Exposure to BPA and DEHP following myocardial infarction affects gut dysbiosis, systemic inflammation and body weight in a sex-specific and dose-dependent manner.

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I. Abstract

Exposure to endocrine disruptors such as bisphenol A (BPA), the monomer which makes up hard plastics, and di-ethylhexyl phthalate (DEHP), the phthalate plasticizer that makes hard plastics soft and flexible, affects most of the population given the extensive use of plastics, notably in food containers. Less well known is that more than 50% of medical devices contain plastic components. Notably, devices such as endotracheal tubes, IV bags and catheters contain these substances that escape to contaminate tissues and solutions. Studies carried out on mice showed that those exposed to bisphenols and phthalates during recovery from an induced myocardial infarct had greater cardiac dilation, worse cardiac function and greater numbers of pro-inflammatory macrophages than control mice. These effects were more prominent in males than females. Chronic phthalate and bisphenol exposure was linked to gut dysbiosis in mice, as it induced obesity and diabetes. However, the link between acute exposure to BPA & DEHP and gut dysbiosis following a myocardial infarction is not well established. To study the effects of acute exposure to bisphenols and phthalates on the intestinal composition, and whether it leads to reduced cardiac function and inflammation, we exposed male and female mice of different ages to vehicle or BPA+DEHP treatment. Some of the mice underwent cardiac surgery, and physiological data (body weight, spleen weight and cecum weight) were collected for all. To determine the gut microbiome composition of the mice, cecal contents were extracted at euthanasia and r16s amplification and sequencing was performed on extracted DNA. Metabolomics analyses were also performed on cecal contents to determine the metabolic profile of the mice. Furthermore, inflammation was characterized by flow cytometry of bone marrow, spleen and heart samples. Among all groups, male mice were consistently losing more weight than their female counterparts and coping less well to the surgery. Preliminary data shows that the AD treatment tends to decrease spleen inflammation in the different groups. DNA sequencing analyses show that the treatment induces gut dysbiosis and causes a shift in

Firmicutes/Bacteroidetes ratio. Finally, metabolomics analyses indicate that several metabolites are affected by the surgery and treatment.

L'exposition aux perturbateurs endocriniens dont le bisphénol A (BPA), le monomère qui compose les matières plastiques dures, et le phtalate de di-2-éthylhexyle (DEHP), qui les rend flexibles et malléables, touche une majorité de la population étant donné leur vaste utilisation dans les objets en plastique, notamment les conteneurs de nourriture. De plus, il est moins reconnu que plus de 50% des équipements médicaux contiennent ces mêmes matières. Spécifiquement, les dispositifs comme les tubes endotrachéaux, les sacs intra-veineux, et les cathéters contiennent du BPA et du DEHP qui s'échappent et contaminent les tissus et solutions. Des études faites sur des souris ont montré que celles qui ont été exposées aux bisphénols et phthalates pendant leur récupération suite à un infarctus du myocarde induit avaient une dilatation cardiaque augmentée, une fonction cardiaque réduite, ainsi qu'un plus grand nombre de macrophages pro-inflammatoires que les souris contrôles. Ces effets étaient plus communs chez les males que les femelles. L'exposition chronique aux bisphénols et phthalates a été liée à la dysbiose intestinale qui induit l'obésité et le diabète. Cependant, le lien entre l'exposition aigüe au BPA & DEHP et la dysbiose intestinale dans le cadre d'un infarctus du myocarde n'est pas clairement établi. Afin d'étudier les effets de l'exposition aigüe aux bisphénols et phthalates sur la composition microbienne de l'intestin, et de déterminer si elle induit une réduction de la fonction cardiaque et l'inflammation, on a exposé des souris mâles et femelles d'âges différents à une solution contrôle ou à un traitement à base de BPA et DEHP. Certaines de ces souris ont subi un infarctus du myocarde, tandis que des données physiologiques (masse corporelle, masse de la rate, masse du caecum) ont été notées pour toutes les souris. Afin de déterminer la composition intestinale des souris, le contenu caecal a été extrait après l'euthanasie, l'ADN bactérien en a été isolé et une amplification r16s suivie d'un séquençage ont été performés. Des analyses métabolomiques ont également été faites afin de déterminer le profile métabolique des souris. De

plus, l'inflammation a été caractérisée par cytométrie en flux d'échantillons de la moelle osseuse, de la rate et du cœur. Parmi tous les groupes, les males ont régulièrement perdu plus de poids que leurs homologues femelles et ont moins bien réagi à la chirurgie. Les données préliminaires montrent que le traitement induirait une réduction de l'inflammation dans les différents groupes. L'analyse des données génétiques indique que le traitement au BPA et DEHP induit une dysbiose intestinale et altère la composition microbienne en changeant le ratio *Firmicutes/Bactéroidètes.* Enfin, les analyses métabolomiques indiquent que plusieurs métabolites sont affectées par l'infarctus du myocarde et le traitement.

II. Acknowledgments

I would like to express my sincere gratitude to everyone who supported and helped me throughout my training, leading to the submission of the following thesis. Firstly, I would like to thank my supervisor, Dr. Lorraine Chalifour, for welcoming me into her laboratory and for her continuous support and guidance. None of this would have been possible without her. Secondly, I extend my gratitude to the LDI AQ staff for their admirable professionalism and help throughout the project. I also thank my brothers, Christopher and Karl, for their constant support and for their unwavering positivity. Finally, I would like to thank my parents, Jeanine and Salim, to whom I dedicate this thesis and future accomplishments. Their unconditional support and love fuel my dedication and motivation to keep going regardless of the circumstances.

III. Contribution to original knowledge

Our study directly compares the sex-specific impact of acute DEHP and BPA exposure on the gut microbiome, heart regeneration and inflammation following a surgically-induced myocardial infarction. The data is consistent with the available scientific literature in that males are generally more affected and cope less well in several ways than their female counterparts. On another hand, our findings suggest that the exposure decreases inflammation which agrees with some studies and contradicts others. We are among the first to study the effects of acute exposure to BPA and DEHP on the gut microbiome and possible dysbiosis following cardiac surgery, and to characterize the microbiome in the setting of BPA/DEHP exposure similar to that following surgery.

IV. Contribution of authors

M.Sc. candidate Joey-Bahige Chammas carried out the experiments, collected data, performed data analysis and drafted the manuscript. Dr. Lorraine Chalifour conceived the study, participated in research coordination, assisted with experimental data collection and analysis, and edited the thesis manuscript. Cecum contents extracted by the candidate were sent to Dr. Jeff Xia's lab for metabolomics analyses performed by Ms. Yao Lu and Mr. Charles Viau. In addition, DNA extracted by the candidate was sent to the McGill Genome Center for amplification and sequencing, then analysed in collaboration with Dr. Ken Dewar. Finally, Mr. Alex Nissenbaum helped in data manipulation of histology and echocardiography results.

V. Abbreviations

BPA, Bisphenol A ; DEHP, di(2-ethylhexyl) phthalate ; PVC, polyvinyl chloride ; CDC, Center for Disease Control and Prevention ; NHANES, National Health and Nutrition Examination Survey ; CVD, cardiovascular disease ; DEP, di-ethyl phthalate ; BBzP, butyl benzyl phthalate ; CABG, coronary artery bypass graft ; AVR, aortic valve replacement ; CPB, cardiopulmonary bypass ; SCFA, short-chain fatty acids ; IL-1β, Interleukin-1β ; DAMP, damage-associated molecular patterns ; NLR, nod-like receptors ; NLRP3, NLR family pyrin domain containing 3 ; DMEM, Dulbecco's modified eagle medium MI, myocardial infarction ; VEH, vehicle ; AD, BPA+DEHP ; RB, retired breeder ; YG, young ; M, male; F, female ; BW, body weight ; SPL, spleen ; BM, bone marrow ; RP, red pulp ; WP, white pulp ; MZ, marginal zone ; LV, left ventricle ; *F/B*, *Firmicutes/Bacteroidetes* ; GI, gastrointestinal ; FAC, fractional area contraction ; PBS, phosphate buffer saline ; ACK, ammonium-chloride-potassium lysing buffer ; FACS, flow cytometry staining buffer ; EDTA, Ethylenediaminetetraacetic acid ; TBA, tris-borate-EDTA ; ASV, amplicon sequence variant ; PCA plot, principal component analysis plot.

VI. Introduction

A. **Bisphenols as endocrine disruptors**

i. Overview

Plastic use began in the mid-20th century and its use has grown since to become predominant in many sectors. According to the Independent Commodity Intelligence Service (ICIS) Supply and Demand database, global plastic demand is projected to reach 308 million metric tons between 2021 and 2025 (2). This means that exposure to the components of plastics, whether in the workplace during their manufacture, as they are used for their designated purpose or during recycling or disposal, is rising. The increase in production and use of plastics in everyday life has led to an increase in the release of such additives into our environment.

Plasticizers, such as phthalates, are an essential component in the production of plastics. These are liquid substances that are incorporated into plastics, such as polyvinyl chloride (PVC), to make the end product softer and more flexible (3). As shown in Figure 1, heat increases the intermolecular distance between PVC molecules allowing the introduction of plasticizers between them (1). Plasticizers are not fixed within the PVC and can escape into the environment. There are concerns that the increase in exposure may impact consumer health (4). Hundreds of tons of plasticizers are released each year into the biosphere (5).

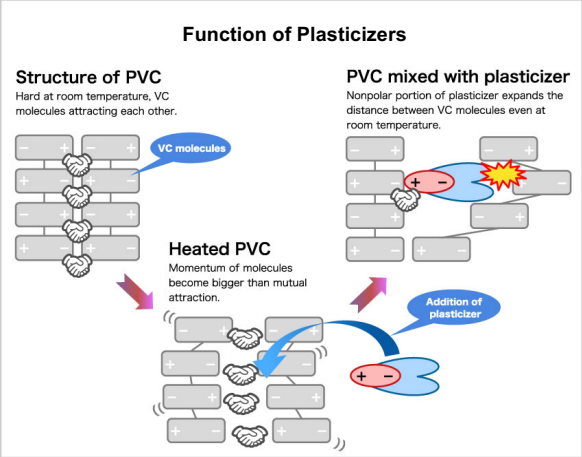


Figure 1 - Function of plasticizers in vinyl chloride softening. From: (1)

Bisphenols are a group of chemical compounds used as monomers in the production of plastics (4). They are used in the manufacture of hard plastics, epoxy resins and are found in a variety of products including medical devices, food containers, water bottles and face makeup. BPA and its analogs are not fixed in the plastic product. Like the plasticizers, they leach from the

plastic to contaminate the environment. As a group, bisphenols have been linked to several adverse health effects in the reproductive system of men and women, and in many species in the environment (6). Population monitoring studies identified bisphenol A (BPA) and bisphenol S (BPS) as the most common bisphenol detected leading to the idea that they are the most widely used in manufacturing (7).

There is great interest in the role of these chemicals in health and disease because research discovered that they are endocrine disruptors (8). Endocrine disruptors are defined as "environmental contaminants that perturb hormonal systems" (9).

ii. Bisphenol A (BPA)

Bisphenol A (BPA) is one of the most produced chemicals worldwide and is the most commonly used bisphenol. It is estimated that more than 8 billion pounds of BPA are produced each year, while more than 100 tons are released in the atmosphere (10). Like all bisphenols, it possesses two hydroxyphenyl functionalities, but is distinguished by the two methyl groups attached to them (Figure 2). It is insoluble in water but soluble in chemical reagents and organic solvents. Given the heavy and steadily increasing production of BPA, it has become an environmental and public health problem. In fact, BPA has been classified as a toxicant and a pollutant, meaning it causes adverse effects on both the environment and biological organisms (11, 12). Humans are exposed to BPA through inhalation, the skin, or ingestion. The European Food Safety Authority set the tolerable daily intake of BPA at 4 µg/kg/day (13). BPA is not only considered a dangerous substance but has been seen in a large majority of the population. CDC-led biomonitoring studies have indicated that BPA is found in nearly all of the U.S. population (14). In Canada, the most recent Health Measure Survey detected BPA in 81.5% of the population (15). These findings highlight BPA's tendency to leak out of common products like food containers or plastic bottles

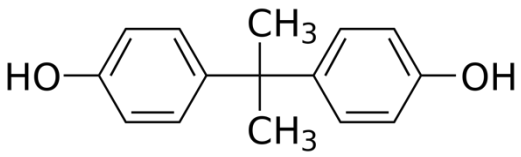


Figure 2 - Bisphenol A chemical structure.

and contaminate the consumers. As a result, the use of BPA in baby bottles has been banned in Canada (16).

iii. Health concerns linked to BPA

Given its extensive use in common goods and products, multiple studies investigated the impact of BPA exposure (17). The goal was to determine if increased exposure increased the potential for adverse effects on consumer health.

U.S. and European authorities consider BPA to be safe for consumers. Other groups promote its prohibition in food-related products (18-20). Studies have identified that BPA causes adverse effects on several organs and systems even in low-dose settings, further confirming the need for a new risk assessment (21).

a. Reproductive system and development

BPA is an endocrine disruptor since it interferes in the normal function of the reproductive system, development and endocrine function (7, 10). Specifically, BPA is a xenoestrogen. It binds to the nuclear estrogen receptor alpha (ERα) and ERβ to mimic estrogen's hormonal properties (22). BPA can also antagonize estrogen, which makes it a selective estrogen modulator of ERs (23). In addition, it is an antagonist of androgenic receptor activity (24). Thus, BPA has the potential to influence male and female steroid hormone function.

In females, BPA exposure is linked to reduced fertility by interfering with the maturing of the oocytes (25). In fact, serum levels of BPA were higher in infertile women compared to fertile women (26). Also, studies suggest that BPA exposure decreases ovarian reserves (27). The many effects and interferences that BPA has on the endocrine system are also translated in a dysregulation of puberty onset, with studies showing that BPA exposure can cause puberty to be advanced, delayed or even eliminated (28, 29). These findings indicate that BPA exposure adversely effects female development, endocrine system functions and fertility.

On the other hand, BPA impaired hormonal functions and development in males. First, studies have determined that BPA disrupts spermatogenesis (30, 31). In a large proportion of infertile men, higher BPA levels in urine samples were linked to lower sperm count and motility (32). The same observations were seen in studies on rats and mice (6).

Nonetheless, findings highlight that BPA is a disruptor of development and impairs proper function of the reproductive system in both males and females.

b. Immune effects

Aside from reproductive and developmental effects, studies linked BPA to increased stimulation of immune cells to promote diseases and inflammation. *In vitro* studies determined that cell culture in BPA-containing medium caused macrophages to develop a pro-inflammatory phenotype (33, 34). Specifically, BPA activated interferon signaling in myeloid cells, and activated inflammasome activity leading to a pro-inflammatory phenotype (34). In addition, we have previously reported greater monocyte infiltration into the cardiac wound of male mice following chronic exposure to BPA (35). Other studies have reported that BPA exposure leads to a downregulation of macrophages' activity in mice (36, 37).

c. Cardiovascular system

In addition to the reproductive system, BPA exposure was linked to adverse effects in the cardiovascular system in both a "low-dose" setting and a supra-physiological dose. In fact, among all endocrine disruptors, BPA is one of the most commonly investigated ones in regards to the incidence of cardiovascular diseases (38). Several epidemiological and experimental studies have focused on characterizing the consequences of BPA exposure on the cardiovascular system.

Data from the National Health and Nutrition Examination Survey (NHANES) compared the level of BPA in urine to the incidence of cardiovascular disease (CVD). Here, an increase in BPA

concentration was linked to an increase in CVD prevalence defined as coronary heart disease, myocardial infarction and angina (39). Some longitudinal studies confirmed this finding such that an increase of one standard deviation in BPA concentration caused a significant increase in CVD risk (40).

Experimental studies attempted to characterize the relation between BPA concentration and cardiovascular diseases. In physiologically relevant doses (low-dose setting) and using an *ex vivo* heart model, exposure to BPA promoted ventricular arrythmias in female rats, but not in their male counterparts. Specifically, BPA destabilized the handling of Ca^{2+} in cardiomyocytes in presence of estrogen that allowed a significant leak of calcium from the sarcoplasmic reticulum (41). On the other hand, chronic exposure was shown to induce cardiac concentric remodelling and increase systolic and diastolic arterial pressures, also in a sex-dependent manner. In that case, effects were more pronounced in male mice than female mice (42). A study focusing solely on male rats determined that BPA possesses a significant cardiotoxicity. BPA-exposed rats displayed increased lipid peroxidation, and decreased catalase activity, suggesting that BPA exposure impairs mitochondrial function in male hearts (43). Other indicators of BPA's cardiotoxicity in males include a decrease in vasodilators' levels such as nitric oxide which leads to increased vasoconstriction and reduced blood flow to the heart. Acute exposure to low doses of BPA interfered with anti-cancer treatments such as doxorubicin by increasing the production of pro-inflammatory interleukins to promote cardiotoxicity (38). Chronic exposure to BPA was also shown to accelerate the development of atherosclerosis in mice, which was also seen in humans (44). These findings highlight the role of BPA in inducing oxidative stress which leads to adverse effects on the cardiovascular system.

In much higher doses, not similar to the everyday exposure of the general population, BPA was shown to weaken pacemaker activity and therefore cause a significant decrease in atrial contractility (45). Furthermore, the adverse effects of BPA were also observed on ion channels. A high dose of BPA induced an activation of Maxi-K voltage-dependent potassium channels in

human coronary smooth muscle cells (46), and deactivated sodium and calcium channels in ventricular myocytes (47, 48).

In conclusion, epidemiological and experimental studies point to a role for BPA exposure in the impairment of cardiovascular function and the promotion of CVDs. It has emerged as a major disruptor of the cardiovascular system even in low doses similar to the exposure of the general population. As seen for the reproductive system and development, sex appears to be a major factor in determining to what extent BPA affects the cardiovascular system.

B. **Phthalates: common plasticizers**

i. Overview

Phthalates are a large group of chemicals commonly used in the production of plastics as liquid plasticizers. As plasticizers, phthalates make otherwise hard plastics more flexible, malleable and durable. Aside from products containing PVC such as food containers, they are found in medical devices and equipment, self-care products, electrical items, fabrics and other common products found in consumers' homes (49). Like bisphenols, plasticizer production has consistently increased to meet the needs of plastic production worldwide. In fact, the annual production of phthalates went from 470 million pounds in 2006 to 11 billion pounds in 2011 (50, 51). Phthalates, like all plastics additives, leak from products into the environment, and contaminate consumers' tissues and solutions. Phthalates are diverse and differ in their chemical structure. Some have low molecular weight and are water soluble, while others are larger and have longer R chains greatly reducing their solubility in water. Phthalates are esters of phthalic acid, and therefore possess ester groups instead of the carboxylic acid functionalities found in phthalic acid (Figure 3).

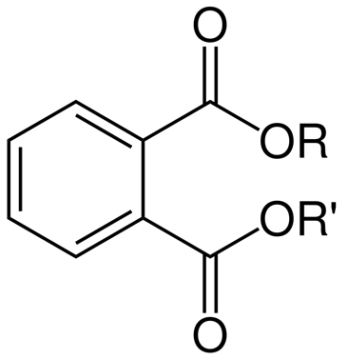


Figure 3 - Chemical structure of phthalates.

Many phthalates are linked to adverse health effects in human or animals. For instance, di-ethyl phthalate (DEP) is suggested to be a toxic substance. Increased exposure was linked to reduced sperm count and motility by targeting Sertoli cells (52). Other studies show that DEP can also influence organ weight and histopathology of both the liver and kidneys (53, 54). Another phthalate, butyl benzyl phthalate (BBzP) possesses carcinogenic properties and increases prevalence of asthma and airway inflammation in humans (55, 56).

ii. Di-ethylhexyl phthalate (DEHP)

Among all phthalates, DEHP is the most abundant in the environment and commonly used in products (57). It is the most produced phthalate and one of the most produced chemicals worldwide; its production reaches approximately 2 million tons per year (58). DEHP consists of two eight-carbon esters linked to a benzene-dicarboxylic acid ring (Figure 4), which makes it insoluble in water and soluble in organic reagents and solvents. DEHP is toxic to consumers and to the environment, even at the low doses that are common in everyday products (59). This led to a permanent ban of DEHP in children's toys in the United States, and in cosmetic products in Canada. Nevertheless, despite efforts to control the exposure and minimize it, biomonitoring studies in the US have determined that the breakdown products of DEHP are found in nearly all participants (60). In fact, DEHP is found in human serum, milk and urine (61) and the daily exposure to it is estimated to be between 3 and 30 µg/kg/day (62). This widespread exposure to DEHP is problematic given its toxic properties and the many adverse health effects that have been linked to it.

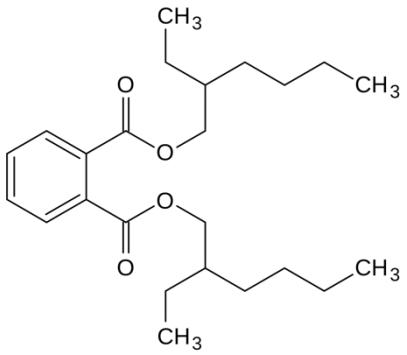


Figure 4 - DEHP chemical structure

iii. Health concerns linked to DEHP

Although health authorities attempted to reduce the exposure through limiting the use of DEHP in some products, it is still contaminating food and therefore contaminates consumers through their diet (57). Therefore, it is important to characterize its toxicity and how it affects human systems to establish an accurate risk assessment of DEHP. Given its prominent use and reach within populations, a large number of studies performed on laboratory cells and animal models were carried out to determine DEHP's health risks. These studies were also complemented by epidemiological studies on DEHP exposure. Findings indicate that DEHP has

adverse effects on development as well as the reproductive, endocrine and cardiovascular systems, amongst others.

a. Reproductive system and development

Like BPA, DEHP is an endocrine disruptor and impairs hormonal regulation and balance (63). It is a known reproductive toxicant and carcinogen and its interference with the endocrine system induces developmental and reproductive toxicity (64).

In females, DEHP exposure caused several adverse effects on the reproductive system. Studies performed on rats and mice determined that DEHP decreased serum estradiol level, reduced the number of primordial follicles, prolonged estrous cycles and prevented ovulation (65-67). Epidemiological studies agree with these findings and confirm the adverse effects of DEHP on female reproductive functions and development. In fact, urinary levels of DEHP were correlated with pregnancy complications such as anemia and toxemia (68). Another epidemiological study confirmed the positive correlation between DEHP's breakdown metabolites and precocious puberty in females, leading to breast cancer (69, 70).

Other studies have focused on the effects of DEHP exposure on males' reproductive and endocrine systems and development. First, increased DEHP exposure was linked with a significant decrease in testosterone suggesting an anti-androgenic action and reduced Leydig cells function (71). Mainly, exposure to high doses have been found to delay puberty in males, as opposed to females in which puberty is advanced following exposure (72). Epidemiological studies have confirmed the aforementioned findings and have established a negative correlation between DEHP exposure and semen count and motility (73-75).

In short, the effects of DEHP on the reproductive and endocrine system, as well as development of both males and females have been established through experimental and epidemiological studies. However, given DEHP's highly sex-specific effects, it seems that it impairs male reproductive, developmental and endocrine functions more than female (76-80). For

instance, males exhibited more vulnerability to neurodevelopmental effects while females showed neuroprotective properties that allowed them to cope with the exposure and be less affected by it (81).

b. Immune effects

In addition to interfering with development and reproduction, DEHP was shown to pose immune risks to contaminated individuals. Specifically, it was linked to enhanced immune reactions as well as inflammation-associated pathologies such as asthma and other respiratory diseases (82). This is manifested as an increase in immune cell proliferation and infiltration, as well as an increased production of cytokines from monocytes, macrophages and T cells. Therefore, DEHP promotes inflammation and impacts both innate and adaptive immunity and immune cells differentiation and inflammatory processes (83).

c. Cardiovascular effects

As part of the risk assessment of DEHP exposure, the cardiovascular system was investigated. Robust experimental and epidemiological studies confirm this and point to reduced cardiac function following exposure.

