of cardiovascular disease.[36] A meta-analysis showed that higher testosterone levels are associated with lower and higher T2DM risks in men and women, respectively.[37] In addition, androgen deprivation therapy for prostate cancer patients leads to a worsening of T2DM.[38] In our recent study with HFD-fed mice, fasting glucose levels increased after castration, whereas castration did not affect insulin resistance.[16] On the other hand, plasma adiponectin levels increased with castration, irrespective of diet (Fig. 2C). Increased blood adiponectin levels may ameliorate insulin sensitivity and suppress the development of insulin resistance.

Adiponectin levels are lower in men than in women,[39-42] whereas the relationship between adiponectin levels and androgen status remains controversial in men and male mice. Several studies, including our results, showed that testosterone levels are negatively associated with adiponectin levels in males,[40,43] whereas several studies indicated a positive association.[20,41,42] A similar discordance was also observed in androgen receptor knockout male mice.[20,44,45] On the other hand, adiponectin levels are higher in subjects with gut bacterial richness (*i.e.*, abundant in the number of gut microbial genes) than in subjects with low bacterial richness,[46] and some *Lactobacillus* strains increased adiponectin levels in HFD-fed mice.[47,48] The gut microbiota affects adiponectin levels, and sex difference and sex hormone affect gut microbiota composition; [49] however, it remains unclear whether the gut microbiota is associated with changes in adiponectin levels by androgen status in males.

The gut microbiota is associated with T2DM prevalence.[50] The pancreatic $\beta$-cell is a direct target of androgen,[51] and the loss of its function is one of the causal factors in androgen-deprivation induced T2DM.[52] The gut-vagus-brain-pancreas axis with its glucagon-like peptide-1 affects $\beta$-cell function.[53] Gut microbe-derived metabolites may also link between changes in the gut and prevention of T2DM through the enhancement of $\beta$-cell function. For example, *S*-equol, which is produced from the soy isoflavone daidzein by gut bacteria, enhances pancreatic $\beta$-cell function.[54] Further studies are needed to clarify the association between androgen deprivation caused changes in the gut microbiota and the occurrence of T2DM.

## Thigh muscle mass

Thigh muscle mass is inversely related to the risk of cardiovascular disease.[55] Our recent study showed

that castration decreases thigh muscle (*i.e.*, quadriceps and hamstring) mass only when the mice were fed the HFD, and this phenotype was not induced by castration when mice were administered antibiotics.[16] Although the gut microbiota is involved in muscle wasting,[56] the gut-muscle axis remains poorly understood.

## Conclusion

Androgen deprivation poses a risk for cardiovascular disease in men. Our studies showed that androgen deprivation via castration altered cecal and fecal microbiota and exacerbated risk factors for cardiovascular disease, including obesity, impaired fasting glucose, excess hepatic triglyceride accumulation, and thigh muscle weight loss in HFD-fed mice (Fig. 3). Androgen–androgen receptor action directly affects the tissue physiology that plays a pivotal role in carbohydrate and lipid metabolism in the liver,[57] fat tissue,[58] muscle,[59] and pancreatic $\beta$-cells.[51,52] On the other hand, these direct androgen actions could not explain its effects on lipid and carbohydrate metabolism fully, suggesting the existence of indirect effects. Our recent results indicate that androgen deficiency can alter the gut microbiota in a diet-dependent manner.[16] The change may, at least in part, explain the indirect action of androgen.

## Disclosure of potential conflicts of interest

The authors have no conflicts of interest.

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