First, epidemiological studies investigated possible correlations between physiological levels of DEHP and cardiovascular disease. Using NHANES data, a positive correlation was established between increased DEHP exposure and increased blood pressure in children between 6 and 19 years old (84). Another cross-sectional study confirmed the correlation between DEHP and hypertension in adults (85). Other studies focused on other cardiovascular diseases and identified a correlation between DEHP levels and coronary artery disease (86) or atherosclerosis (87).

As for experimental studies, most have used animal models to characterize the effects of DEHP exposure on cardiovascular health. A study on rat cardiomyocytes found that DEHP caused a reduction in conduction velocity and synchronicity of the cellular network through

disruption of connexin 43 (88). In addition, DEHP induced changes in mRNA expression of genes involved in calcium handling, cell electrical activity, adhesion and microtubular transport in cardiomyocytes (89). Other studies confirmed the epidemiological findings that increased DEHP exposure increased systemic blood pressure (90, 91). *In vitro*, neonatal rat cardiomyocytes treated with environmentally relevant doses of DEHP altered metabolic pathways such as fatty acid substrate utilization, oxygen consumption, extracellular acidosis and mitochondrial mass (92). Metabolism was also affected in mice treated with DEHP in that exposure caused fat reduction in fat reserves and an increase in hepatic fatty acid oxidation (89). Finally, a study performed on human stem cell-derived cardiomyocytes determined that exposure to environmentally relevant doses of DEHP reduced the transient amplitude of calcium, the decay time constant and the spontaneous beating rate (93).

In conclusion, epidemiological and experimental studies indicate that DEHP causes adverse effects on the cardiovascular system and promotes the development of cardiovascular dysregulation and diseases. Consistently, males seemed to be more affected than females to the same exposure to DEHP, highlighting clear sex-specific effects of DEHP (94).

C. **Medical equipment**

The studies performed to assess the risk of BPA and DEHP exposures to consumers were able to characterize their adverse effects on the different systems as well as physiological and metabolic functions. Findings clearly encourage the complete removal of BPA and DEHP from common products in order to minimize exposure, and authorities in Canada, the US and Europe have acted in that direction by limiting their use in industries. It is widely accepted that bisphenols and phthalates leach out of products into consumers, tissues and solutions.

However, leaching of BPA and DEHP from medical devices is much less appreciated. For instance, cardiac surgeries are common major surgeries that necessitate the use of medical devices and may therefore pose a risk to the patient's health. In fact, isolated coronary artery

bypass graft (CABG) surgery and aortic valve replacement (AVR) are the most frequently performed cardiac surgeries in adults (95). These interventions necessitate the use of a cardiopulmonary bypass (CPB machine) to allow the surgeon to operate on an arrested heart. Patients therefore require several devices such as IV bags, catheters, endotracheal tubes, draining tubing, enteral feeding tubes, during and after surgery or treatment at the hospital. This intensive tubing can remain in place for hours, if not days at a time.

Most of these medical devices contain several plasticizers to render them flexible and durable. Among those substances are significant quantities of BPA and DEHP. As a matter of fact, flexible medical tubing can contain up to 40% w/w DEHP, and can lose up to 20% of its DEHP, especially in lipophilic solutions such as blood (96, 97). Furthermore, a clinical study of ICU patients found a slight increase in BPA concentrations in patients, and a significant 10 to 100-fold increase in the levels of DEHP metabolites (98). Similarly, a recent study quantified the leaking of several phthalates from medical devices in a clinical setting and determined that within 12 hours following surgery, DEHP levels increased 1600-fold (95). In neonates in neonatal ICU, DEHP exposure increased to 4000 to 160,000 times more than the safe amounts (99). These findings point to the fact that the most intensive exposure to BPA and DEHP occurs in a medical setting and comes from the extensive use of equipment and tubing.

We previously found that only 12 hours post-surgery, the amount of DEHP in the urine of patients reached 98,000 µg/g creatinine compared to 150 µg/g creatinine before the surgery. As for BPA, we found that the urine concentration could reach up to 42 µg/g creatinine after 12 hours compared to 1.3 µg/g creatinine before surgery. The direct implications of an exposure to average doses of BPA and extreme doses of DEHP on patients' health and recovery are yet to be elucidated.

D. **Gut microbiome**

i. Gut composition and dysbiosis

Human adults harbor in their GI tract around 100 trillion bacteria, known as the gut microbiome (100). The microbiome has been labelled “forgotten organ” because of its underappreciated roles beyond the GI tract. In fact, it is directly involved in the pathogenesis of metabolic disorders, such as obesity, as well as inflammatory bowel diseases, cardiovascular diseases, immune disorders, neurological diseases and others (100-102). Furthermore, the importance of the microbiome in promoting obesity was shown in a study in which inoculating healthy mice with the microbiome of obese mice induced obesity (103).

The human microbiome is extremely diverse, and consists of 8 phyla, 18 families, 23 classes, 38 orders, 59 genera and 109 species (104). Having said so, the microbiome is significantly dominated by only 4 phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* which make up more than 80% of the human microbiome (104, 105). Overall, the gut microbiome plays a central role in maintaining systemic homeostasis, and is therefore strictly maintained and regulated itself (105). The adverse effects of any imbalance in the composition of the microbiome can be appreciated by studying the effects of antibiotic treatments, such as antibiotic-associated diarrhea (106-108). It can last days after the end of the treatment, indicating that long-lasting dysfunction is due to changes in the microbiome’s composition.

Alterations in the bacterial profile of the gut’s microbiome are referred to as “dysbiosis”. Several factors can lead to dysbiosis, such as changes in diet, antibiotic use, or physical and psychological stress (109). Dysbiosis can be quantified by the *Firmicutes*/*Bacteroidetes* (*F/B*) ratio, which is a biomarker for several pathological conditions (110). For instance, the *F/B* ratio was shown to increase in patients suffering from coronary artery disease or obesity (100, 111). In addition, microbial diversity and equilibrium in the gut decrease in hypertensive rats and humans (105). Hypertension was also linked to an increased *F/B* ratio, and a targeted decrease of the

ratio with probiotic treatments was used to lower blood pressure in rats (112). On another hand, a decrease in ratio is associated with inflammatory bowel disease (113).

ii. BPA and DEHP effects on gut microbiome

As part of the factors that can influence the gut microbiome's composition is the exposure to toxic substances, such as BPA and DEHP (114). Gut dysbiosis was not studied as part of their initial risk assessments. Several recent studies have focused on the gut microbiome-related consequences of BPA and DEHP exposures.

Recent studies investigated the effects of chronic exposure to high BPA concentrations on the microbiome. Findings demonstrate that BPA exposure promoted gut dysbiosis in mice and led to a decrease in the overall size of the microbiome. Specifically, the abundance of *Proteobacteria* increased, whereas *Akkermansia*, which is associated with increased barrier in the gut and reduced inflammation decreased (115). Comparative metagenomics analyses determined that BPA exposure led to gut dysbiosis characterized by a significant loss of species diversity, an increase in *Proteobacteria* prevalence and a decrease in *Firmicutes* and *Clostridia* abundance (116). BPA also leads to a significant reduction in short-chain fatty acid (SCFA) producers like *Oscillospira* and *Ruminococcaceae* leading to reduced immune cell proliferation (117).

On another hand, DEHP has similarly been linked to gut dysbiosis in recent studies. First, chronic exposure to environmentally relevant doses of DEHP was shown to induce gut dysbiosis and an increase in the *F/B* ratio (118). A study conducted on human newborns and studying DEHP exposure through IV infusions determined that DEHP induced gut dysbiosis and promoted inflammation in exposed patients. The dysbiosis was characterized by a significantly reduced species diversity (119). In mice, DEHP also induced dysbiosis as it was linked to an increase in *Lachnoclostridium* and a decrease in *Clostridium*, leading to neurodevelopmental disorders (120).

These findings clearly indicate that both BPA and DEHP induce gut dysbiosis and a significant change in *F/B* ratio. This capacity to cause an imbalance in gut microbiome composition accentuates the need to rethink their use, especially in medical equipment and in a clinical setting in general.

iii. Gut dysbiosis and cardiac surgery recovery

As mentioned, cardiac surgery is the most common surgery, and myocardial infarction (MI) remains the top cause of mortality in developed countries. Establishing a clear correlation between AD exposure and gut dysbiosis raises the question of their effect on patients who undergo surgery as far as their recovery and systemic health goes. Specific bacterial species in the gut can have immunomodulatory properties which affect the proliferation of different immune cells subpopulations (121). Given this significant role in immune response modulation, there is basis to think that gut dysbiosis can impair wound repair following cardiac surgery. Additionally, a healthy gut microbiome could be a major factor aiding recovery. A study conducted on mice found that antibiotic-treated mice were much more likely to die following a myocardial infarction (121, 122). The study linked increased mortality to a significant decrease in abundance of SCFA producers, such as *Lactobacillus*, leading to a greatly reduced number of myeloid cells (121). This is consistent with other studies' findings, which state that *Lactobacillus* and other SCFA producers have cardioprotective properties during cardiac recovery from a MI (123-125). The decrease in *Lactobacillus* due to dysbiosis would be translated in a decreased *F/B* ratio.

Although relatively under documented, the drastic effect of gut dysbiosis on cardiac repair and patient recovery following surgery has the potential to impact outcomes. The findings point to a clear link between loss of microbial diversity and significantly reduced wound repair, which should be addressed and avoided.

E. **Wound healing**

Following cardiac surgery, or any other injury for that matter, the body activates a wound healing process. It is conserved in mammalians throughout evolution and is equivalent for mice and humans (126, 127). Monocytes, neutrophils and macrophages all play essential roles in injury healing and tissue repair. The process is divided into several phases: haemostasis, inflammation, proliferation and remodelling. The immediate reaction to injury is haemostasis: a coagulation cascade and the constriction of vessels are initiated to prevent excessive blood loss (128). This step is therefore characterized by clot formation and platelets aggregation. Inflammation starts when chemoattractants allow the neutrophils to infiltrate the wound. They are further attracted by damage-associated molecular patterns (DAMPs) released from dying cells and damaged tissue (129). Neutrophils produce pro-inflammatory cytokines upon arrival in the wound. Chemokines also attract monocytes whose phenotype changes to become macrophages. DAMPs are also recognized by signaling receptors such as the NLR Family Pyrin Domain Containing 3 (NLRP3), predominantly found on monocytes and macrophages (130). Macrophages are the principal phagocytic cells and the primary producer of growth factors necessary to start proliferation (128). Proliferation is characterized by the formation of granulation tissue. The last phase is the remodelling of the extracellular matrix.

Given the highly regulated and controlled process of wound healing, studies have investigated the effects of disruptors like BPA and DEHP on tissue repair. Experimental and epidemiological studies determined that DEHP exposure increases inflammation-related diseases in presence of CVDs (131, 132). On another hand, BPA activated interferon signaling in myeloid cells, and activated inflammasome activity leading to a pro-inflammatory phenotype (34). These findings show that both BPA and DEHP promote inflammation and impair tissue healing by increasing immune cells proliferation and infiltration.

F. **Sex-specific responses to cardiac surgery**

As mentioned earlier, exposure to BPA and DEHP induced sex-specific effects on the reproductive and cardiovascular systems. In fact, they were generally more pronounced in males, who tend to cope less well than their female counterparts to the exposure (80, 81). Similarly, cardiac surgery has sex-specific ramifications and effects. In fact, a study conducted on cardiac patients determined that male peripheral blood mononuclear cells (PBMCs) express significantly higher mRNA levels of AIM2, NLRP3, ASC (PYCARD), Caspase-1, and IL-1β than females (133). This designates that PBMCs adopt a proinflammatory state which might enhance pathogenesis. Hormonal differences between male and female can also explain the sex-specific responses to surgical interventions. In the nervous system, estrogen was shown to suppress NLRP3-mediated inflammation and protect against it (134). On the other hand, testosterone was shown to promote proinflammatory cytokines production (135).

G. **Conclusion and hypothesis**

In conclusion, the high production of bisphenols and phthalates still answers to an ever-growing demand of plastics. Despite their established risk assessments and their known adverse health and environmental effects, they are still heavily used in common products that consumers use on a daily basis. Among them, BPA and DEHP are the most common and most problematic. Summarizing the effects of each would be too complex because of their many targets within the human body and their sex-specific effects. In fact, sex appears to be a major factor in the toxicology of these substances and how they are dealt with.

Although these effects are studied and acknowledged, less well appreciated is that the most extensive exposure to BPA and DEHP comes from medical devices. Health authorities and government bodies attempt to regulate their use in common products, food containers and children's toys, but limited steps have been taken to find a suitable replacement for plasticizers in medical equipment. BPA and DEHP were found to leach out of such devices in significant

quantities and contaminate the patients, who require the use of tubes, catheters, IV bags and others. Moreover, some studies suggested that endocrine disruptors, and especially DEHP, act in a non-monotonic way, which means that even more attention should be given to the specific concentrations that leak out of devices into patients (136-138). This raises the question of how extreme exposures to DEHP following surgery affect the patient compared to average exposures.

In addition to their well-documented effects, BPA and DEHP also alter the gut microbiome in a way to impair recovery from surgery and cardiac repair. The gut dysbiosis in itself has several adverse effects in the body but can dysregulate the immune response following surgery through loss of beneficial microbes and metabolites. This can significantly change the outcome of surgery and endanger the patients' well-being and recovery.

Given that microbiome studies are relatively recent and that the link between gut dysbiosis and acute exposure to environmentally relevant doses of BPA and DEHP during cardiac surgery is under-documented, this project aims at characterizing the effect of such an exposure on cardiac repair. By doing so, the study would provide more insight into the necessity to rethink medical devices, as well as more information on the gut's role in sex-specific recovery from cardiac surgery, which could be used to control inflammation and promote wound repair.

The pharmacokinetics of both DEHP and BPA in mice are similar to humans, which allows for the use of mice models to study their exposures on humans (139, 140). This project therefore uses a mouse model of cardiac surgery to test the effects of acute exposure of low-dose BPA and high-dose DEHP on heart regeneration, gut dysbiosis and systemic inflammation for males and females. The objectives were to characterize these sex-specific effects on physiological parameters, immune cells proliferation, metabolome variations and microbiome alterations.

Finally, the aforementioned findings led me to hypothesize that acute exposure to doses of BPA and DEHP that resemble those in patients post-surgery will cause gut dysbiosis and metabolome alterations in a way to impair cardiac recovery following an induced wound. I hypothesize that effects will be highly sex-specific and that males will be more affected than

females to both the surgery and the treatment. In addition, I hypothesize that the effects on inflammation and physiological parameters will differ from previous findings about low-dose exposures given that DEHP and BPA act in a non-monotonic matter.

VII. Materials and Methods

A. **Materials**

Peanut oil (CAS 8002-03-07) was purchased from SpectrumChemical. Di-(2-ethyl hexyl) phthalate (DEHP, >99.5% pure, CAS 117-81-7) and bisphenol A (BPA, >99% pure, CAS 80-05-7) were purchased from Sigma-Aldrich. Ethanol was purchased from Commercial Alcohols.

VEH water was prepared by diluting 1mL of absolute ethanol in 1L of distilled and sterilized water. BPA was dissolved in absolute ethanol to a concentration of 25mg/ml. To prepare mouse drinking water, 1ml of BPA:alcohol solution was diluted in 1L of distilled and sterilized water resulting in a final concentration of 25μg/L. Peanut oil was used as VEH oil for DEHP delivery. DEHP stock solution was prepared by mixing 1mL DEHP to 6.6mL of peanut oil.

Percoll was purchased from GE Healthcare, and Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (ThermoFischer).

The formula, human equivalent dose = [mouse equivalent dose x (Km for mice/Km for humans)], where the correction factor (Km) for mice is 3 and the Km for humans is 37, was used to calculate the mouse equivalent of the human exposure for BPA (141). The final concentration of the BPA in the drinking water was 25ng/mL. Since mice drink approximately 5mL per day (142), this delivers the human equivalent of 0.40 μg/kg/day BPA.

As for DEHP, the concentration of the treatment was similar to extreme cases of exposure following surgery. The final concentration of the DEHP was such that the treatment would deliver the human equivalent of 4mg/kg/day.

B. **Animal manipulation and isolation**

The animal use protocol was reviewed and approved by the Lady Davis Institute Animal Care Committee. The mouse experiments were performed according to the guidelines of the Canadian Council on Animal Care. Male and female C57bl/6N mice were purchased (Charles River, St.

Constant, Quebec) and kept in polycarbonate cages with ¼' corncob bedding in a 12:12h light-dark (LD) cycle. The mice were fed a Harlan Teklad Global 2018 (irradiated) diet.

A week before the scheduled treatment or surgery date, mice were separated into single cages and the drinking water changed to acidified municipal tap water delivered in glass bottles. They were weighed and fecal pellets collected. This day is referred to as D-7.

Mice were separated into groups according to 4 criteria: their sex (M or F), the presence or absence of an induced myocardial infarction (MI or no-MI), the treatment (VEH or AD) and the age (YG or RB).

C. **Surgery and treatment post-MI**

The surgically-induced myocardial infarction was performed by the surgery core of the Lady Davis Institute (35, 143). The mice were anesthetized with isoflurane and intubated. Analgesia for 3 days was obtained by an injection of slow-release buprenorphine. The surgery consists of a permanent ligation of the left anterior descending coronary artery using a 7-0 silk suture. This surgery places approximately 40% of the left ventricle at risk for infarction (144). Given that prolonged anesthesia may cause corneal damage and eye trauma, an ophthalmic ointment is applied on the eyes of the mouse for the duration of the surgery. Following the surgery, animals were kept in a heated incubator to optimize recovery before being put back in their cages. The day of the surgery is referred to as D0. Figure 5 illustrates the experimental model for the treatment of mice of different sex, age, and treatment.

Mice were randomly assigned by the Surgery core to VEH or AD treatments before surgery. To avoid batch effects, VEH and AD groups were assigned each surgery day. For mice which underwent surgery, treatment started the same day (D0), immediately after the surgery. Phthalate treatment was administered with micro-pipettor by oral delivery of 25µL of either VEH oil or DEHP oil for 3 days (D0, D1 and D2). In addition to that, the bottled water of the mice was changed on

D0 to either VEH water, or to BPA added water. All mice were weighed on each of the three days to monitor the weight fluctuation.

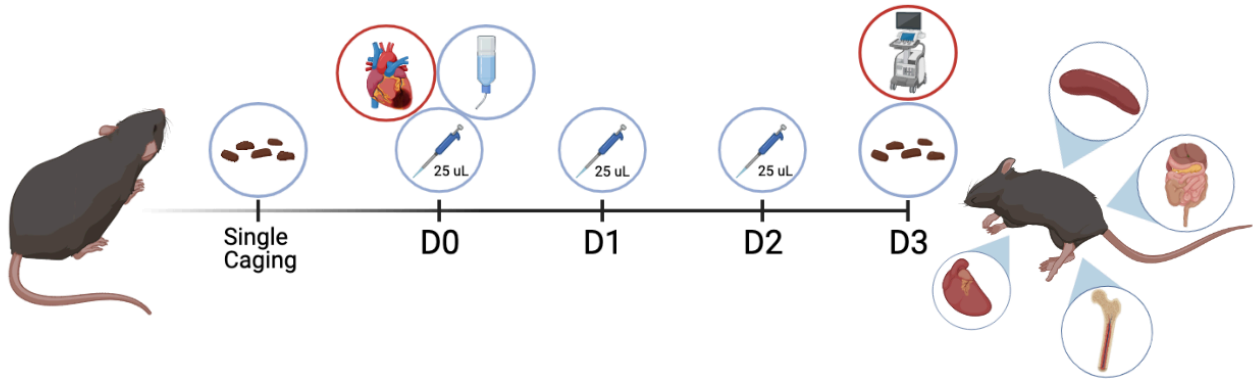


Figure 5 - Experimental design for the treatment of mice from different sexes and ages with or without surgery. The fecal pellets are collected on the day of single caging. D0 is the day on which the MI surgery is performed, and the treatment and water are administered according to the group. Treatment is given daily until euthanasia on D3, which is also the day of the echocardiography. Created on: BioRender.com

D. **Echocardiography**

On day 3 post-MI (D3), echocardiography was performed on the mice before they were euthanized. Echocardiography was only done for the mice which underwent surgery 3 days earlier, and not for groups that did not have surgery. They were anesthetized with isoflurane and the echocardiography was carried out using a VEVO 3100 sonograph (VisualSonic, Toronto, Ontario). Electrocardiogram-gated Kilohertz Visualization (EKV-gated) acquisitions of both the long and short axes were collected. The long axis acquisitions allowed to outline the left ventricular (LV) wall in diastole and systole (35, 95, 143) in order to estimate LV area, LV volume in systole and diastole, stroke volume, ejection fraction and cardiac output (145). The short axis acquisitions at the level of the papillary muscles permitted calculations of the fractional area contraction (FAC). The data obtained from the echocardiography was analyzed by VEVO Lab (VisualSonic) in order to determine all the aforementioned parameters.

E. **Euthanasia and sampling**

On D3, mice of all groups were weighed, then isolated in small containers for approximately 5 to 10 minutes in order to collect their fecal pellets. Then, they were anesthetized with isoflurane and euthanized by cervical dislocation.

The mice which had previously undergone surgery would undergo a terminal cardiac puncture to collect blood before euthanasia. The cardiac puncture was performed by first anesthetizing the mouse, then collecting the blood. The mouse was immediately euthanized by cervical dislocation afterwards.

After euthanasia, mice were opened, and the heart and bone marrow (from one femur) were collected for flow cytometry. The spleen was collected and used for flow cytometry as well as histology. The intestine was cut from the duodenum to the colon and isolated. The cecum was separated from the intestine, and cecum contents collected for DNA extraction and metabolomics while the rest of the intestine was used for histology.

F. **Physiological measurements**

To better assess the physiological fluctuations of the mice throughout and after the treatment, a set of measurements were taken along the experiment. As mentioned, fecal pellets were collected on the day of single caging and on the day of euthanasia (D3). These pellets were weighed and counted. The body weight of the mice was measured on D-7, D0, D1, D2 and D3. Upon euthanasia, the spleen's total weight was noted, as well as the weight of the cecum contents that were extracted from the cecum. The data was kept in a database where the mice were divided in 16 groups depending on their sex (M or F), the presence or absence of surgery (MI or no-MI), the treatment (VEH or AD) and the age (YG or RB). The mice which died during surgery were not counted in the survival rate calculations.

G. **Flow cytometry**

To establish the immunological profile and immune cells proliferation in each mouse, flow cytometry was performed on spleen, heart and bone marrow samples. The cells that were extracted from spleen and bone marrow were isolated and stained differently than those that were extracted from hearts.

i. Spleen and bone marrow cells

Upon spleen collection, a piece of approximately 15mg was cut and put in 1x PBS. The spleen was crushed using the plunger of a 1mL syringe and the mixture was then passed through a 100 micron filter (SureStrain). As for the bone marrow, a 1mL syringe attached to a 26G needle was filled with 1mL of 1x PBS and used to flush the bone marrow out of the left femur of the mouse. In both cases, the cells were centrifuged and 1mL of Ammonium-Chloride-Potassium (ACK) lysing buffer was added to the pelleted cells for 5 minutes to lyse the red blood cells. The cells were centrifuged again afterwards and the washed. The spleen and bone marrow cells were transferred to a 96-well plate and were incubated with AmCyan Live/Dead Fixable Dead Cell Stain (Molecular Probes, Carlsbad, California) for 30 minutes at 4°C then 2.4G2 hybridoma (FC Receptor Block, ATCC: HB-197) for 30 min at 4°C. Samples were then stained with fluorescently labeled antibodies for 30 min at 4°C. The antibodies used to prepare the antibody cocktail were the following: Brilliant Violet 785-conjugated anti-CD45 (30-f11, BioLegend), Brilliant Violet 650-conjugated anti-MHCII (M5/114.15.2, BD Biosciences), phycoethythrin/Dazzle 594-conjugated CD64 (x54-5/7.1, BioLegend), efluor450-conjugated anti-CD11b (MI/70, eBioscience), APC-efluor780-conjugated anti-CD11c (N418, eBioscience), Alexa 488-conjugated anti-Ly6G (RB6-8c5, eBioscience), APC-conjugated anti-Ly6C (AL-21, BD Biosciences), and phycoethythrin-conjugated anti-MerTK (2b10c42, BioLegend). Finally, the cells were fixed in 1.6% paraformaldehyde for 15 minutes at 4°C and stored in 300µL of 5% horse serum in 1x PBS (FACS buffer).

ii. Heart cells

To determine the infiltration of immune cells in the heart, single-cell suspensions of the heart tissue were prepared to be analyzed by flow cytometry. The heart was extracted from the mouse, cut into small pieces and placed in a collagenase cocktail made of 675 U/ml collagenase I, 18.75 U/ml collagenase XI, and 9 U/ml hyaluronidase (Sigma-Aldrich) at 37°C for 90 min. The resulting mixture was filtered with a 100 micron filter (SureStrain). A Percoll gradient was used to isolate the myeloid cells. Specifically, the initial mixture was passed through the filter with 3mL of 40% Percoll in 5% horse serum in Dulbecco's Modified Eagle Medium (DMEM), called wash media. Using a Pasteur transfer pipette, 2mL of 80% Percoll in wash media were added under the 3mL of 40% Percoll, creating the desired gradient. The samples were then centrifuged at 2000g for 20 minutes at 4°C in a centrifuge with a zero-deceleration setting. The cells were collected from the interface, pelleted and transferred to a 96-well plate. Each sample was divided into two wells because two different antibody mixtures are used for each sample. Similar to spleen and bone marrow cells, the heart cells were incubated in AmCyan Live/Dead Fixable Dead Cell Stain (Molecular Probes, Carlsbad, California) for 30 minutes at 4°C, then 2.4G2 hybridoma (FC Receptor Block, ATCC: HB-197) for 30 min at 4°C, and then stained with the fluorescently labeled antibodies for 30 min at 4°C. One of the cocktails was identical to the previously described one used for bone marrow and spleen cells. The second cocktail was made of the following: Brilliant Violet 785-conjugated anti-CD45 (30-f11, BioLegend), Brilliant Violet 650- conjugated anti-MHCII (M5/114.15.2, BD Biosciences), phycoethythrin/Dazzle 594-conjugated CD64 (x54-5/7.1, BioLegend), efluor450-conjugated anti-CD11b (MI/70, eBioscience), phycoethythrin- conjugated anti-MerTK (2b10c42, BioLegend) and anti-Mo CD38 (eBioscience). The cells incubated in the first cocktail were then fixed and stored as described previously for spleen and bone marrow cells. As for the cells that were incubated with the second cocktail, they were fixed in paraformaldehyde and left in the 96-well plate overnight. The next day, they were first permeabilized by incubating in 0.05% Tween20 in PBS for 5 minutes at room temperature. They were then centrifuged and

incubated with Anti-Mo CD206 (MMR) (eBioscience) and fixed again. The CD38 and CD206 antibodies were used to characterize the macrophage infiltration by discriminating between M1 macrophages (CD38+) and M2 macrophages (CD206+).

Finally, 123count e-beads (cat No. 01-1234, Affymetrix) were added to the samples from the heart, spleen and bone marrow. The samples were analyzed using an LSR Fortessa Cell Analyzer (BD Biosciences, San Jose, California). Data were analyzed using FlowJo software v10.7.1 (Tree Star, Inc, Ashland, Oregon).

H. **DNA extraction and quality control**

The microbial DNA was extracted from the cecum contents of the mice. Approximately 50mg of content were used. The DNA extraction was performed using QIAGEN's QIAamp PowerFecal DNA kit (reference: 12830-50) (QIAGEN, Hilden, Germany), according to the manufacturer's guidelines and protocol. Briefly, approximately 50mg of cecum contents were added to kit-provided bead tubes, heated at 65°C for 10 minutes and then vortexed for 10 minutes. The resulting mixture was then incubated with kit-provided solutions to allow DNA extraction away from unwanted polysaccharides, cell debris and proteins. It was centrifuged at 13,000g for 1 minute between each incubation.

The extracted DNA's quality was assessed by using a DS-11 Nanodrop^{TM} spectrophotometer (DeNovix, Delaware, United States) to determine the A260/280 ratio, then by running a DNA gel. The A260/280 ratio is an indicator of the DNA's purity and the ideal value indicating a highly pure sample is around 1.80.

The size of the isolated DNA was verified using agarose electrophoresis. The gel was prepared by boiling 1% agarose in 1x Tris-Borate-EDTA buffer (TBE), and adding a 1:10,000 dilution of GelRed Nucleic Acid stain (Biotium; Cat: 41003) before pouring it in the gel plate for solidification. The DNA samples were prepared by mixing 5µL of the extracted DNA to 3µL of Tris-EDTA and 2µL of 6x gel-loading buffer (25mg of bromophenol blue dissolved in 30% glycerol in

water). The resulting 10µL were loaded in the wells when the gel solidified. 5µL of a 1Kb DNA ladder (GeneDireX) were also added to 3µL of Tris-EDTA and 2µL of 6x gel-loading buffer and loaded into one well to obtain the ladder. The gel was electrophoresed at 90V for approximately 40 minutes. Images were taken with a ChemiDoc Touch Gel Imaging System (BioRad Laboratories).

I. DNA sequencing

After undergoing quality control as described above, the DNA was stored in -20°C. Aliquots were then sent to the McGill Genome Center for amplification and sequencing. The bacterial DNA was amplified with r16S primers and sequenced. Specifically, the bacterial V4 region was amplified from bases 515F to 806R (Table 1). The sequencing was done with the MiSeq Reagent Kit v3 (600-cycle) and ran on the Illumina MiSeq.

An initial data sheet indicating how many times each DNA sequence, as an amplicon sequence variant (ASV), appeared in each sample was obtained. The analyses of the results were done using Microsoft Excel. First, the total number of ASV reads was normalized to 10,000 reads per mouse. The ASV were sorted from most represented to least. ASVs that appeared fewer than 10 reads per mouse were designated as 0 reads.

The average of cohorts was calculated. To select for ASVs predominating on one cohort versus another, we used the difference of means. We sorted this difference of means from high to low and low to high. Data manipulation and reorganizing the sequences generated heatmaps for males and females showing differences in ASV sequences found for each group. Finally, the sequences were analysed against a database using BLAST (NCBI), against the refseq_rna database.

Table 1 - Target primers sequences

Base		Sequence
515F	Forward Primer	GTGCCAGCMGCCGCGGTAA
806R	Reverse primer	GGACTACHVGGGTWTCTAAT

J. **Metabolomics**

In order to characterize the variation in metabolites found in the gut, metabolomics analyses were performed on cecum contents taken from each mouse. Approximately 20mg of cecum per mouse for 32 mice in total were sent to Dr. Jeff Xia's laboratory, McGill. Briefly, the samples were lyophilized for 2 hours, then dissolved in 500µL of 80% methanol at -20°C (146, 147). The cecum materials were then broken down using Bead Mill homogenizers, centrifuged at 14,000g for 20 minutes and dissolved in 50% acetonitrile (147). Metabolite profiling was obtained from cecal samples by using an untargeted UPLC–MS/MS platform (Thermo Scientific Q-Exactive Orbitrap mass spectrometer). MS raw data processing was performed by the spectral processing module on MetaboAnalyst and MS/MS data was annotated by R package metID. MetaboAnalyst and R studio were further used for statistical analysis, pathway enrichment and visualization.

K. **Histology**

In order to appreciate structural changes following surgery and treatment, spleen and intestine samples were collected and processed for microscopy analysis. After collecting the part of the spleen to be used for flow cytometry, the rest of the spleen was stored in 4% formaldehyde in 1x PBS for 48 hours, then transferred to 70% ethanol in water.

As for the intestine (duodenum to the end of the colon), it was collected from the mouse and kept in 1x PBS during manipulation. It was flushed with Bouin's solution (50% ethanol and 5% acetic acid in water) in order to fix the tissue and flush the contents. It was then cut in two or three separate pieces of approximately the same length. Each piece was opened up along its length to

expose the interior and then rolled on itself to create a swiss roll of the intestine (148, 149). Like the spleen, the rolls were stored in 4% formaldehyde for 48 hours, then transferred to 70% ethanol.

The spleen and intestine rolls were sent to the Pathology Core of the Lady Davis Institute to be processed. They were embedded in paraffin, cut and stained with hematoxylin and eosin (H&E). The samples were observed using a Leica Fluo III microscope (Leica, Germany) under a magnification of 1x for the intestine rolls and both 2x and 8x for spleen. The software that was used to capture pictures and adjust picture settings was Infinity Capture (Lumenera). The spleen pictures were further analyzed by using FIJI (ImageJ). To determine spleen follicle number and area, the perimeter of each follicle within a cross section was manually traced, and the number of pixels was measured. Then, the perimeter of the entire cross section was traced manually, and number of pixels measured in order to calculate what percentage of the spleen was made up by follicles. Finally, the number of follicles within each spleen was manually counted.

L. **Statistical analyses**

Student's *t* tests were used to determine the significance in difference in pairwise comparisons, and one-way or two-way ANOVA tests were done for groups of >2. The Student-Neuman-Keuls method was used to further determine results significance. A two-tailed p value of <0.05 was considered significant. These tests were performed using SigmaStat 3.1 (Systat Software).

VIII. Results

A. DNA extraction from cecum contents

All the mice from which DNA was extracted and sequenced were retired breeders. They were divided into males (n=21) and females (n=21). Of the males, 10 did not undergo surgery (5 VEH and 5 AD) and 11 underwent surgery (5 VEH and 6 AD). Of the females, 8 did not have a MI (4 VEH and 5 AD) and 12 had a MI (6 VEH and 6 AD).

Once extracted from cecum contents, the quality of the bacterial DNA was examined prior to amplification and sequencing. Further, the amount of DNA was measured to determine whether the surgery or treatment affect the DNA yield, which can be a potential indicator of the size of the microbiome. Figure 6A shows the average DNA yields obtained for the 41 retired breeders for which DNA was sequenced. Of the females which did not undergo surgery, those that were treated with VEH yielded 109.07±14.66 ng DNA/mg cecum on average, while those treated with BPA and DEHP yielded 144.12±22.60 ng DNA/mg cecum. The trend was different for the females which underwent surgery. The amount of DNA extracted from the cecum contents of VEH-treated female mice averaged 125.54±20.47 ng DNA/mg cecum, while the average yield for AD mice in this group was 102.76±29.40 ng DNA/mg cecum ($p=0.56$). For the males, those that did not have a MI yielded the highest concentrations of DNA with 166.87±15.46 ng DNA/mg cecum for VEH males and 150.53±9.70 ng DNA/mg cecum for AD males ($p=0.40$). In contrast, males which had a MI yielded the lowest amounts of DNA with 104.71±18.99 ng DNA/mg cecum for VEH males and 81.53±21.95 ng DNA/mg cecum for AD ($p=0.46$). A two-way ANOVA, followed by a Neuman-Keuls test, indicated that surgery is a determining factor and that it causes a significant difference ($p=0.002$) which is not the case for the treatment.

DNA purity was evaluated by the A260/280 ratio. The quality control of the DNA revealed that all the samples that were sequenced had acceptable purity, which is indicated by an A260/280 ratio of around 1.80. The average for all samples was 1.77 and indicates acceptable purity of the

sample. One significant outlier in the “F no-MI AD” group was found and was removed from the experiment because of very low purity, bringing the number of samples from female mice to 20. All 8 groups had average A260/280 values close to 1.8 with the highest being 1.85±0.02 for the “F no-MI VEH” group and the lowest being 1.63±0.12 for the “F no-MI AD” (Table 2). No significant difference was measured between A260/280 values.

Gel electrophoresis of aliquots of the extracted DNA was performed to ensure that large molecular weight bacterial DNA was isolated. Figure 6B shows the DNA gel that was performed for YG males (9 samples) that were treated with VEH or AD treatments. The majority of the DNA isolated was ~ 10Kb and showed little degradation.

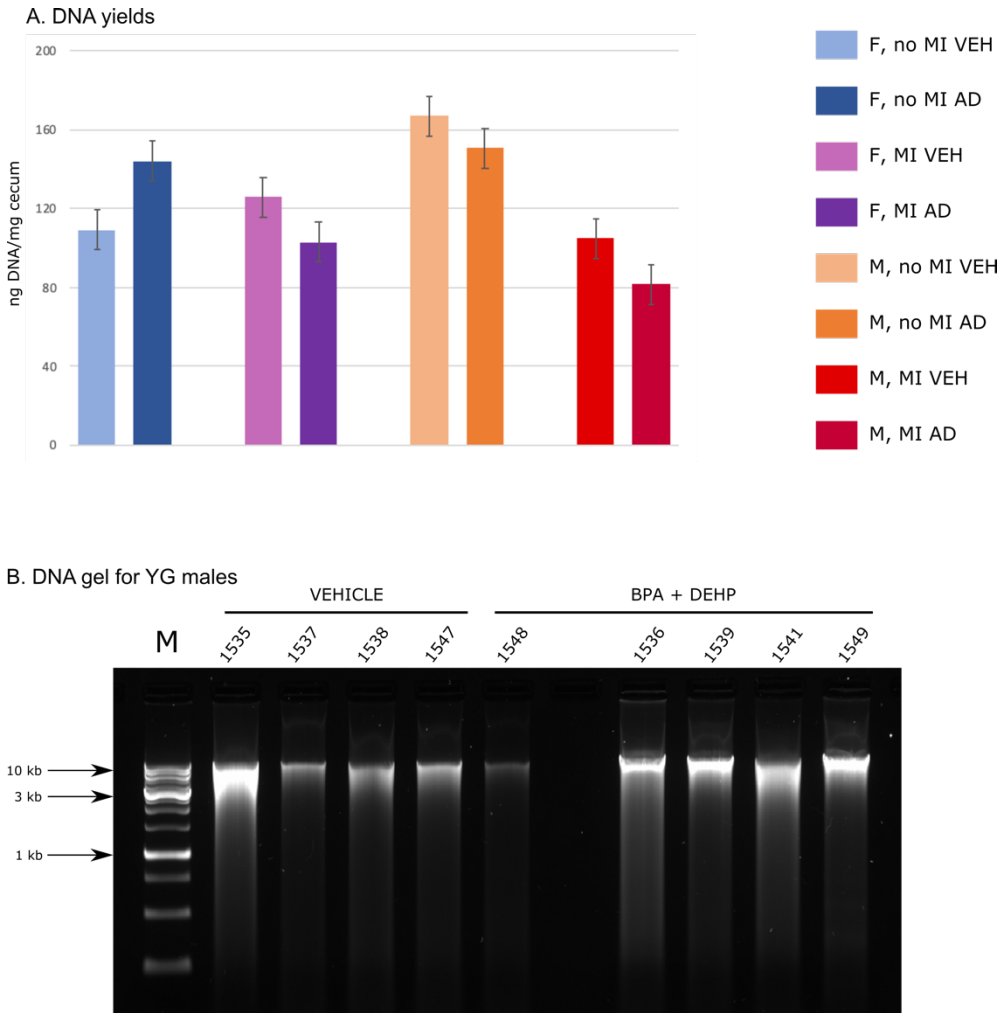


Figure 6 - DNA concentrations and quality control.
Concentrations and quality control of extracted bacterial DNA from cecum contents. A, DNA yields for sex, surgery and treatment groups obtained by Nanodrop analysis. B, DNA gel of samples extracted from YG males (VEH and AD). Gel was 1% agarose in TBA, stained in GelRed, and marker was a 10kb DNA ladder.

Table 2 - Average A260/280 ratios for the DNA samples extracted from different groups of mice.
No significant difference was measured between the groups.

Group	A260/280
F no-MI VEH	1.85±0.02
F no-MI AD	1.63±0.12
F MI VEH	1.75±0.05
F MI AD	1.79±0.02
M no-MI VEH	1.81±0.02
M no-MI AD	1.80±0.03
M MI VEH	1.78±0.03
M MI AD	1.77±0.05

B. **Gut microbiota alterations**

To characterize the microbiome of each of the mice and determine the alterations that might be caused by surgery and treatment, the extracted DNA was amplified and sequenced. The most abundant and representative sequences were identified and linked to specific species and their phyla. The sequences were sorted based on the highest difference between MI and no-MI groups, then between VEH and AD. Following the sorting, the top 50 most representative sequences were identified and linked to a genus or species of bacteria using BLAST. Further analyses were performed within the male and the female cohorts.

i. Treatment and surgery induce alterations of the microbiota in males

In males, there were some considerable differences in microbiome composition based on the abundance of the most abundant species. A heatmap was generated based on the largest differences between MI and no-MI groups, then between VEH and AD groups. As shown in the heatmap, sorting of the most read ASVs revealed ASVs more prominent in either the MI or the no-MI group. Other ASVs were more present in one of the treatment groups compared to the other (Figure 7).

To identify the bacteria associated with the abundant ASVs, the sequences were compared with the “refseq_rna” database on BLAST. A more detailed analysis of the data allows to determine the relative abundance of phyla within each group. The bar graph in Figure 8A indicates that there is a slight decrease of *Bacteroidetes* compared to *Firmicutes* following AD treatment, leading to an increase in the ratio. However, the opposite effect is seen in mice which underwent surgery. Here, *Firmicutes* are slightly decreased compared to *Bacteroidetes*. Also, there is a significant domination of *Verrucomicrobia* in the MI groups whereas the no-MI groups have few bacteria of this phylum. The relative abundances of the genus or species shown in Figure 8B show that *Akkermansia muciniphila* is the *Verrucomicrobium* bacterium that predominates in the male MI groups. The bar graph shows the high microbiota diversity in all groups regardless the

surgery and treatment, but indicates significant alterations between the 3 test groups when compared with the VEH no-MI group, which serves as a control.

The pie charts showing the relative abundance of the most represented bacteria within each group confirm this change in composition and major changes in the microbiota composition (Figure 8C). In the male no-MI group, the VEH and AD mice have a similar microbiome composition except for a significant loss of the *Bacteroidetes*, *Bacteroides vulgatus* and *Muribaculum intestinale* in the AD group. This agrees with the phyla composition shown in Figure 8A indicating an increase of the *F/B* ratio after treatment. Within the no-MI groups, the dominating genera are *Enterocloster*, *Parabacteroides*, and *Lachnoclostridium*. As for the MI groups, the dominating species in both VEH and AD groups is *Akkermansia muciniphila*, although it is more dominating in the AD group compared to the VEH group. In fact, *A. muciniphila* constitutes 44.1% of the microbiome of AD mice compared to 22.8% in VEH mice. *Muribaculum intestinale* is equally present in both groups and accounts for 15% of the microbiome in both treatment groups. Lastly, there is a considerable loss of *Lachnoclostridium* (a *Firmicute*) and an increase of *Prevotella* (a *Bacteroidete*), which agrees with the decrease of *F/B* ratio in the “MI AD” group compared to the “MI VEH” group seen in Figure 8A.

ii. Females have less obvious alterations following treatment and surgery

DNA analyses on females highlighted the sex-specific differences of an induced myocardial infarction and of the AD treatment. The heatmap of the most representative sequences in the gut microbiota of females showed that some sequences are biased towards a specific treatment or surgery group, but the overall conclusion is that the differences in the females are less obvious than those in the males. The heatmap is more scattered and less indicative of what the effects of the myocardial infarction and the treatment are on the gut microbiota of female mice (Figure 9).

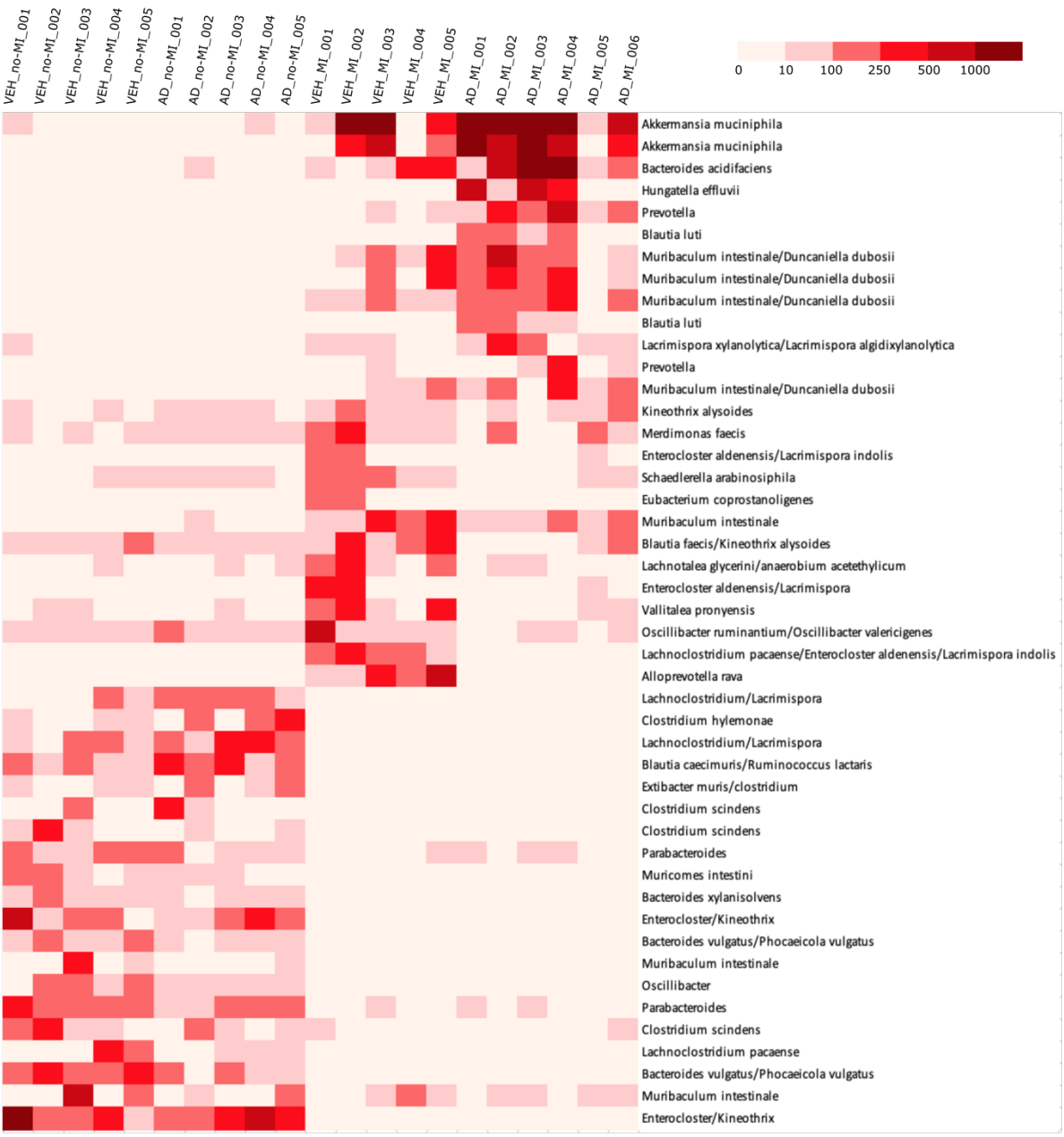


Figure 7 – Heatmap of the most represented sequences in males.

Heatmap representing the number of times each sequence was read within a sample, and the identification of each sequence to a specific bacteria species or genus. Each row represents one mouse as identified according to surgery and treatment groups.

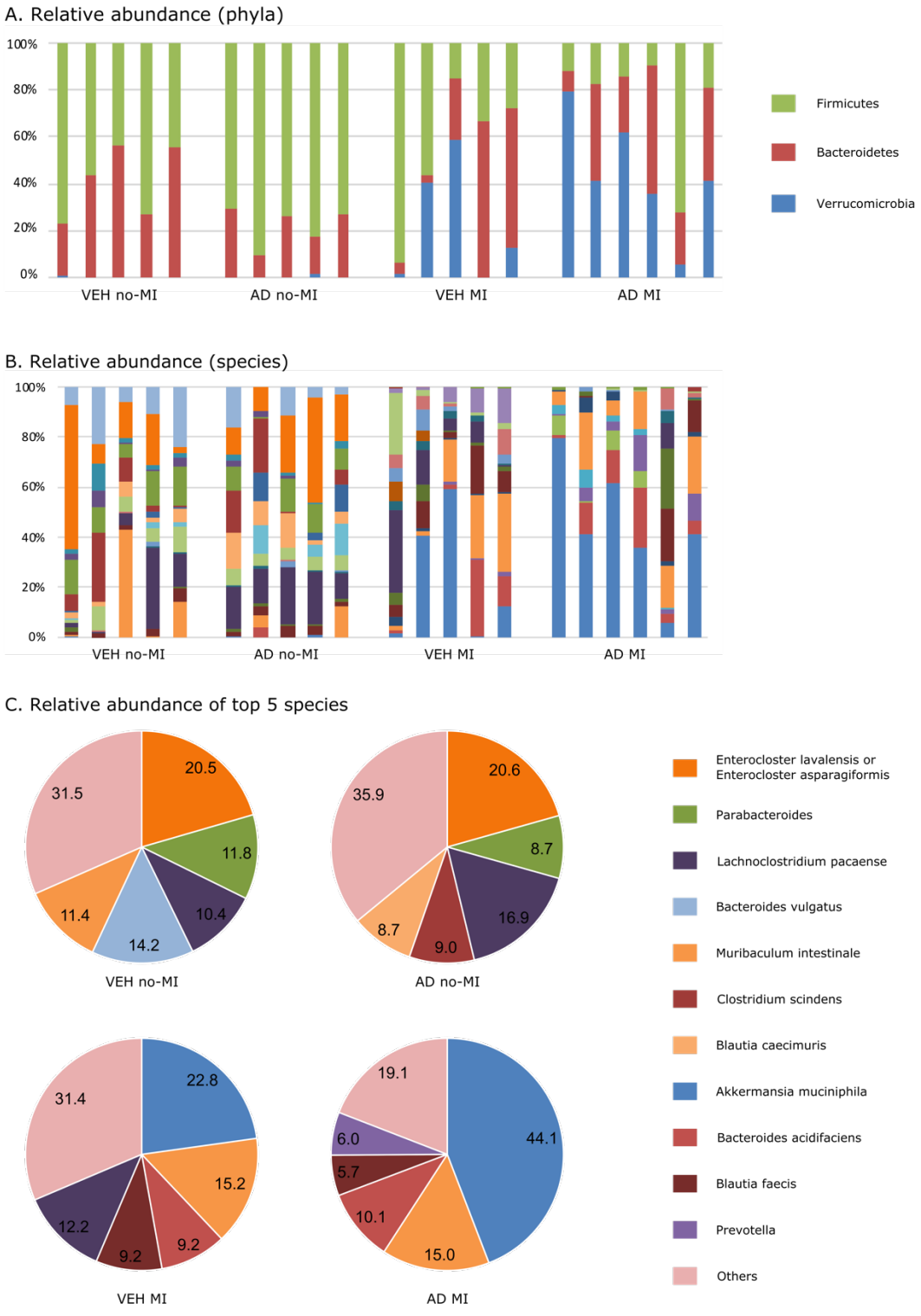


Figure 8 – Microbiome composition of males.
Relative abundances of bacteria phyla and species in RB males. A, Relative abundance of phyla in each group. Every column represents a mouse within the group. B, Relative abundance of species in each group. Every column represents a mouse in the group. C, Pie charts showing the relative abundance of the most present genera/species in each group.

Further analyses of the DNA sequences in female samples allowed for a more detailed characterization of their microbiota and allowed a comparison with the males. The relative abundances of bacterial phyla indicate that the *F/B* ratio tends to slightly decrease because of a significant increase of *Verrucomicrobia* and *Proteobacteria* at the expense of *Firmicutes* (Figure 10A). The relative abundance of *Bacteroidetes* was conserved after surgery and treatment and they constitute around 15% of the microbiota present in female mice, compared with 30 to 40% of male microbiomes. Also, there was no *Proteobacteria* among the most abundant species in the males as opposed to the considerable colonization of the female gut by this genus for some of the female mice. The relative abundance of species in the female mice point to the high diversity of the females' microbiomes, which is more-or-less conserved across the groups, as opposed to the pronounced differences seen in males (Figure 10B). Also, *Akkermansia muciniphila* is among the dominant species in all groups, as opposed to its presence only in MI groups for the males. Next, the pie charts for each of the groups confirm the dominant presence of *Akkermansia muciniphila* in all groups, and its little variation following surgery or treatment (Figure 10C). Within the "no-MI" groups, there is a significant increase in proportion of *Ligilactobacillus* in the AD mice compared to the VEH mice ($p=0.05$), and a decrease in the relative abundance of *Muribaculum intestinale* (18.8% in the VEH vs 6.7% in AD mice). Also, "no-MI AD" mice gain a significant amount of *Anaerobium* which is not present among the most represented genera in VEH mice ($p=0.01$). As for mice which underwent surgery, they also possess a considerable amount of *Muribaculum intestinale*. As opposed to the "no-MI" groups, *Ligilactobacillus* is only present in the VEH mice and not the AD ones. Finally, there is an increase in the relative abundance of *Klebsiella* in "MI AD" mice, which is not present in large proportions in any of the other groups.

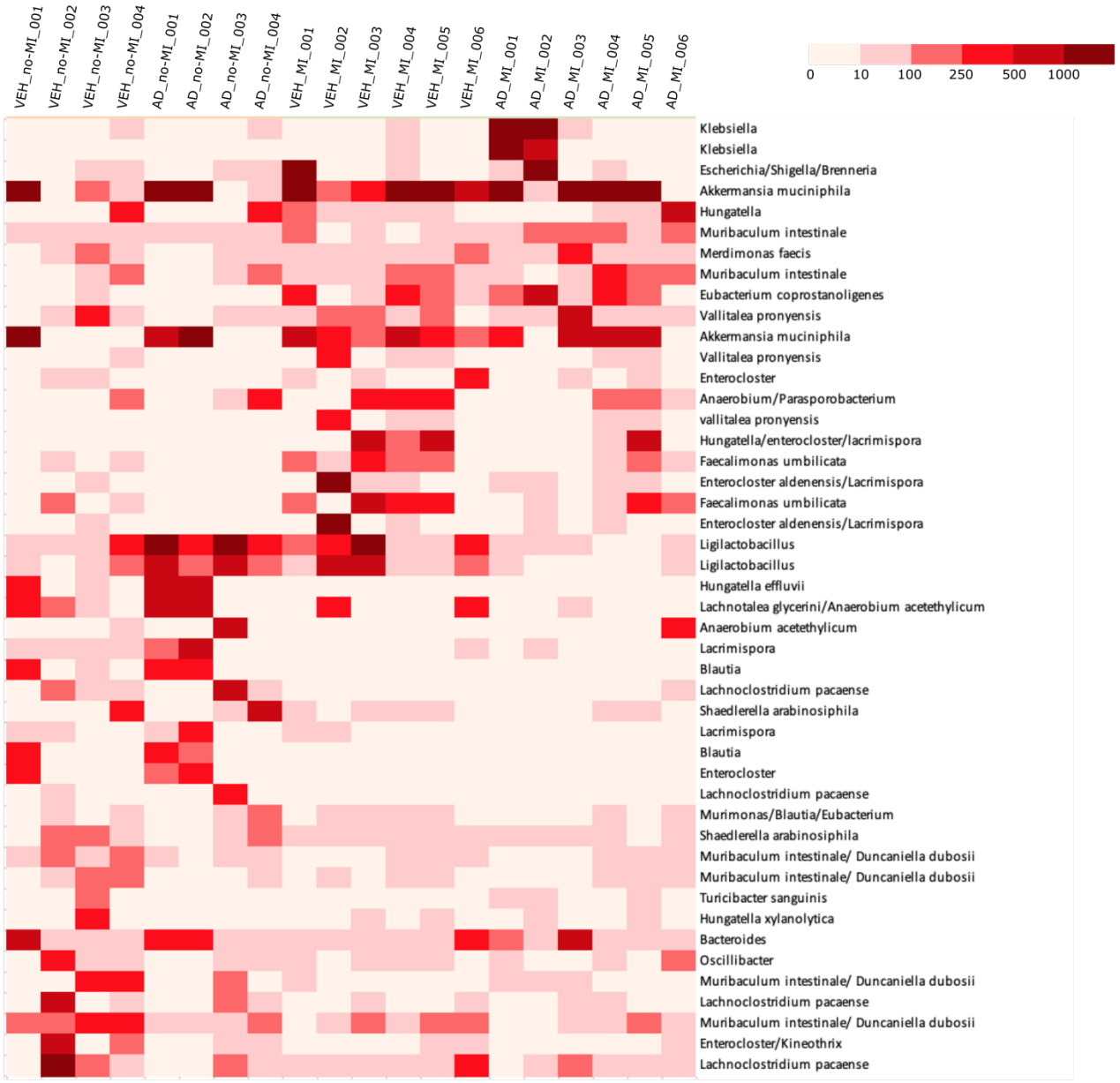


Figure 9 - Heatmap of the most represented sequences in females.
Heatmap representing the number of times each sequence was read within a sample, and the identification of each sequence to a specific bacteria species or genus. Each column represents one mouse as identified according to surgery and treatment groups.

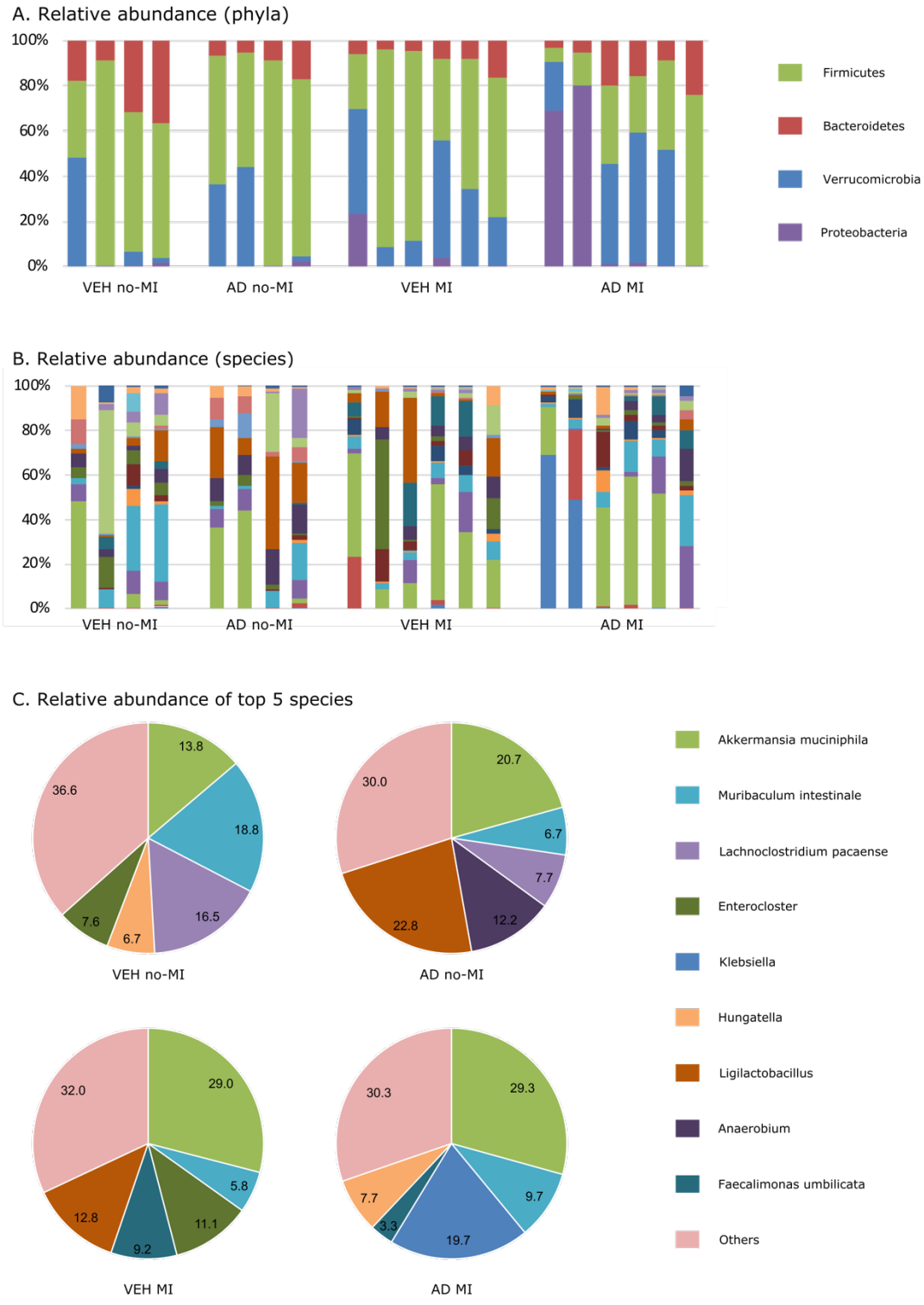


Figure 10 - Microbiome composition of females.
Relative abundances of bacteria phyla and species in RB females. A, Relative abundance of phyla in each group. Every column represents a mouse within the group. B, Relative abundance of species in each group. Every column represents a mouse in the group. C, Pie charts showing the relative abundance of the most present genera/species in each group.

C. **Effects of surgery and treatment on metabolome**

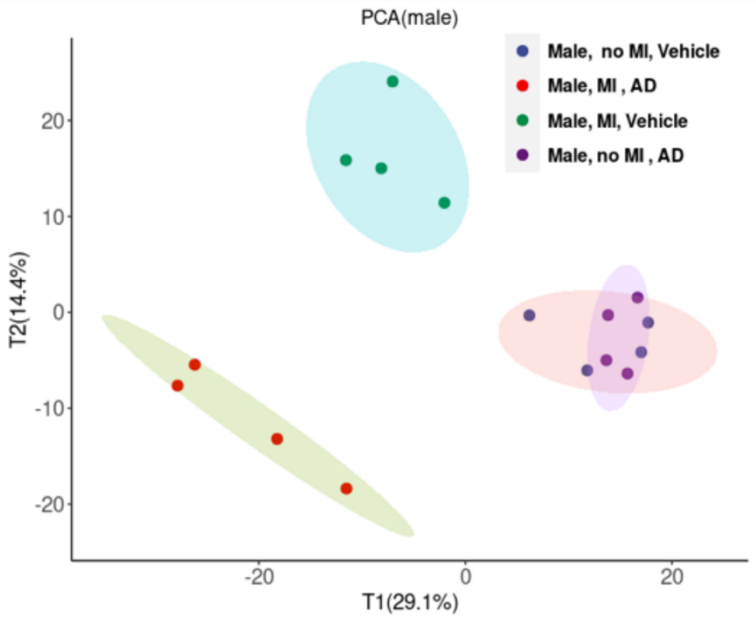
As a means to characterize the effects of surgery and treatment on the metabolic profile and to better appreciate the consequences of gut dysbiosis, the metabolomes of 16 retired breeder male and 16 retired breeder female mice were analyzed.

The different metabolic profiles were first compared. The PCA plot indicates that males which did not undergo surgery have similar metabolomes regardless of the treatment. However, the PCA plot shows that VEH and AD mice differ significantly after surgery (Figure 11A). PCA plots indicate that female metabolomes are not affected by treatment regardless of surgery. However, the surgery groups differed from each other (Figure 11B).

Further analysis within each of the groups allowed for a detailed metabolic profile in terms of significantly upregulated and downregulated metabolites. For the males, Figure 12A shows the metabolites that were significantly upregulated or downregulated after the MI compared to the no-MI mice in VEH groups. The surgery led to a considerable increase in the levels of jasmonic acid and a decrease in the levels of azelaic acid, which were the most affected metabolites. Figure 12B indicates the metabolites significantly upregulated or downregulated in the AD surgery compared to AD no-MI mice. In this case, more metabolites are significantly impacted than following VEH treatment. Isoschaftoside was the most upregulated metabolite while nicotinic acid was the most downregulated. Figure 12C shows the metabolites affected by the AD treatment compared to VEH within no-MI mice. Only three metabolites are involved and as expected, phthalic acid is considerably upregulated between the AD and VEH as a result of the DEHP treatment. Finally, Figure 12D shows the metabolites affected by AD compared to VEH in the MI groups. In this case, many more metabolites are involved. Here, isoschaftoside is the most upregulated while nicotinic acid is the most downregulated. Phthalic acid is also among the significantly upregulated metabolites as a result of the treatment. Microbial pathways analysis determined that mucin-o-glycan degradation was significantly increased following AD treatment.

On another hand, females' metabolomes are affected in a similar way to surgery and treatment, although many of the metabolites differ from male metabolomes. Within the VEH mice, the MI significantly impacted many metabolites, of which oleoyle ethanolamide was the most upregulated and isoschaftoside the most downregulated (Figure 13A). Within the AD mice, the MI significantly impacted more metabolites (Figure 13B). Similarly to the males, the AD treatment only affected three metabolites within the no-MI groups, but phthalic acid was not one of them in that case (Figure 13C). Finally, the AD treatment within the mice which had a MI affected several more metabolites, of which phthalic acid was one of the most upregulated (Figure 13C).

A. Males metabolome



B. Females metabolome

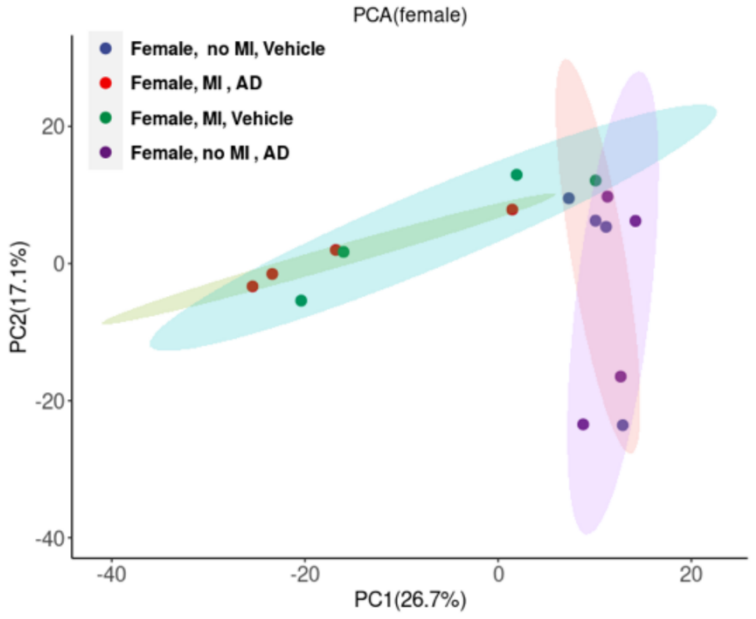


Figure 11 – PCA plots of metabolome compositions for males and females.
PCA plots showing the metabolome compositions of mice from different groups. Each dot represents one mouse's metabolome. A, PCA of male mice's metabolomes. B, PCA of female mice's metabolomes.

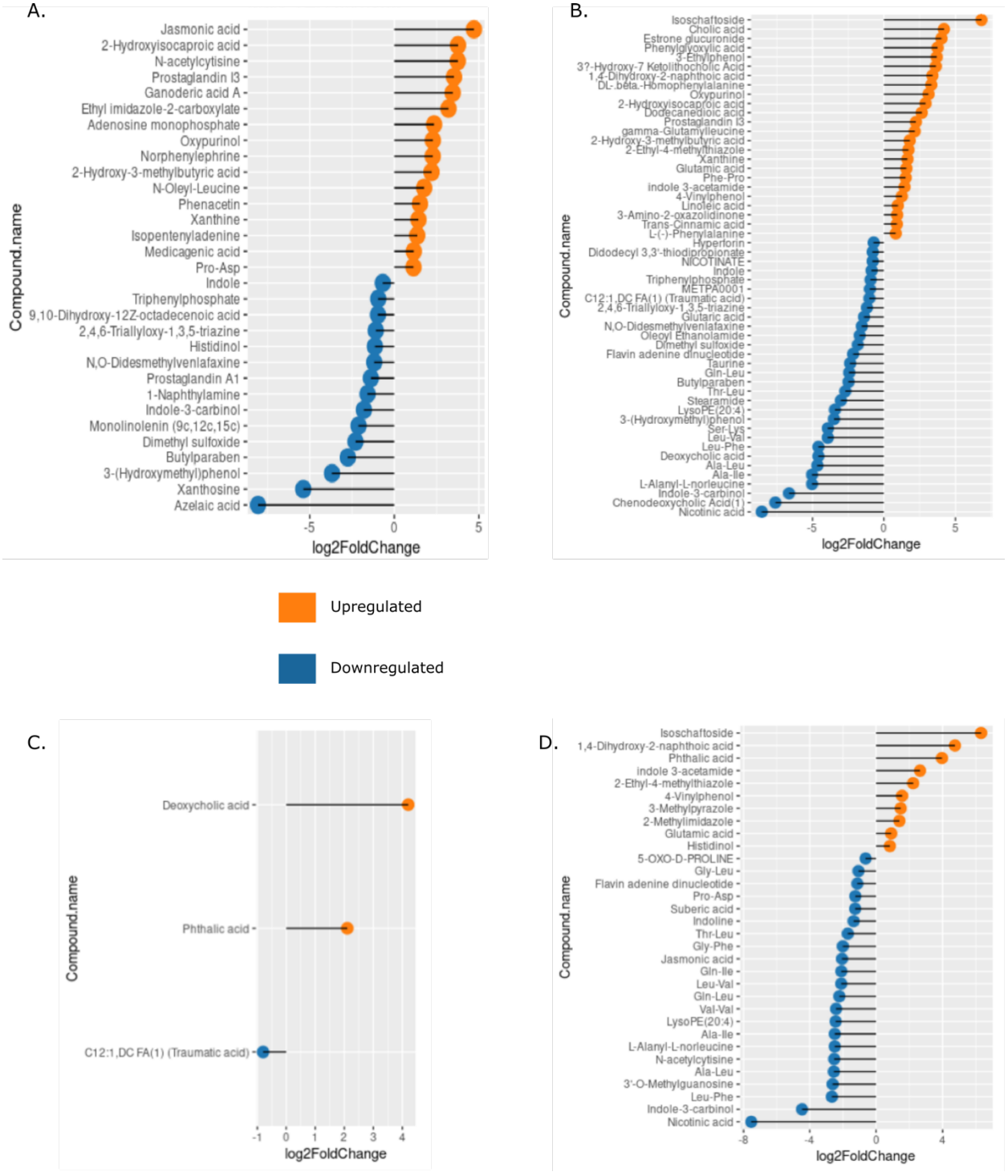


Figure 12 - Metabolites differential in the different male groups according to treatment or surgery.
Graphs showing the impact of surgery or treatment on metabolites presence within the males. A, Impact of MI compared to no-MI within VEH mice. B, Impact of MI compared to no-MI within AD mice. C, Impact of AD compared to VEH within no-MI mice. D, Impact of AD compared to VEH within MI mice. An absolute value log_2FoldChange ≥ 1 is considered significant.

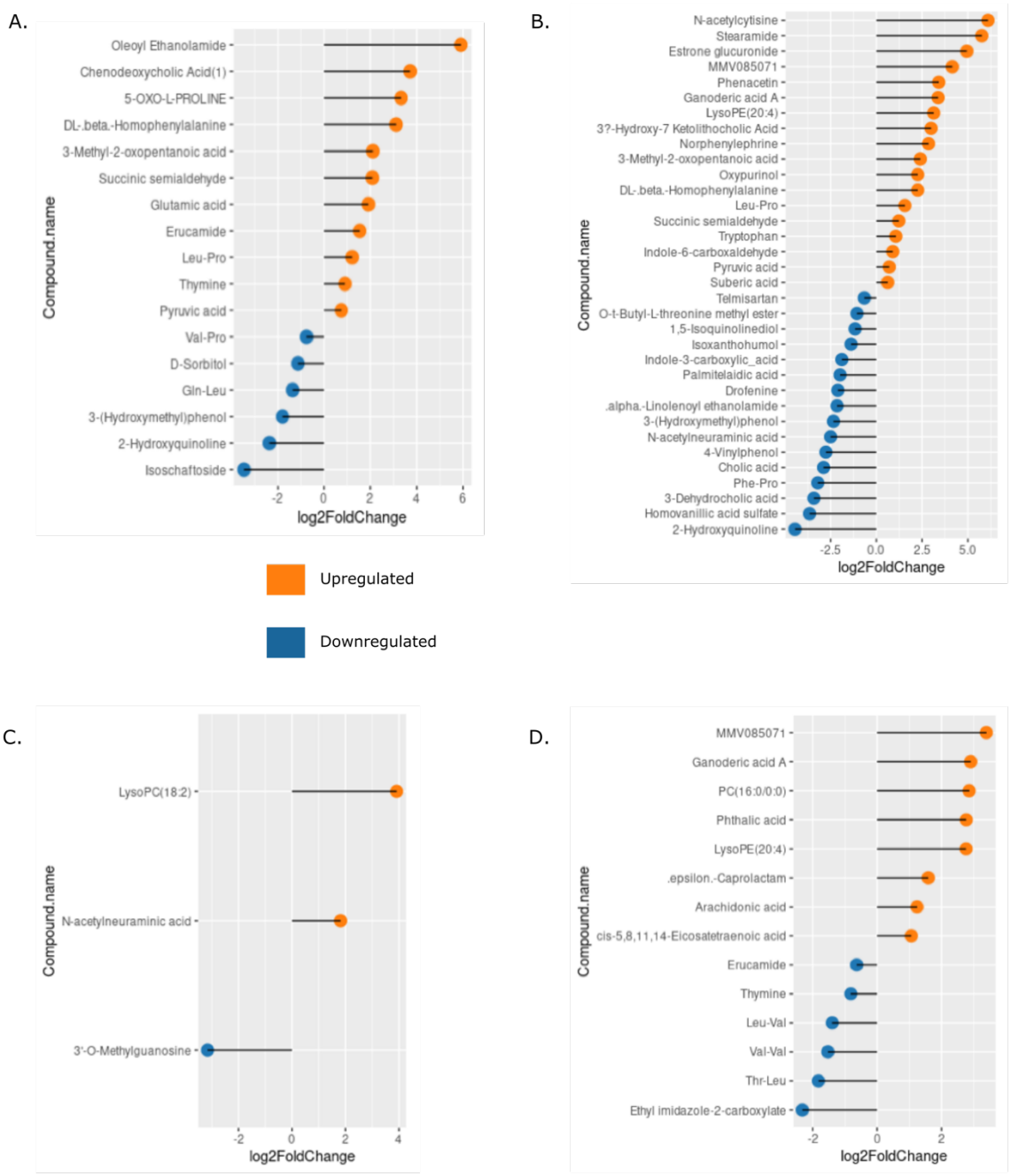


Figure 13 - Metabolites differential in the different female groups according to treatment or surgery.
Graphs showing the impact of surgery or treatment on metabolites presence within the females. A, Impact of MI compared to no-MI within VEH mice. B, Impact of MI compared to no-MI within AD mice. C, Impact of AD compared to VEH within no-MI mice. D, Impact of AD compared to VEH within MI mice. An absolute value $\log_2$FoldChange ≥ 1 is considered significant.

D. **Retired breeder males**

i. Impact of MI and treatment on survival and weight loss

In order to characterize the effect of the surgery and the AD exposure on the mice, we collected several physiological parameters from the mice before and after the treatment and the surgery. These parameters included survival, body weight, spleen weight, cecum content weight, and fecal pellets weight and count. A total of 51 retired breeder males were divided into two groups: one that would undergo surgery (n=35) and one that would not undergo surgery (n=16). Within these two groups, mice would either be treated with VEH or AD. Two mice died during surgery and were excluded from survival rate calculation.

Of the 4 groups of retired breeder males, all those which did not have a MI survived (Figure 15A). The MI groups had survival rates of 81% (13 out of 16) for the VEH mice and 88% (15 out of 17) for the AD group (Figure 14A). The body weights were reduced after the surgery. In fact, on average the RB males which had a MI lost more than 11% of their initial weight compared to less than 4% for the “no-MI” group (Figures 14B, 15B). Within the MI group, the AD treatment had a significant difference in body weight loss compared to VEH mice. This is not the case for the mice which didn’t have surgery. Specifically, mice which were exposed to AD lost less body weight than VEH mice following surgery.

In regard to spleen weights, all 4 groups had similar spleen weights of between 2 and 3 mg/g of body weight meaning neither the surgery nor the treatment had a significant impact (Figures 14C, 15C). On another hand, cecum weights were significantly impacted by the surgery. Here, there was a statistically significant difference between the VEH groups as well as between the AD groups with MI ($p=0.008$) and without MI ($p=0.003$) (Figures 14D, 15D). The treatment did not have an impact on the cecum weights within the MI or no MI groups, although AD mice tended to have slightly heavier ceca than VEH mice regardless of the surgery.

Finally, all groups had smaller fecal pellets on D3 compared to D-7 albeit not significantly (Figures 14E, 15E). Treatment did not have an impact on the weight of fecal pellets for the group

that had surgery. However, fecal pellets in the AD no MI group weighed less than those obtained from VEH mice. As for the number collected, all groups had fewer pellets on D3 compared to D-7. More fecal pellets were obtained on D3 in mice which did not have a MI and were treated with AD (Figures 14F, 15F).

ii. Impact of MI and treatment on immune cell number and infiltration

After a myocardial infarction, a rapid immune reaction involving several types of cells occurs to remove cell debris and promote cardiomyocytes regeneration (150). In order to determine the effects of the surgery or the AD treatment on the immune response and inflammation, we characterized the immune profile of mice in our 16 different groups (males and females, old and young, with and without surgery, with and without AD treatment). Specifically, flow cytometry was performed on single cells isolated from the heart, spleen and bone marrow using an established gating strategy (35). This strategy allowed us to determine the number of macrophages, dendritic cells (DC), monocytes ($Ly6C^{high}$ and $Ly6C^{low}$) and neutrophils for all samples. B and T cells were enumerated in the spleen.

Surgery was a significant factor in the immune cells' proliferation in spleen and bone marrow of retired breeder males. In fact, the same types of immune cells were similarly affected. In the spleen, all of the monocytes, neutrophils, B cells and T cells were significantly upregulated after the MI in the VEH groups (Figure 16A, B, C & D). In the bone marrow, the surgery caused a significant difference between the "MI" and "no-MI" groups regardless of the treatment in 4 of the 6 types of cells of interest. Specifically, macrophages, dendritic cells, monocytes and $Ly6C^{low}$ monocytes were all upregulated after the surgery compared to the "no-MI" groups (Figures 17). Other cells for which no significant difference was seen between groups are shown in Tables 3 and 4.

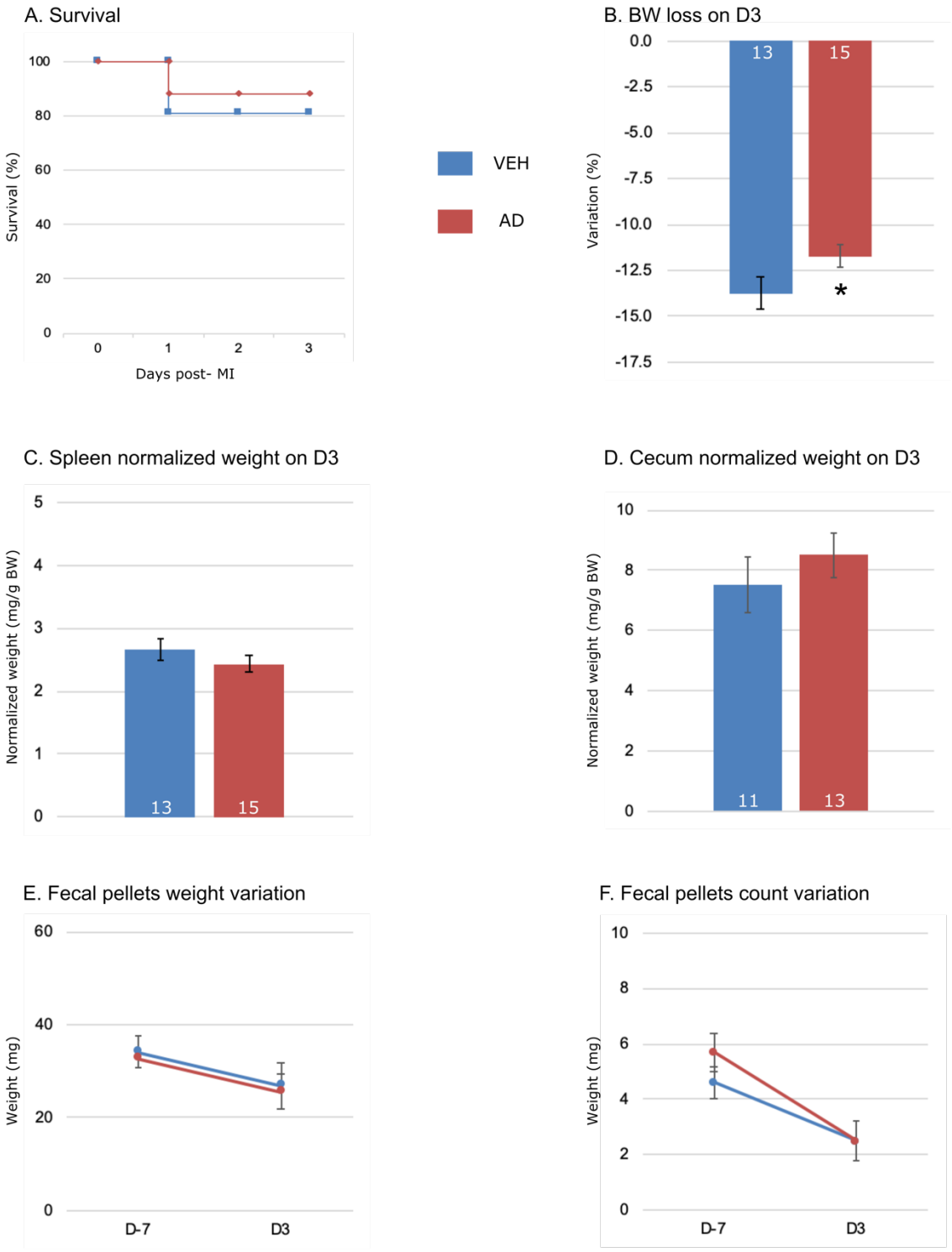


Figure 14 - Physiological data for retired breeder males with surgery (VEH & AD).

Impact of the VEH and AD treatments on the physiological parameters of retired breeder males which underwent surgery. A, Survival rate of mice after surgery. B, Body weight variation from D0 to D3. C, Spleen normalized weight per body weight on D3 following euthanasia. D, Cecum normalized weight per body weight on D3 following euthanasia. E, Fecal pellets weight variation between D-7 and D3. F, Fecal pellets count variation between D-7 and D3. N=16 for the VEH group and N=17 for the AD group.

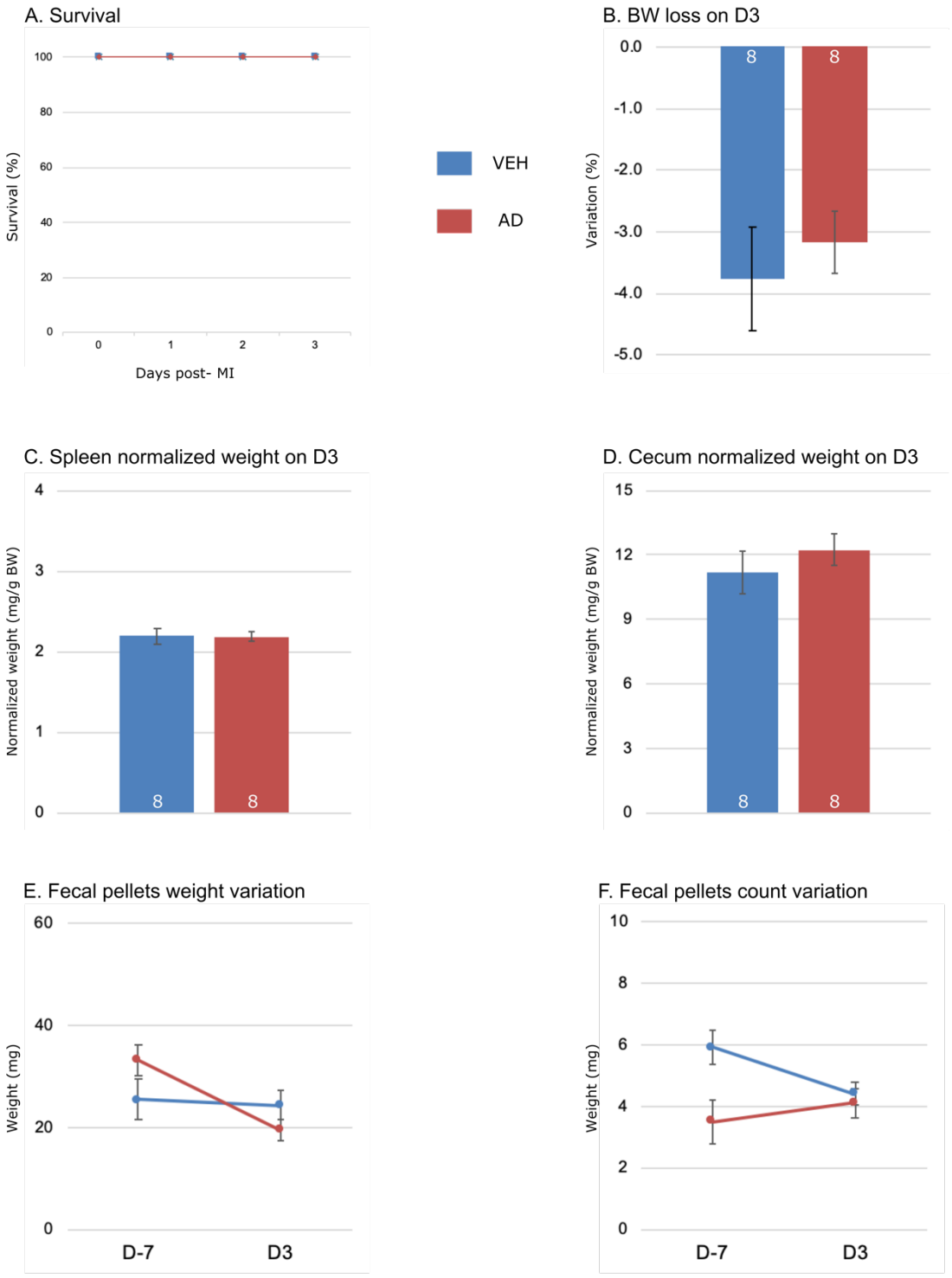


Figure 15 - Physiological data for retired breeder males without surgery (VEH & AD).
Impact of the VEH and AD treatments on the physiological parameters of retired breeder males which did not undergo surgery. A, Survival rate of mice after surgery. B, Body weight variation from D0 to D3. C, Spleen normalized weight per body weight on D3 following euthanasia. D, Cecum normalized weight per body weight on D3 following euthanasia. E, Fecal pellets weight variation between D-7 and D3. F, Fecal pellets count variation between D-7 and D3. N=8 for the VEH group and N=8 for the AD group.

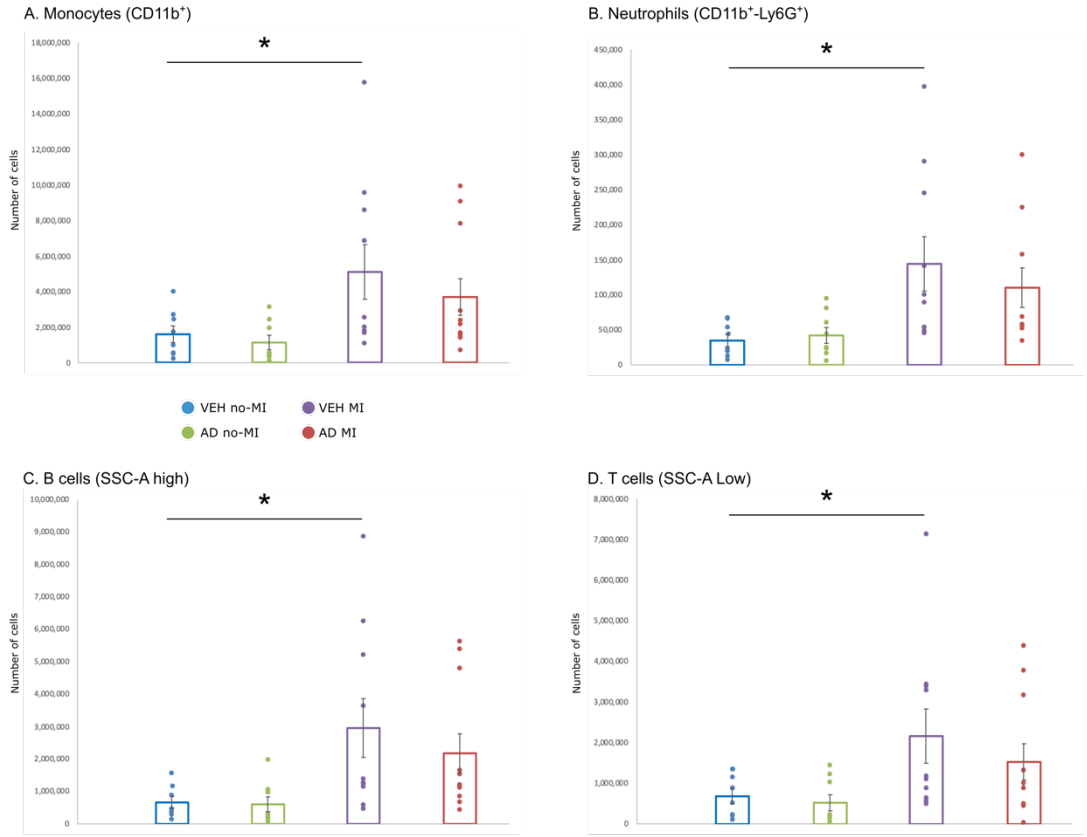


Figure 16 – Impact of surgery and treatment on immune cell numbers in RB male spleen.
Impact of surgery and treatment on the immune cell numbers in the spleen of retired breeder males. A, Monocytes ($CD11b^+$) count per spleen. B, Neutrophils ($CD11b^+Ly6G^+$) count per spleen. C, B cells (SSC-A high) count per spleen. D, T cells (SSC-A Low) count per spleen. Each dot represents an individual mouse. The bars show the averages and standard error of mean for each group. * denotes a statistically significant difference between two groups. A *p* value of <0.05 was considered significant.

Table 3 – Immune cell numbers in RB male spleen.
Average counts and standard error of mean (SEM) in spleen of RB males.

Cell types	Group	Average number of cells
Macrophages (CD64+MerTK+)	VEH no-MI	2,103,863 ± 663,796
	AD no-MI	1,447,179 ± 530,521
	VEH MI	7,467,472 ± 2,323,773
	AD MI	6,931,010 ± 2,805,318
Dendritic cells (MHC II^+)	VEH no-MI	19,575 ± 8,010
	AD no-MI	11,906 ± 3,492
	VEH MI	19,319 ± 6,194
	AD MI	29,588 ± 7,245
Monocytes ($CD11b^+$-Ly6C High)	VEH no-MI	15,155 ± 5,454
	AD no-MI	11,768 ± 4,190
	VEH MI	49,940 ± 20,919
	AD MI	138,816 ± 106,002
Monocytes ($CD11b^+$-Ly6C Low)	VEH no-MI	156,336 ± 57,442
	AD no-MI	105,137 ± 38,325
	VEH MI	204,428 ± 63,018
	AD MI	770,854 ± 539,748

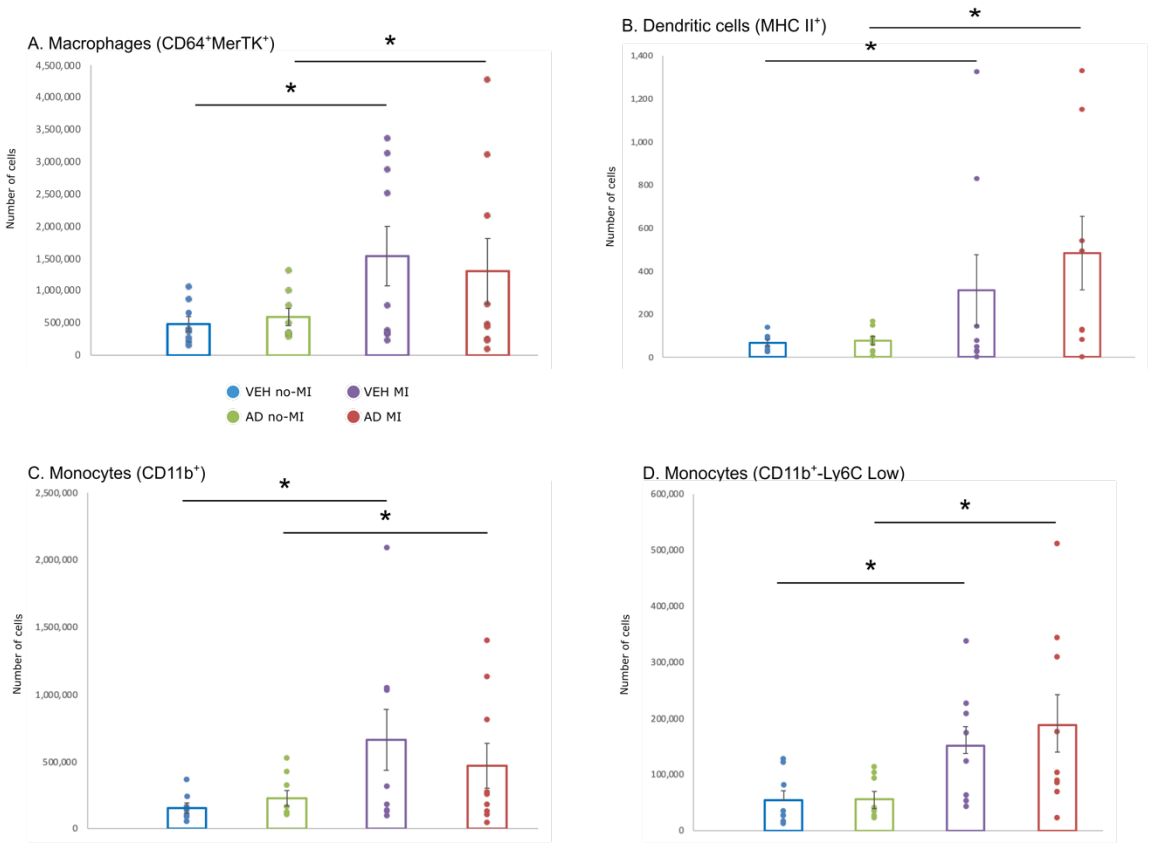


Figure 17 – Impact of surgery and treatment on immune cell numbers in the bone marrow of RB males.
Impact of surgery and treatment on the immune cell numbers in the bone marrow (femur) of retired breeder males. A, Macrophages ($CD64^+MerTK^+$) count per femur. B, Dendritic cells ($MHC\ II^+$) count per femur. C, Monocytes ($CD11b^+$) count per femur. D, Monocytes ($CD11b^+$-Ly6C Low) count per femur. Each dot represents an individual mouse. The bars show the averages and standard error of mean for each group. * denotes a statistically significant difference between two groups. A p value of <0.05 was considered significant.

Table 4 - Immune cell numbers in RB male bone marrow.
Average counts and standard error of mean (SEM) in bone marrow of RB males.

Cell types	Group	Average number of cells
Monocytes ($CD11b^+$-Ly6C High)	VEH no-MI	26,255 ± 14,938
	AD no-MI	11,489 ± 4,631
	VEH MI	52,514 ± 26,301
	AD MI	30,269 ± 10,789
Neutrophils ($CD11b^+$-$Ly6G^+$)	VEH no-MI	221,612 ± 42,801
	AD no-MI	297,023 ± 58,971
	VEH MI	459,016 ± 187,773
	AD MI	536,094 ± 203,326

E. **Young males**

i. Impact of MI and treatment on survival and weight loss

A total of 34 young males were divided into a group which would undergo surgery (n=22) and a group which would not undergo surgery (n=12). Within each group, mice would either be subjected to a VEH treatment or to the AD treatment. Two mice died during surgery and were excluded from survival rate calculation.

Of these 4 groups, only the young males which had both a MI and the AD treatment did not have a 100% survival rate (Figures 18A, 19A). On D3, the survival rate for the "YG M MI AD" group dropped to 90% (9 of 10 mice survived). In regard to the body weight, the AD mice consistently lost more weight than the VEH mice, although the difference was not statistically different with MI ($p=0.11$) and without MI ($p=0.9$). However, the most significant difference lies between the no-MI and MI groups, regardless of the treatment ($p<0.001$) (Figures 18B, 19B). The mice which had a MI lost more than 10% of their BW between D0 and D3 while the mice which didn't undergo surgery lost less than 4% of their BW.

The spleen and cecum weight were normalized to the body weight of the mice to have a more accurate comparison between groups. For the spleen, among the mice which underwent surgery, the AD mice had a significantly lighter spleen than the VEH group (Figure 18C). Without the surgery, there was no significant difference between the VEH and AD groups (Figure 19C). As for the cecum weights, although the "MI" mice had smaller ceca when they were treated with the AD compared to the VEH, the difference was not significant (Figure 18D). For the "no-MI" group, the cecum weights were very similar regardless of the treatment (Figure 19D). In contrast, cecum weights were significantly different in the MI and no MI groups, regardless of the treatment ($p<0.001$). The MI mice had a cecum weighing less than 12mg/g BW while the no-MI mice had a cecum weighing more than 22mg/g BW.

Finally, fecal pellets weight and number obtained were noted to serve as an indicator of GI function of the mice. The fecal pellets collected on D3 weighed less than those collected at D-7

for all groups except the no-MI VEH group (Figures 18E, 19E). As for the number of the pellets obtainable, all 4 groups had fewer pellets on D3 than they did on D-7 except for the “MI AD” mice that gave the same amount on both days (Figures 18F, 19F).

ii. Impact of MI and treatment on immune cells proliferation and infiltration

In the spleen of YG males, several types of cells were significantly affected by either the surgery or the treatment. In fact, the number of macrophages per spleen was significantly lower in AD groups than in VEH groups, regardless of the surgery (Figure 20A). $Ly6C^{high}$ monocytes were upregulated in the presence of a surgery only within the VEH groups (Figure 20B). Similarly, neutrophils were also upregulated in the presence of an infarct, regardless of the treatment (Figure 20C). Finally, the treatment had a significant impact on B cells in that AD-treated mice had fewer B cells in their spleen than VEH mice, regardless of surgery. Other cells for which no significant difference was seen between groups are shown in Table 5.

On another hand, the immune profile of the bone marrow showed different changes than those detected in the spleen. Specifically, only the macrophages and dendritic cells were significantly affected by the surgery or the treatment. Macrophages were significantly downregulated in the MI groups for both VEH and AD groups (Figure 21A). Dendritic cells were downregulated in the bone marrow of mice which underwent surgery but only between the AD groups (Figure 21B). Other cells for which no significant difference was seen between groups are shown in Table 6.

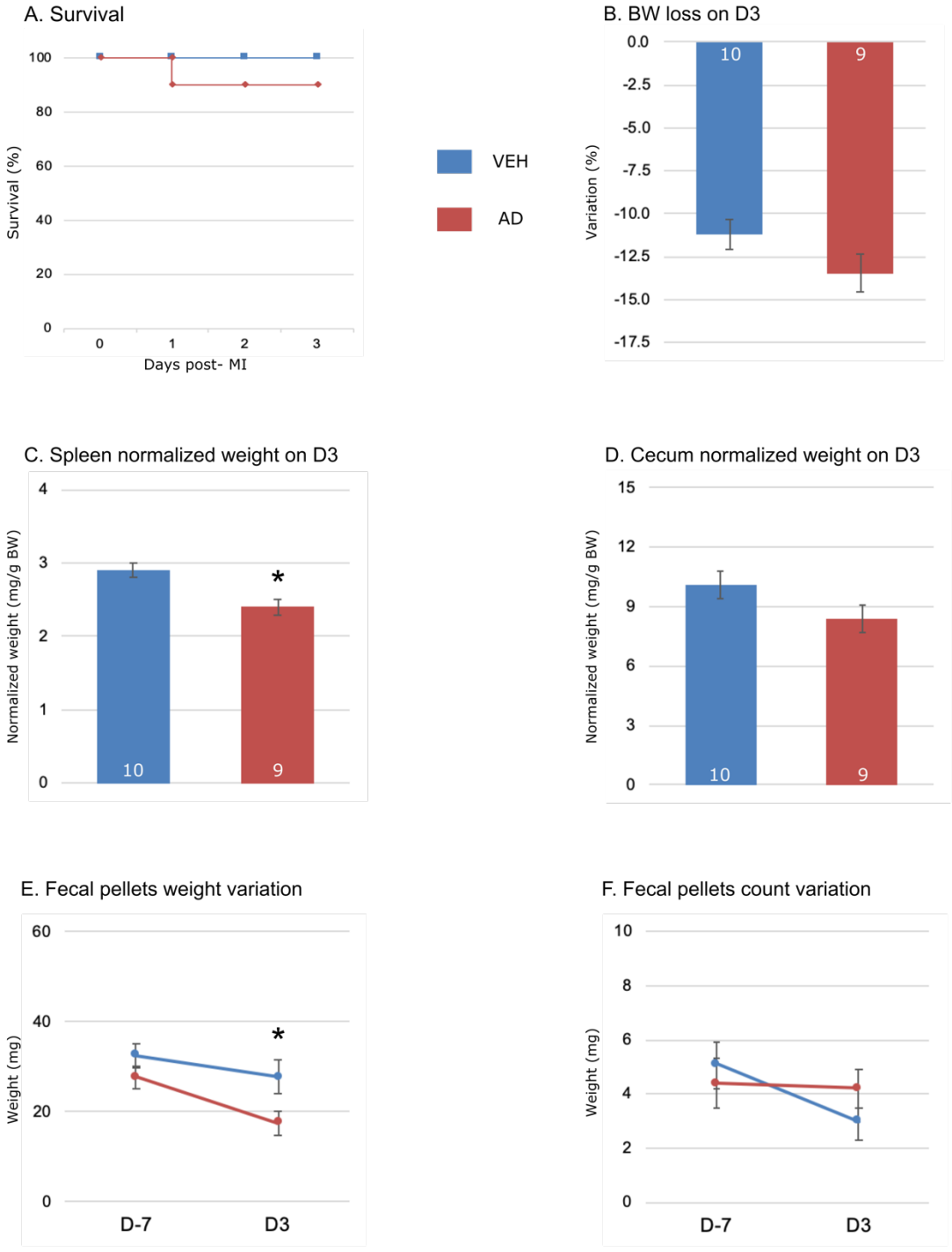


Figure 18 - Physiological data for young males with surgery (VEH & AD).

Impact of the VEH and AD treatments on the physiological parameters of young males which underwent surgery. A, Survival rate of mice after surgery. B, Body weight variation from D0 to D3. C, Spleen normalized weight per body weight on D3 following euthanasia. D, Cecum normalized weight per body weight on D3 following euthanasia. E, Fecal pellets weight variation between D-7 and D3. F, Fecal pellets count variation between D-7 and D3. N=10 for the VEH group and N=10 for the AD group.

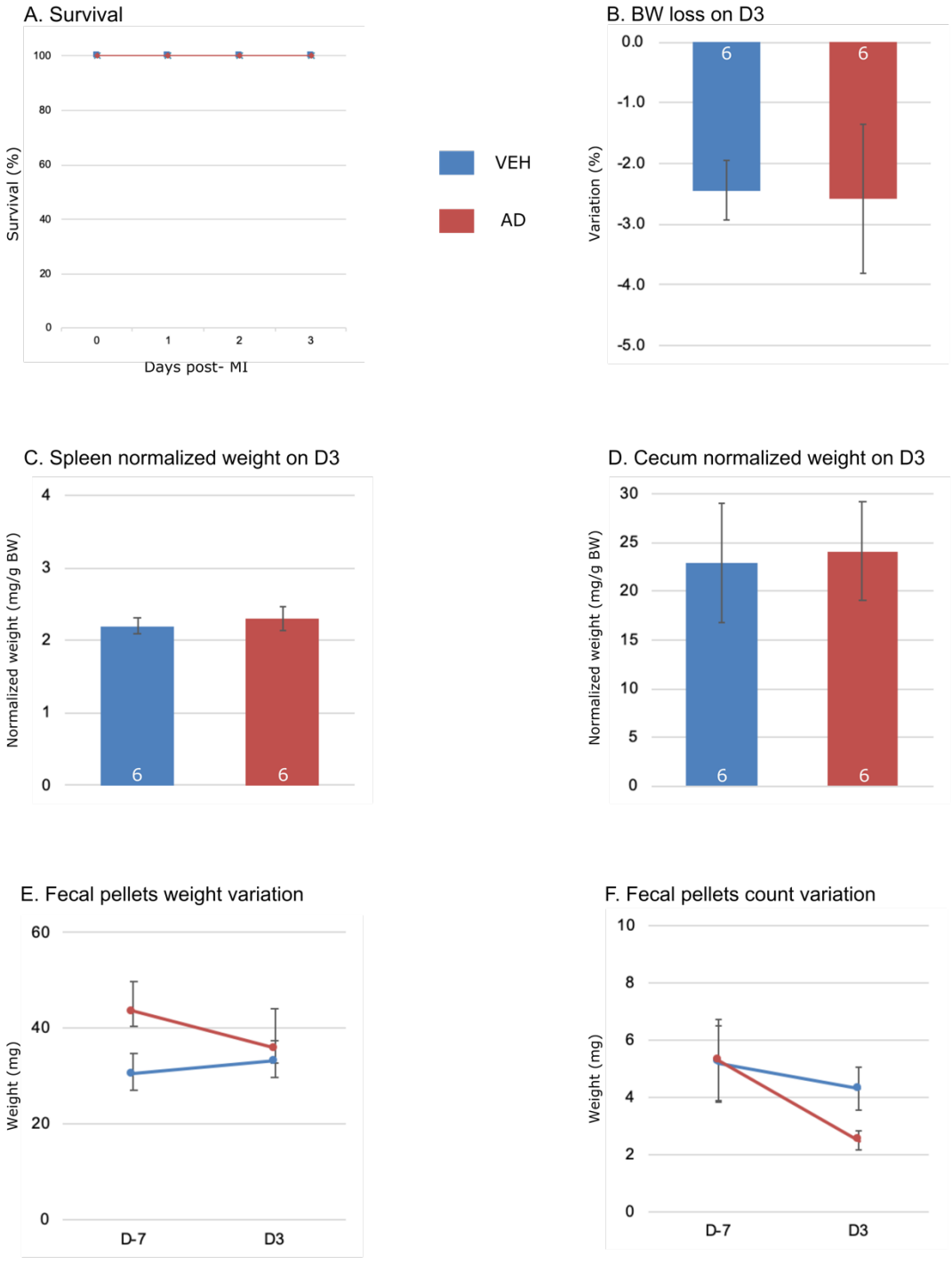


Figure 19 – Physiological data for young males without surgery (VEH and AD).
Impact of the VEH and AD treatments on the physiological parameters of young males which did not undergo surgery. A, Survival rate of mice after surgery. B, Body weight variation from D0 to D3. C, Spleen normalized weight per body weight on D3 following euthanasia. D, Cecum normalized weight per body weight on D3 following euthanasia. E, Fecal pellets weight variation between D-7 and D3. F, Fecal pellets count variation between D-7 and D3. N=6 for the VEH group and N=6 for the AD group.

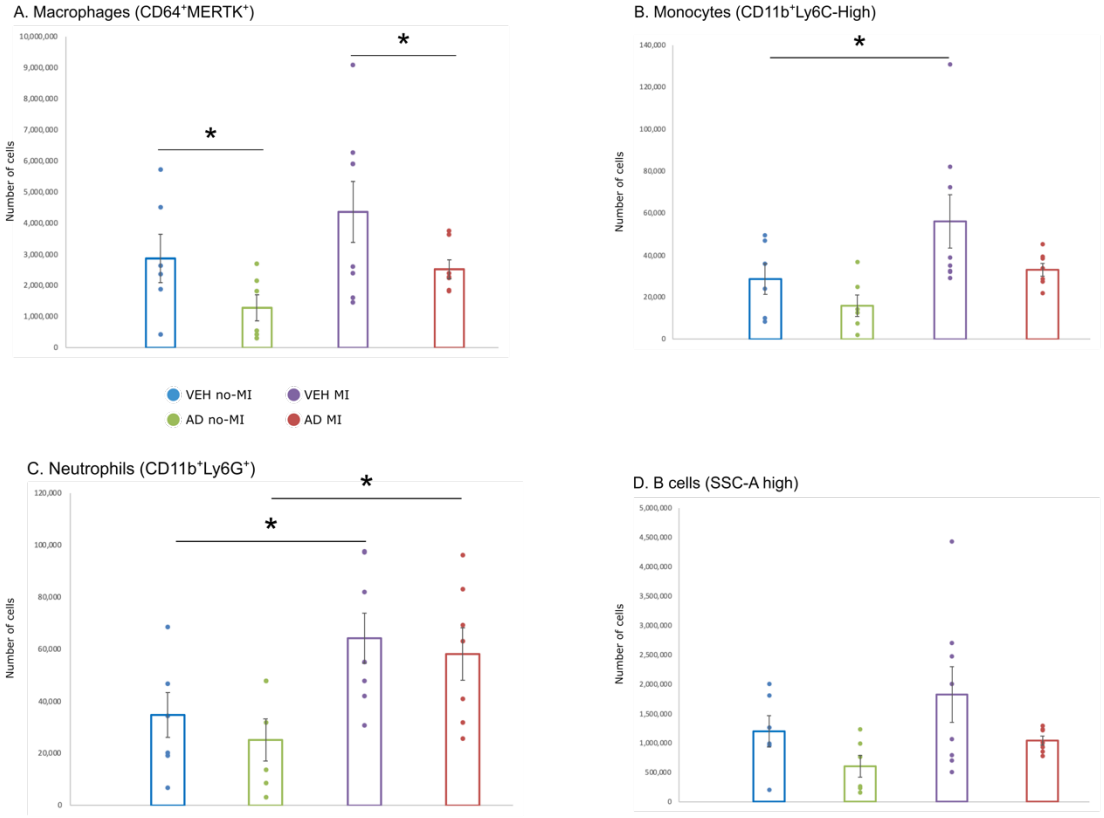


Figure 20 – Impact of surgery and treatment on immune cells proliferation in spleen of YG males.
Impact of surgery and treatment on the immune cells' numbers in spleen of young males. A, Macrophages ($CD64^+MerTK^+$) count per spleen. B, Monocytes count ($CD11b^+Ly6C$-high) per spleen. C, Neutrophils count ($CD11b^+Ly6G^+$) per spleen. D, B cells (SSC-A high) per spleen. Each dot represents an individual mouse. The bars show the averages and standard error of mean for each group. * denotes a statistically significant difference between two groups. A p value of <0.05 was considered significant.

Table 5 – Immune cell numbers in YG male spleen.
Average counts and standard error of mean (SEM) in spleen of YG males.

Cell types	Group	Average number of cells
Dendritic cells ($MHC\ II^+$)	VEH no-MI	12,742 ± 3,901
	AD no-MI	6,358 ± 2,168
	VEH MI	12,383 ± 2,668
	AD MI	11,135 ± 2,789
Monocytes ($CD11b^+$)	VEH no-MI	2,375,394 ± 651,205
	AD no-MI	1,040,387 ± 345,743
	VEH MI	3,294,586 ± 804,748
	AD MI	1,842,233 ± 272,681
Monocytes ($CD11b^+$-Ly6C Low)	VEH no-MI	101,395 ± 26,306
	AD no-MI	51,585 ± 22,740
	VEH MI	128,258 ± 29,030
	AD MI	57,535 ± 7,384
T cells (SSC-A Low)	VEH no-MI	1,214,540 ± 428,750
	AD no-MI	455,116 ± 166,574
	VEH MI	1,515,138 ± 372,834
	AD MI	827,718 ± 210,421

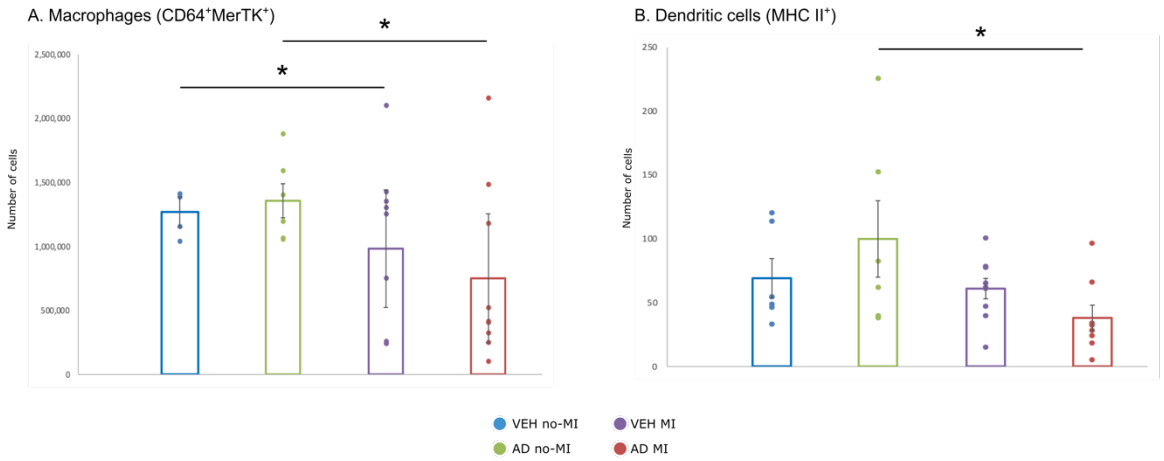


Figure 21 - Impact of surgery and treatment on immune cells proliferation in bone marrow of YG males.
Impact of surgery and treatment on the immune cells proliferation in bone marrow (femur) of young males. A, Macrophages ($CD64^+MerTK^+$) count per femur. B, Dendritic cells ($MHC\ II^+$) count per femur. Each dot represents an individual mouse. The bars show the averages and standard error of mean for each group. * denotes a statistically significant difference between two groups. A p value of <0.05 was considered significant.

Table 6 - Immune cell numbers in YG male bone marrow.
Average counts and standard error of mean (SEM) in bone marrow of YG males.

Cell types	Group	Average number of cells
Monocytes ($CD11b^+$)	VEH no-MI	467,360 ± 106,263
	AD no-MI	491,606 ± 69,881
	VEH MI	357,988 ± 71,416
	AD MI	291,916 ± 81,245
Monocytes ($CD11b^+$-Ly6C High)	VEH no-MI	21,502 ± 5,010
	AD no-MI	20,205 ± 4,701
	VEH MI	32,538 ± 6,553
	AD MI	30,416 ± 10,172
Monocytes ($CD11b^+$-Ly6C Low)	VEH no-MI	82,898 ± 17,265
	AD no-MI	109,478 ± 11,080
	VEH MI	107,200 ± 20,463
	AD MI	82,878 ± 22,928
Neutrophils ($CD11b^+Ly6G^+$)	VEH no-MI	430,204 ± 87,304
	AD no-MI	612,892 ± 45,764
	VEH MI	448,850 ± 85,215
	AD MI	306,436 ± 80,715

F. **Retired breeder females**

i. Impact of MI and treatment on survival and weight loss

A total of 38 retired breeder females were divided into two groups: one that would undergo surgery (n=28) and one that would not undergo surgery (n=10). Within these two groups, mice would either be treated with VEH or AD. 1 mouse died during surgery and was excluded from the survival rate calculation.

Of the RB females which underwent surgery, the survival rates for VEH and AD groups are similar. The VEH group has a survival rate of 92.3% (12 of 13 survived) while the AD treatment group has a survival rate of 92.8% (13 of 14 survived) (Figure 22A). In terms of body weight variation, the MI group lost significantly more weight than the no-MI group, regardless of the treatment ($p=0.001$). Within the same group, treatment had no significant impact on body weight variation (Figures 22B, 23B).

In regard to spleen, the spleen of mice which had surgery were significantly bigger than those of mice which did not have surgery ($p<0.001$). Within the same group, treatment did not have a significant effect on indexed spleen weight (Figures 22C, 23C). The ceca of the RB females were not significantly affected by treatment, but surgery was a significant factor in that mice which underwent surgery had significantly smaller ceca compared to those which did not have a MI ($p<0.001$) (Figures 22D, 23D). Finally, there was no significant difference in the weight and number of fecal pellets on D3 compared to D-7 (Figures 22E, 22F, 23E, 23F).

ii. Impact of MI and treatment on immune cells proliferation and infiltration

Many cell types were affected by surgery and treatment in the bone marrow of RB females, while only one type was significantly impacted in the spleen. Ly6C^{high} monocytes were the only cell type that was considerably impacted by surgery in the spleen of retired breeder females. The “MI” groups had significantly more of these monocytes than “no-MI” mice in both treatment groups (Figure 24). As for the bone marrow, 4 cell types were significantly impacted by surgery only. In fact, macrophages, monocytes, Ly6C^{high} monocytes, and neutrophils were all upregulated in the

surgery groups compared to the groups which did not undergo surgery (Figure 25). Other cells for which no significant difference was seen between groups are shown in Tables 7 and 8.

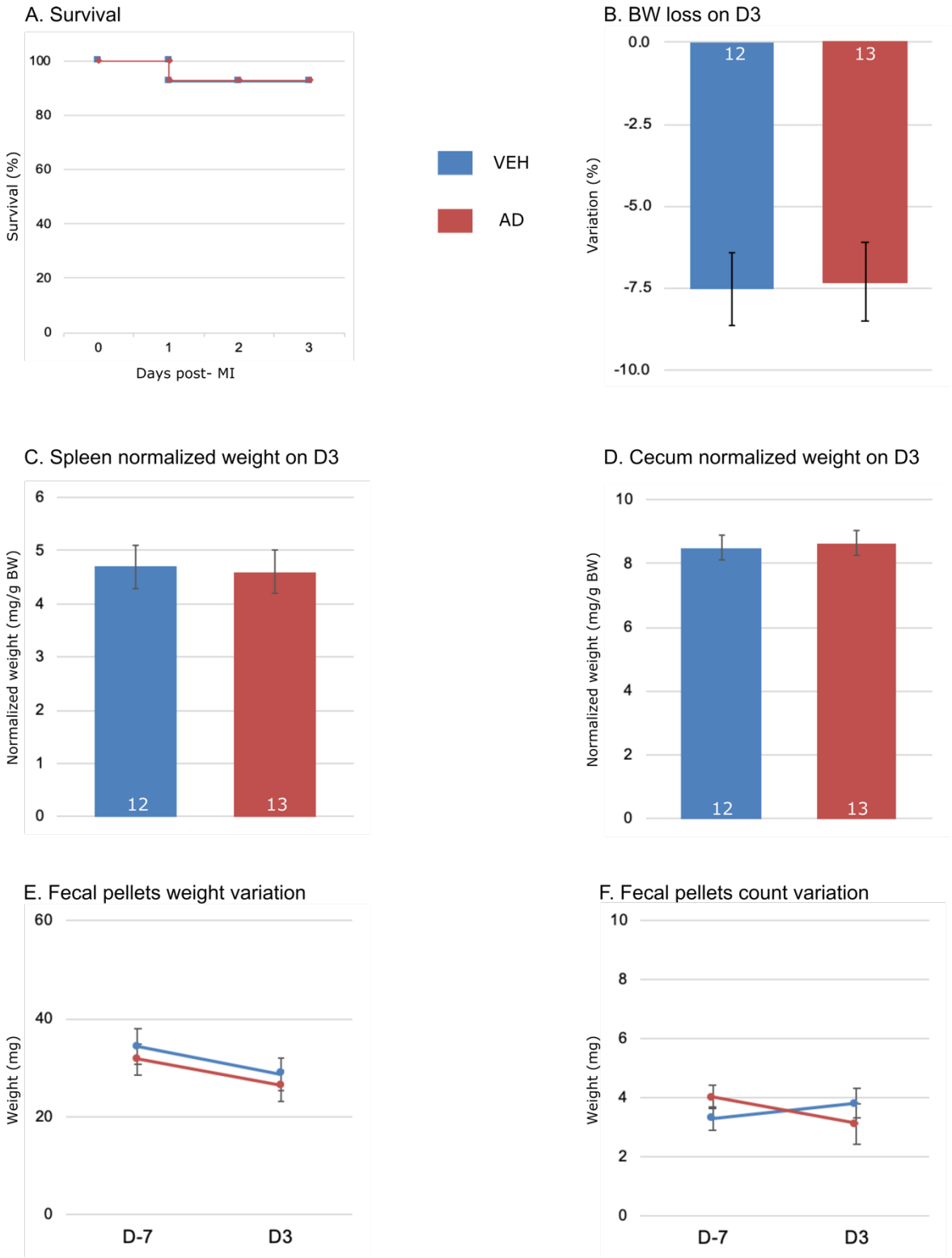


Figure 22 - Physiological data for retired breeder females with surgery (VEH & AD).

Impact of the VEH and AD treatments on the physiological parameters of retired breeder females with surgery. A, Survival rate of mice after surgery. B, Body weight variation from D0 to D3. C, Spleen normalized weight per body weight on D3 following euthanasia. D, Cecum normalized weight per body weight on D3 following euthanasia. E, Fecal pellets weight variation between D-7 and D3. F, Fecal pellets count variation between D-7 and D3. N=13 for the VEH group and N=14 for the AD group.

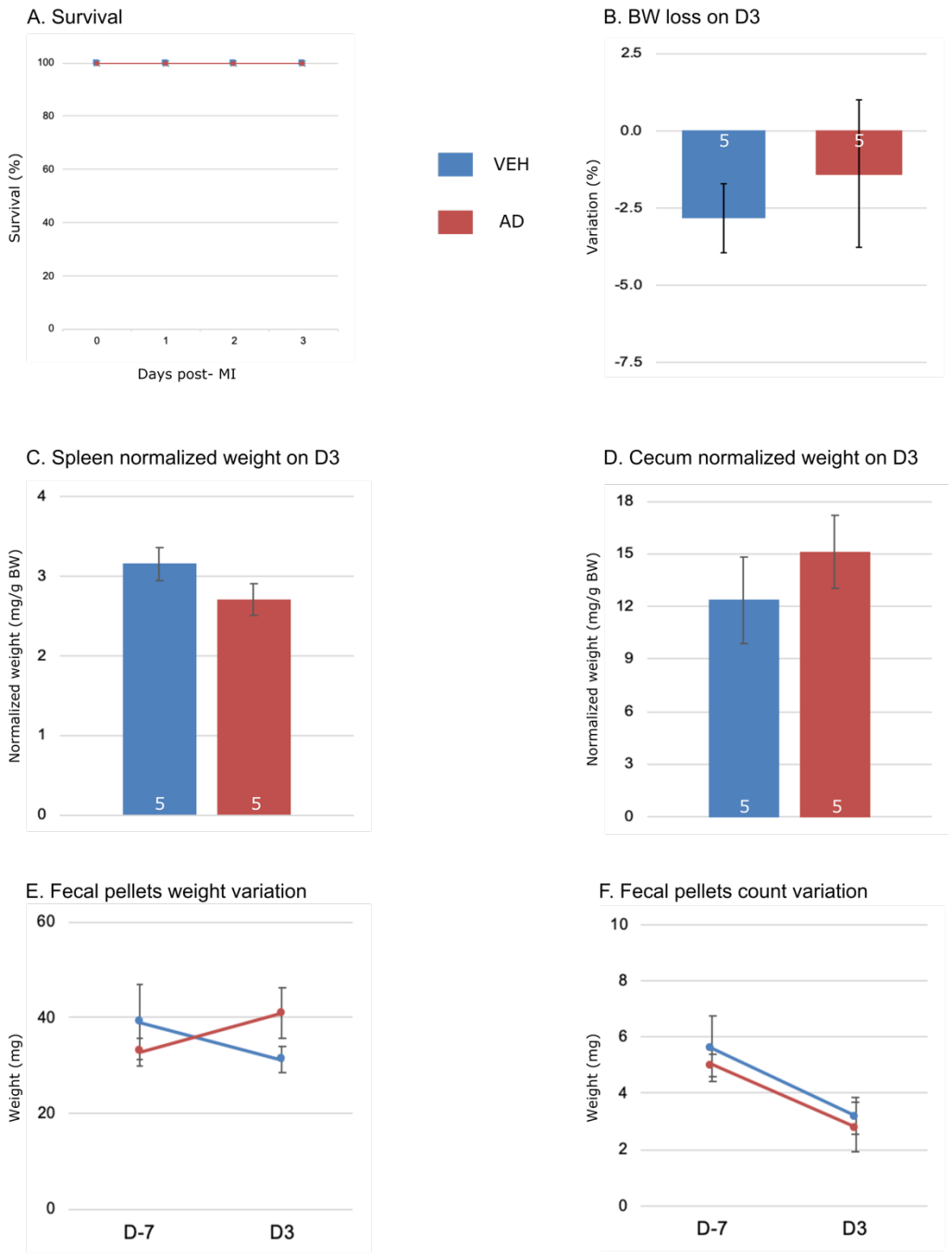


Figure 23 - Physiological data for retired breeder females without surgery (VEH & AD).
Impact of the VEH and AD treatments on the physiological parameters of retired breeder females which underwent surgery. A, Survival rate of mice after surgery. B, Body weight variation from D0 to D3. C, Spleen normalized weight per body weight on D3 following euthanasia. D, Cecum normalized weight per body weight on D3 following euthanasia. E, Fecal pellets weight variation between D-7 and D3. F, Fecal pellets count variation between D-7 and D3. N=5 for the VEH group and N=5 for the AD group.

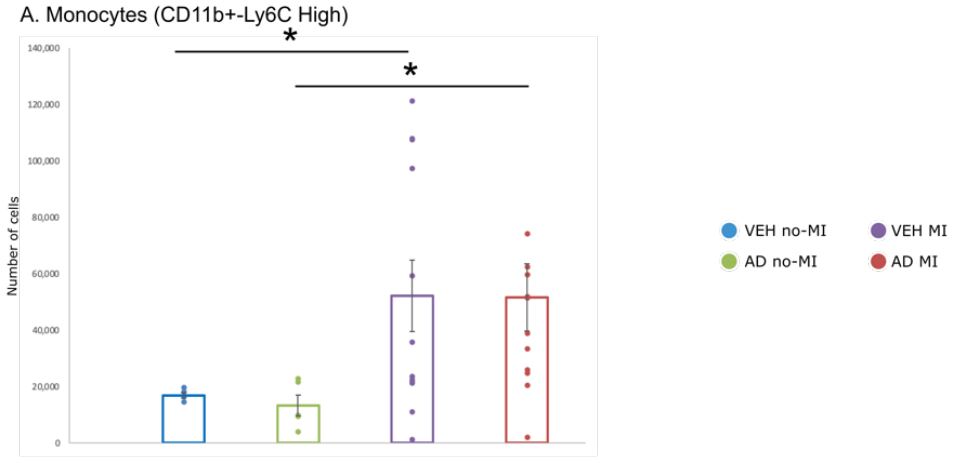


Figure 24 - Impact of surgery and treatment on immune cells numbers in spleen of RB females.
Impact of surgery and treatment on the immune cell numbers in spleen of retired breeder females. A, Monocytes ($CD11b^{+}Ly6C$-high) count per spleen. Each dot represents an individual mouse. The bars show the averages and standard error of mean for each group. * denotes a statistically significant difference between two groups. A p value of <0.05 was considered significant.

Table 7 - Immune cells number in RB female spleen.
Average counts and standard error of mean (SEM) in spleen of RB females.

Cell types	Group	Average number of cells
Macrophages (CD64+MerTK+)	VEH no-MI	3,059,118 ± 746,911
	AD no-MI	2,110,031 ± 849,803
	VEH MI	4,055,388 ± 1,035,473
	AD MI	2,991,972 ± 523,133
Dendritic cells (MHC II^{+})	VEH no-MI	66,188 ± 27,529
	AD no-MI	41,802 ± 25,740
	VEH MI	31,787 ± 7,723
	AD MI	23,413 ± 4,168
Monocytes ($CD11b^{+}$)	VEH no-MI	2,403,375 ± 574,549
	AD no-MI	1,604,076 ± 641,182
	VEH MI	2,699,983 ± 628,378
	AD MI	2,049,958 ± 342,037
Monocytes ($CD11b^{+}$-Ly6C Low)	VEH no-MI	84,585 ± 26,209
	AD no-MI	47,709 ± 23,227
	VEH MI	113,095 ± 27,766
	AD MI	89,671 ± 16,106
Neutrophils ($CD11b^{+}Ly6G^{+}$)	VEH no-MI	56,973 ± 11,809
	AD no-MI	47,662 ± 25,658
	VEH MI	159,892 ± 50,498
	AD MI	174,383 ± 51,091
B cells (SSC-A high)	VEH no-MI	1,628,012 ± 455,595
	AD no-MI	1,145,078 ± 483,340
	VEH MI	1,781,477 ± 416,514
	AD MI	1,387,844 ± 212,077
T cells (SSC-A low)	VEH no-MI	797,968 ± 156,327
	AD no-MI	472,652 ± 165,683
	VEH MI	935,975 ± 218,832
	AD MI	679,163 ± 154,286

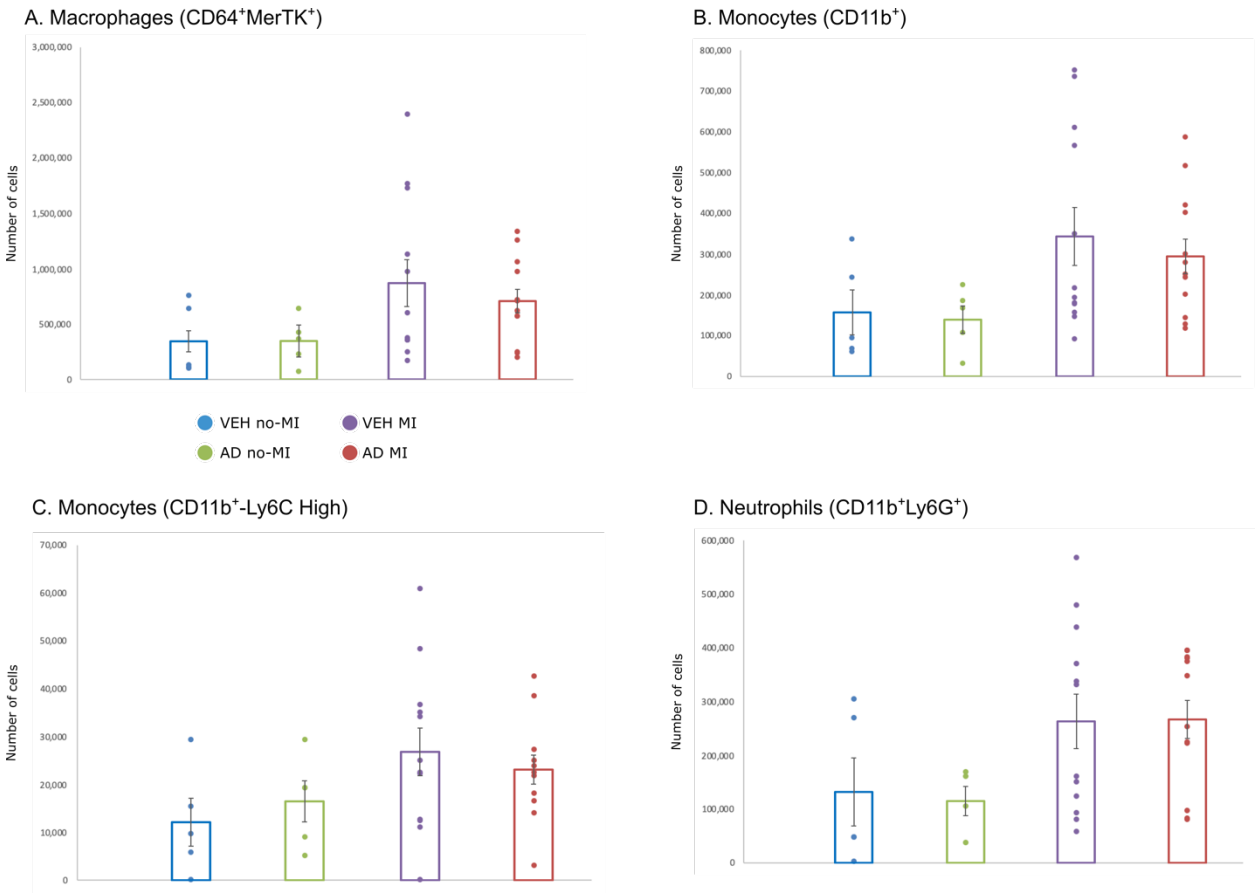


Figure 25 - Impact of surgery and treatment on immune cell numbers in the bone marrow of RB females.
Impact of surgery and treatment on the immune cell numbers in the bone marrow (femur) of retired breeder females. A, Macrophages ($CD64^+MerTK^+$) count per femur. B, Monocytes ($CD11b^+$) count per femur. C, Monocytes ($CD11b^+Ly6C$-high) count per femur. D, Neutrophils ($CD11b^+Ly6G^+$) count per femur. Each dot represents an individual mouse. The bars show the averages and standard error of mean for each group. * denotes a statistically significant difference between two groups. A p value of <0.05 was considered significant.

Table 8 - Immune cell numbers in RB female bone marrow.
Average counts and standard error of mean (SEM) in bone marrow of RB females.

Cell types	Group	Average number of cells
Dendritic cells ($MHC\ II^+$)	VEH no-MI	2,349 ± 1,236
	AD no-MI	1,411 ± 730
	VEH MI	146 ± 51
	AD MI	115 ± 34
Monocytes ($CD11b^+$-Ly6C Low)	VEH no-MI	50,785 ± 22,910
	AD no-MI	55,390 ± 15,471
	VEH MI	89,629 ± 17,252
	AD MI	115,331 ± 17,855

G. **Young females**

i. Impact of MI and treatment on survival rate and weight loss

A total of 30 young females were divided into two groups: one that would undergo surgery (n=16) and one that would not undergo surgery (n=14). Within these two groups, mice were treated with VEH or AD. No mice died during surgery.

All the young females that did not have a MI survived throughout the experiment, as did the ones which had a MI and the AD treatment (Figures 26A, 27A). The young females which had surgery and the VEH treatment survived except for one, bringing the survival rate for the “YG F MI VEH” group down to 87.5% (7 out of 8 survived) (Figure 27A). In terms of body weight, the surgery had a significant impact on body weight loss after 3 days ($p=0.02$). The young females which did not undergo surgery lost less than 1% of their initial body weight while those which had a MI lost 3 to 5 times that (Figures 26B, 27B). The treatment had no significant impact on BW loss in the case of young females. Although the trend is similar between the no-MI and MI groups for both males and females, the males were significantly more affected than the females in both cases ($p<0.001$).

To better characterize the effect of the surgery and treatment on the immune and GI functions of the mice, the weights of the spleen and ceca were noted and normalized to body weight for an accurate comparison. The spleen weight was not significantly impacted by the treatment in both the MI and no-MI groups, but the surgery did have a significant effect on spleen weights ($p=0.002$) (Figures 26C, 27C). In fact, the spleen of the “YG F MI” group was consistently higher than the spleen of the “YG F no-MI” group, regardless of the treatment. The cecum weights followed the same trend: the treatment had no significant impact while the surgery did ($p<0.001$) (Figures 26D, 27D). In comparison with the young males, the spleen were consistently and significantly higher for females than males within the same surgery and treatment groups ($p<0.001$).

Finally, the weights and number of fecal pellets collected on D-7 and D3 were compared to further assess the GI function. There was no significant difference between the MI and no-MI groups as well as between the VEH and AD groups (Figures 26E, 26F, 27E, 27F).

ii. Impact of MI and treatment on immune cell numbers and infiltration

Compared to young males, immune cell numbers in the spleen and bone marrow of young females were considerably less impacted by surgery or treatment. In the spleen, both types of monocytes, as well as T cells were affected by surgery or treatment. $Ly6C^{high}$ monocytes were impacted by surgery. Young females which underwent surgery had more of these monocytes in their spleen compared to the “no-MI” mice (Figure 28A). On another hand, $Ly6C^{low}$ monocytes were impacted by both surgery and treatment in that they were more abundant in VEH mice which did not undergo surgery than in “AD no-MI” mice and VEH mice which had a MI (Figure 28B). Finally, T cells were significantly impacted by surgery within the AD groups. T-cells were downregulated in AD mice which had a MI compared to AD mice which did not have surgery (Figure 28C). Other cells for which no significant difference was seen between groups are shown in Tables 9 and 10.

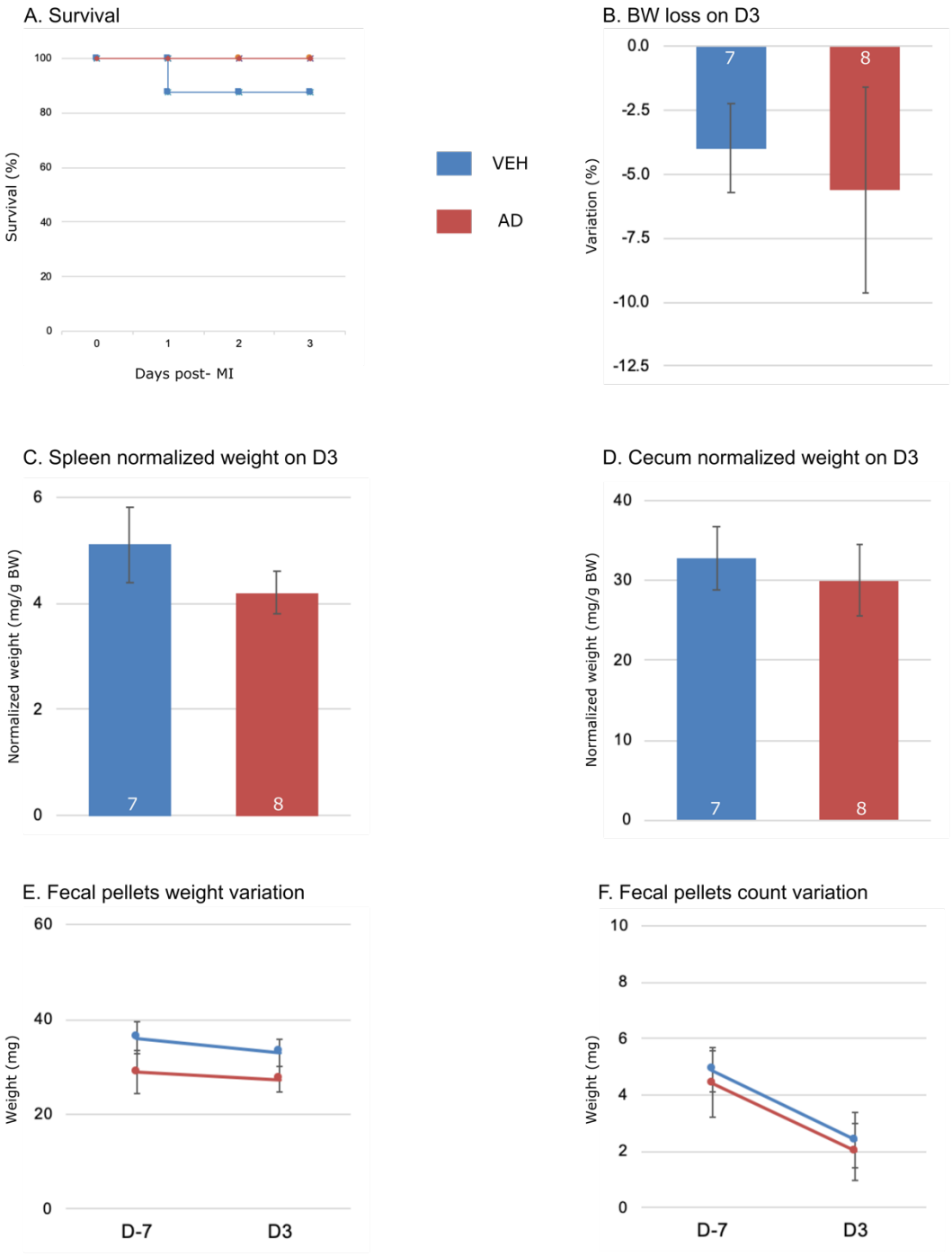


Figure 26 - Physiological data for young females with surgery (VEH & AD).
Impact of the VEH and AD treatments on the physiological parameters of young females which underwent surgery. A, Survival rate of mice after surgery. B, Body weight variation from D0 to D3. C, Spleen normalized weight per body weight on D3 following euthanasia. D, Cecum normalized weight per body weight on D3 following euthanasia. E, Fecal pellets weight variation between D-7 and D3. F, Fecal pellets count variation between D-7 and D3. N=8 for the VEH group and N=8 for the AD group.

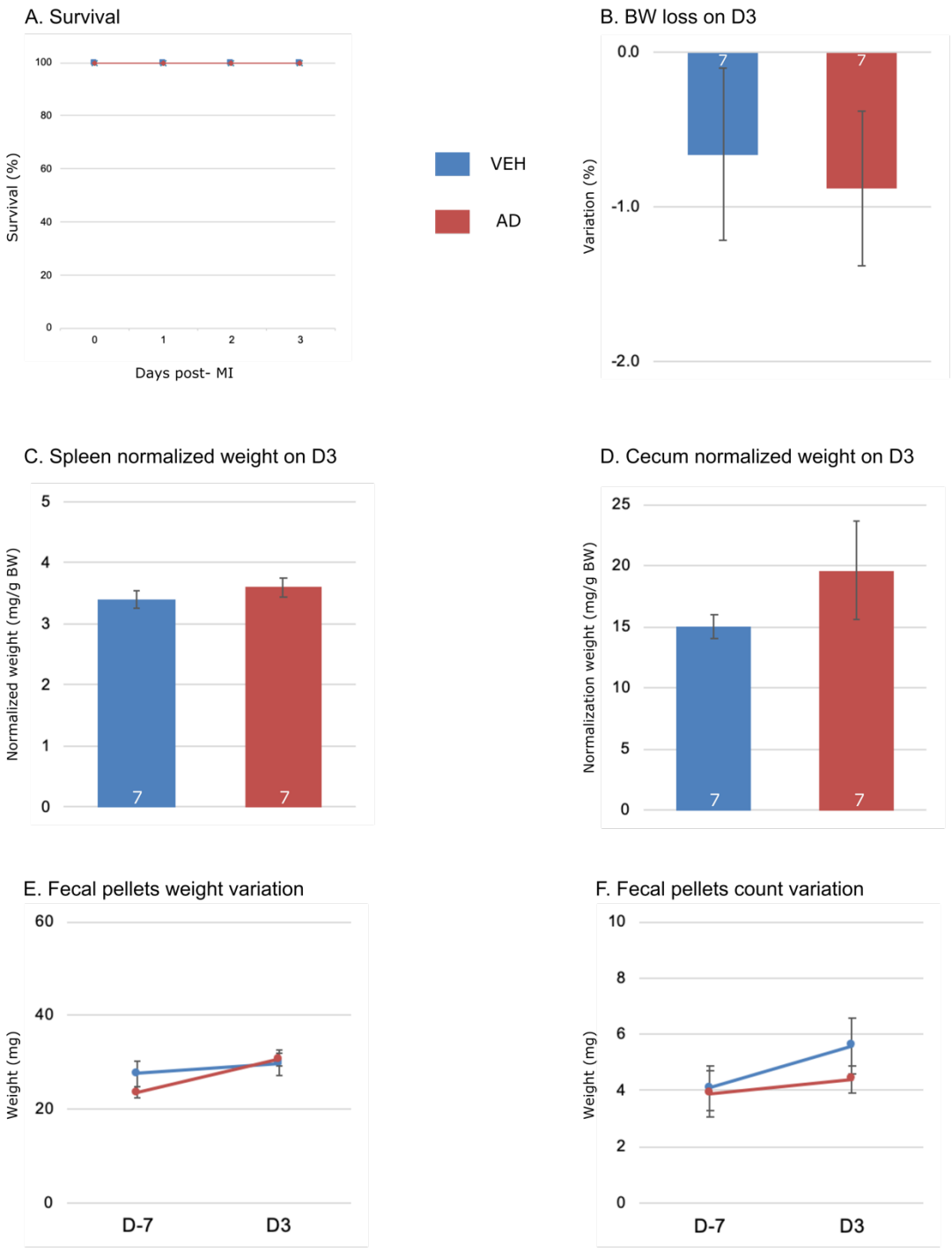


Figure 27 - Physiological data for young females without surgery (VEH & AD).
Impact of the VEH and AD treatments on the physiological parameters of young females which did not undergo surgery. A, Survival rate of mice after surgery. B, Body weight variation from D0 to D3. C, Spleen normalized weight per body weight on D3 following euthanasia. D, Cecum normalized weight per body weight on D3 following euthanasia. E, Fecal pellets weight variation between D-7 and D3. F, Fecal pellets count variation between D-7 and D3. N=7 for the VEH group and N=7 for the AD group.

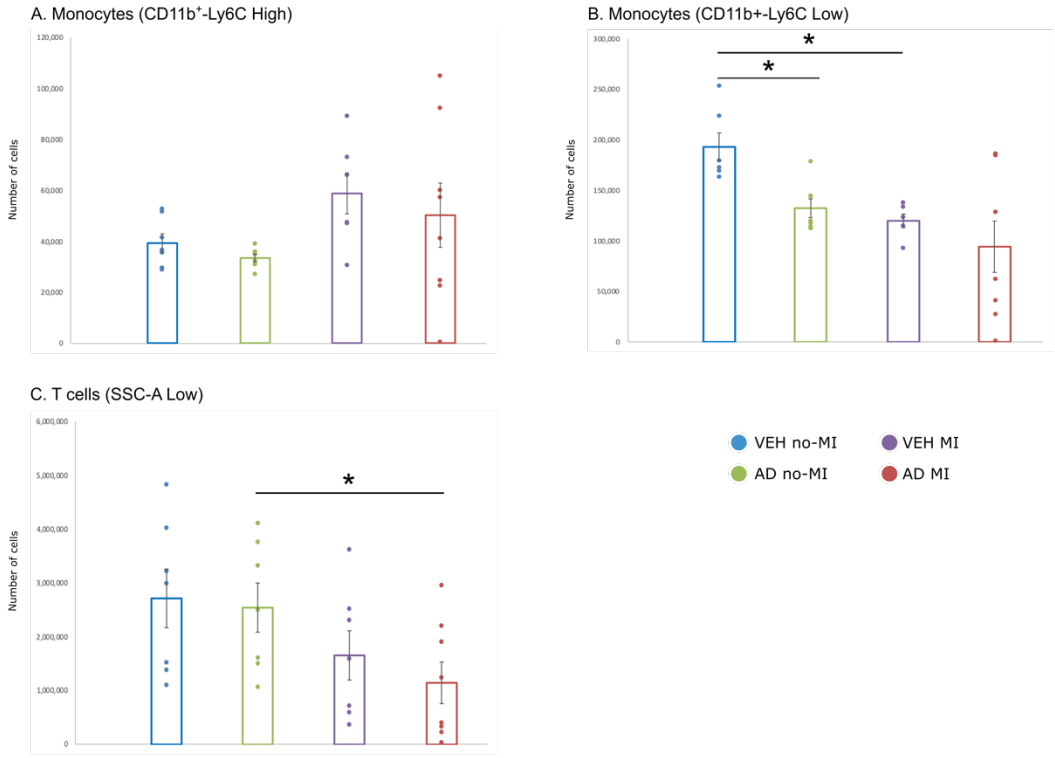


Figure 28 - Impact of surgery and treatment on immune cell numbers in the spleen of YG females.
Impact of surgery and treatment on the immune cell numbers in the spleen of young females. A, Monocytes (CD11b$^+$Ly6C-high) count per spleen. B, Monocytes count (CD11b$^+$Ly6C-low) per spleen. C, T cells (SSC-A low) per spleen. Each dot represents an individual mouse. The bars show the averages and standard error of mean for each group. * denotes a statistically significant difference between two groups. A p value of <0.05 was considered significant.

Table 9 - Immune cell numbers in YG female spleen.
Average counts and standard error of mean (SEM) in spleen of YG females.

Cell types	Group	Average number of cells
Macrophages (CD64+MerTK+)	VEH no-MI	7,799,036 ± 1,338,004
	AD no-MI	6,561,133 ± 756,716
	VEH MI	6,458,843 ± 2,090,244
	AD MI	4,578,201 ± 1,462,341
Dendritic cells (MHC II$^+$)	VEH no-MI	33,090 ± 8,127
	AD no-MI	30,187 ± 4,558
	VEH MI	21,498 ± 6,523
	AD MI	18,774 ± 5,452
Monocytes (CD11b$^+$)	VEH no-MI	6,028,309 ± 1,067,143
	AD no-MI	4,987,661 ± 598,096
	VEH MI	4,506,676 ± 1,289,415
	AD MI	3,373,616 ± 1,064,081
Neutrophils (CD11b$^+$-Ly6G$^+$)	VEH no-MI	104,250 ± 12,409
	AD no-MI	114,386 ± 4,307
	VEH MI	117,607 ± 26,825
	AD MI	91,023 ± 23,005
B cells (SSC-A high)	VEH no-MI	3,400,392 ± 611,430
	AD no-MI	2,521,322 ± 192,823
	VEH MI	2,901,414 ± 860,026
	AD MI	2,257,934 ± 690,632

Table 10 - Immune cell numbers in YG female bone marrow.
Average counts and standard error of mean (SEM) in bone marrow of YG females.

Cell types	Group	Average number of cells
Macrophages (CD64+MerTK+)	VEH no-MI	1,283,974 ± 118,425
	AD no-MI	1,711,341 ± 196,056
	VEH MI	1,335,323 ± 259,047
	AD MI	1,177,053 ± 116,555
Dendritic cells (MHC II$^+$)	VEH no-MI	133 ± 38
	AD no-MI	102 ± 23
	VEH MI	84 ± 13
	AD MI	80 ± 17
Monocytes (CD11b$^+$)	VEH no-MI	534,539 ± 56,819
	AD no-MI	630,876 ± 67,616
	VEH MI	469,082 ± 94,688
	AD MI	444,083 ± 45,896
Monocytes (CD11b$^+$-Ly6C High)	VEH no-MI	47,308 ± 12,474
	AD no-MI	57,007 ± 13,836
	VEH MI	40,441 ± 10,824
	AD MI	34,360 ± 2,402
Monocytes (CD11b$^+$-Ly6C Low)	VEH no-MI	134,572 ± 41,270
	AD no-MI	151,382 ± 25,050
	VEH MI	153,681 ± 27,930
	AD MI	158,410 ± 17,465
Neutrophils (CD11b$^+$-Ly6G$^+$)	VEH no-MI	460,346 ± 41,480
	AD no-MI	630,089 ± 45,962
	VEH MI	553,645 ± 69,165
	AD MI	482,701 ± 39,992

H. **Histology**

In order to determine the structural differences in mice which undergo surgery and are exposed to AD, samples of spleen and intestines were collected after euthanasia, embedded in paraffin and stained with hematoxylin/eosin. Images obtained using a dissecting microscope allowed for a detailed analysis of their structural properties.

i. Marginal zone in spleen is affected by surgery and treatment

Structural properties of the spleen were analysed as a means to better understand the link between the AD treatment and any systemic inflammation that it may cause. In the case of cardiac surgery, immune cells originate mostly from the bone marrow and the spleen. Monocytes and macrophages can proliferate in both, while T cells and B cells originate from the spleen (151, 152). The histology of the spleen offers information about the number of follicles, their area, and the area of white pulp and marginal zone which can be linked to the spleen and immune system's functions.

Images of stained spleen sections from young males are shown in Figure 29 along with data about the number of follicles, white pulp and marginal zone. The images show a number of follicles with the white pulp and marginal zone with no difference found between surgery and treatment groups (Figures 29A, B, C & D). Further analysis showed that there is no significant difference in the number of follicles or percentage of white pulp in spleen of mice which underwent surgery or were treated with AD compared to VEH mice (Figures 29E & F). However, the marginal zone was impacted significantly by both surgery and treatment. Here, "VEH MI" young males had three times more marginal zone than "VEH no-MI" and "AD MI" mice (Figure 29G).

These findings are similar to those for retired breeder males for which no significant difference was seen on the images, in the number of follicles or the percentage of white pulp (Figure 30). The marginal zone was significantly impacted only by the surgery within the VEH groups with the "VEH MI" spleen containing 20% of marginal zone compared to the "VEH no-MI" mice's 10% (Figure 30G).

As for the females, images from both young and retired breeder females did not show a demonstrable structural impact for surgery or treatment (Figures 31 & 32). Also, the number of follicles in both groups were not significantly affected by surgery or treatment. For the young females, the white pulp percentage was lower following surgery within the VEH groups. The marginal zone in young female spleen was significantly impacted by both surgery and treatment. Here, surgery induced an increase in the percentage of marginal zone for both treatments (VEH and AD) and the AD treatment induced an increase of marginal zone percentage within the MI groups (Figure 31G). The white pulp in retired breeder female spleen was not impacted by surgery or treatment whereas the marginal zone was increased after surgery in both treatment groups (Figure 32G).

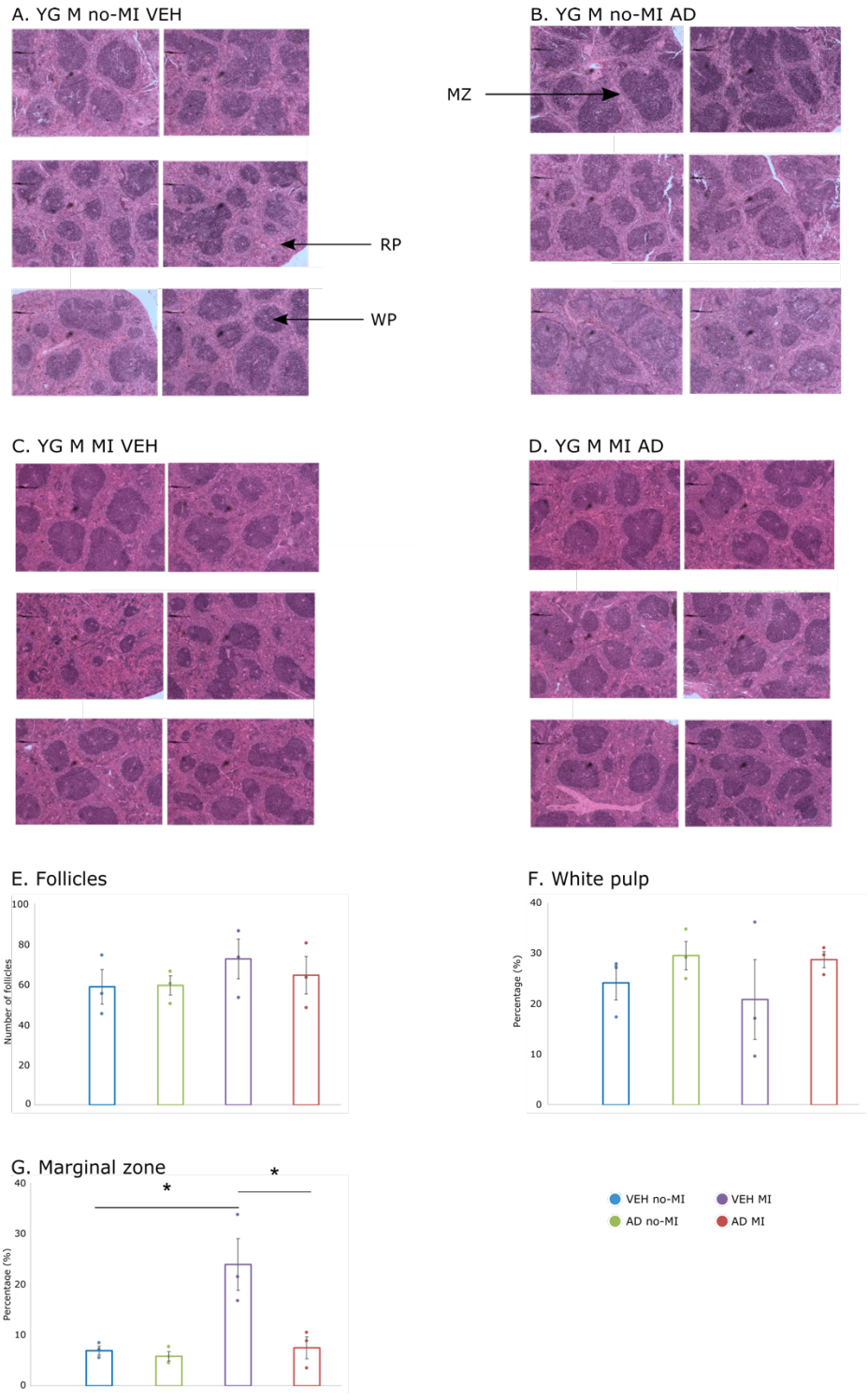


Figure 29 - Spleen histology for young males.
Histology slides and data for YG males. A, Spleen of 3 mice (YG M no-MI VEH) stained with H/E at 8x magnification. B, Spleen of 3 mice (YG M no-MI AD) stained with H/E at 8x magnification. C, Spleen of 3 mice (YG M MI VEH) stained with H/E at 8x magnification. D, Spleen of 3 mice (YG M MI AD) stained with H/E at 8x magnification. E, Total number of follicles per spleen for each of the 4 groups of YG males. F, Percentage of white pulp per spleen for each of the 4 groups of YG males. G, Percentage of marginal zone per spleen for each of the 4 groups of YG males. A *p* value <0.05 was considered significant.

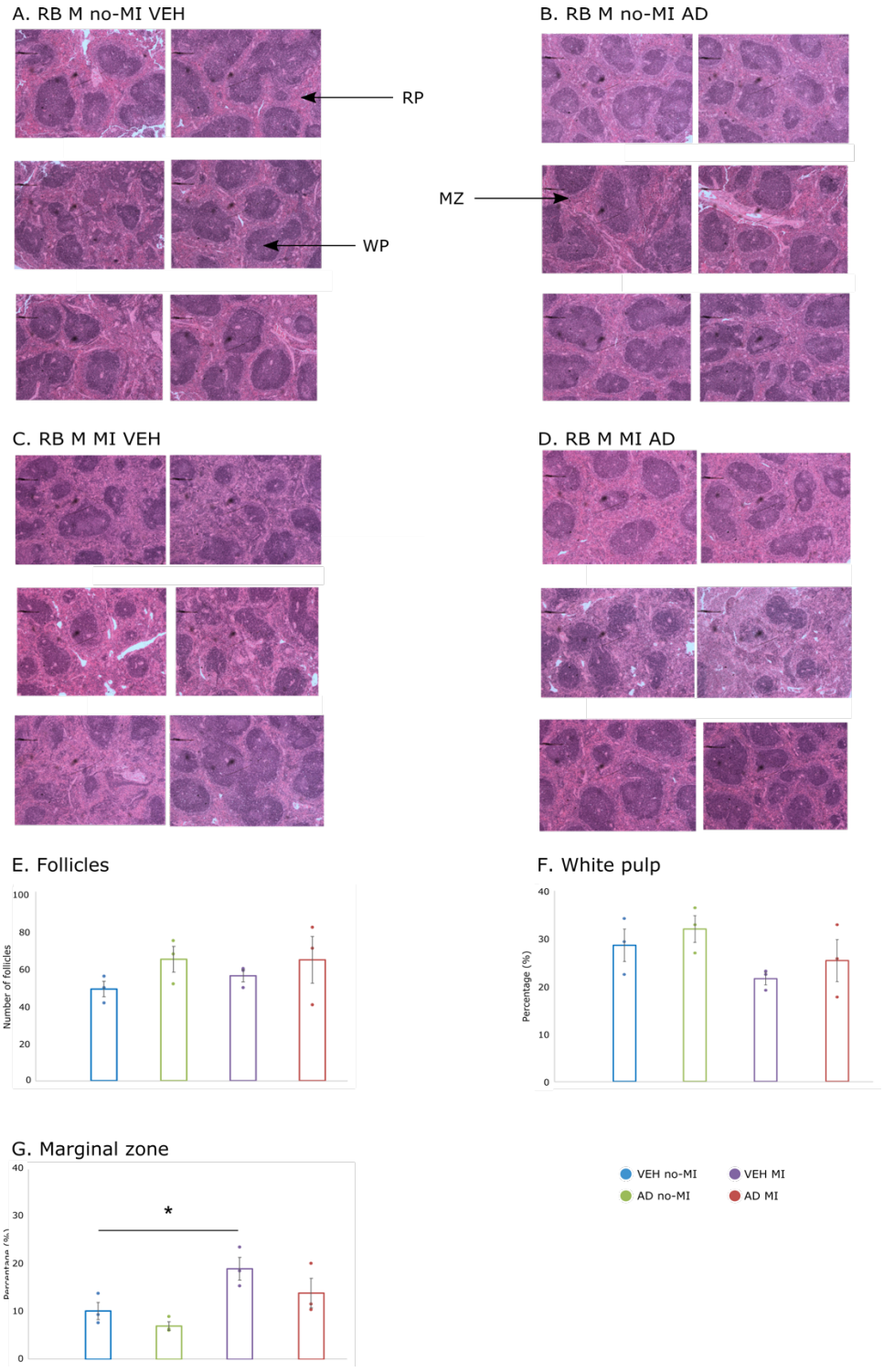


Figure 30 - Spleen histology for retired breeder males.
Histology slides and data for RB males. A, Spleen of 3 mice (RB M no-MI VEH) stained with H/E at 8x magnification. B, Spleen of 3 mice (RB M no-MI AD) stained with H/E at 8x magnification. C, Spleen of 3 mice (RB M MI VEH) stained with H/E at 8x magnification. D, Spleen of 3 mice (RB M MI AD) stained with H/E at 8x magnification. E, Total number of follicles per spleen for each of the 4 groups of RB males. F, Percentage of white pulp per spleen for each of the 4 groups of RB males. G, Percentage of marginal zone per spleen for each of the 4 groups of RB males. A *p* value <0.05 was considered significant.

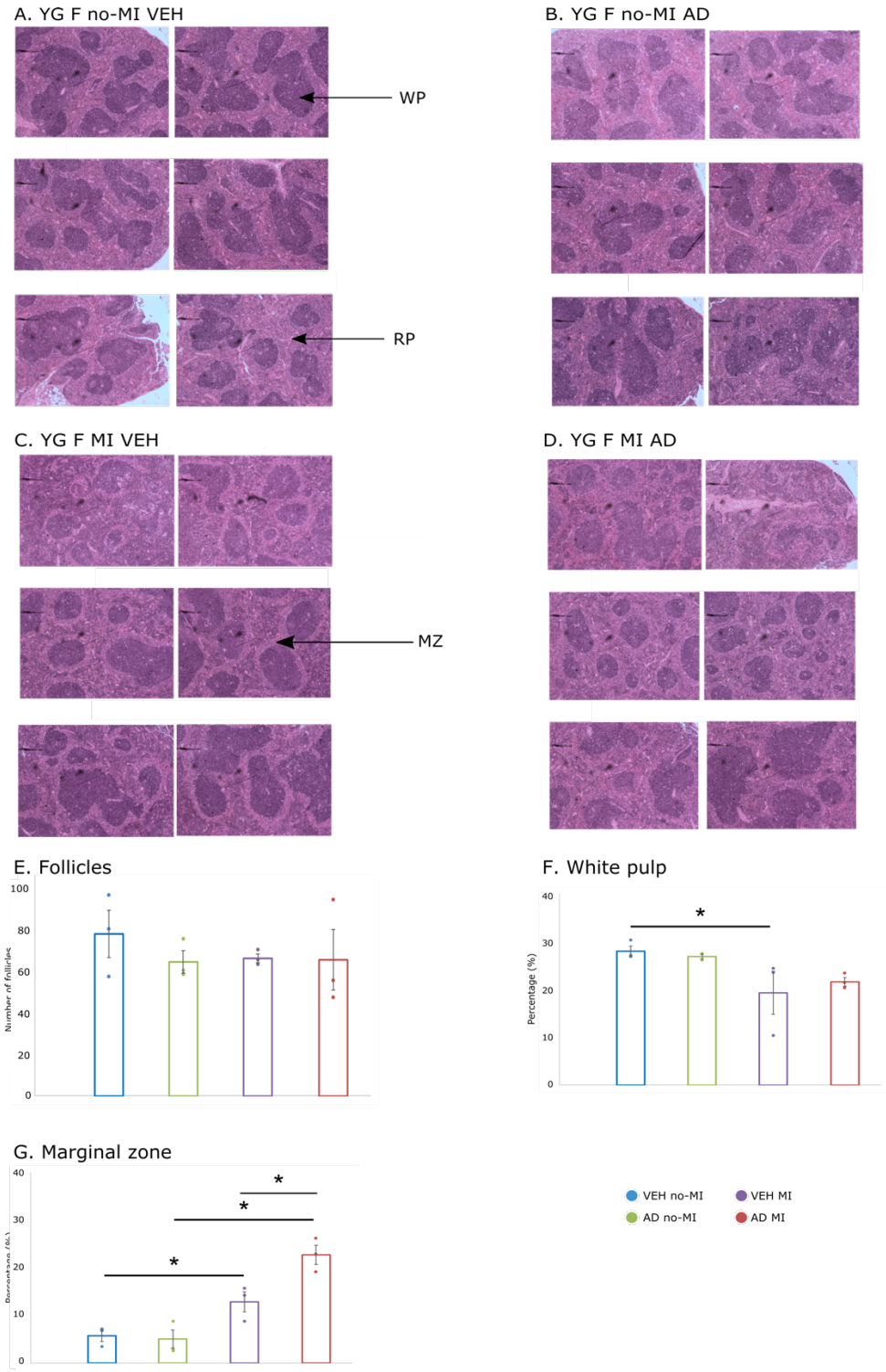


Figure 31 - Spleen histology for young females.
Histology slides and data for YG females. A, Spleen of 3 mice (YG F no-MI VEH) stained with H/E at 8x magnification. B, Spleen of 3 mice (YG F no-MI AD) stained with H/E at 8x magnification. C, Spleen of 3 mice (YG F MI VEH) stained with H/E at 8x magnification. D, Spleen of 3 mice (YG F MI AD) stained with H/E at 8x magnification. E, Total number of follicles per spleen for each of the 4 groups of YG females. F, Percentage of white pulp per spleen for each of the 4 groups of YG females. G, Percentage of marginal zone per spleen for each of the 4 groups of YG females. A p value <0.05 was considered significant.

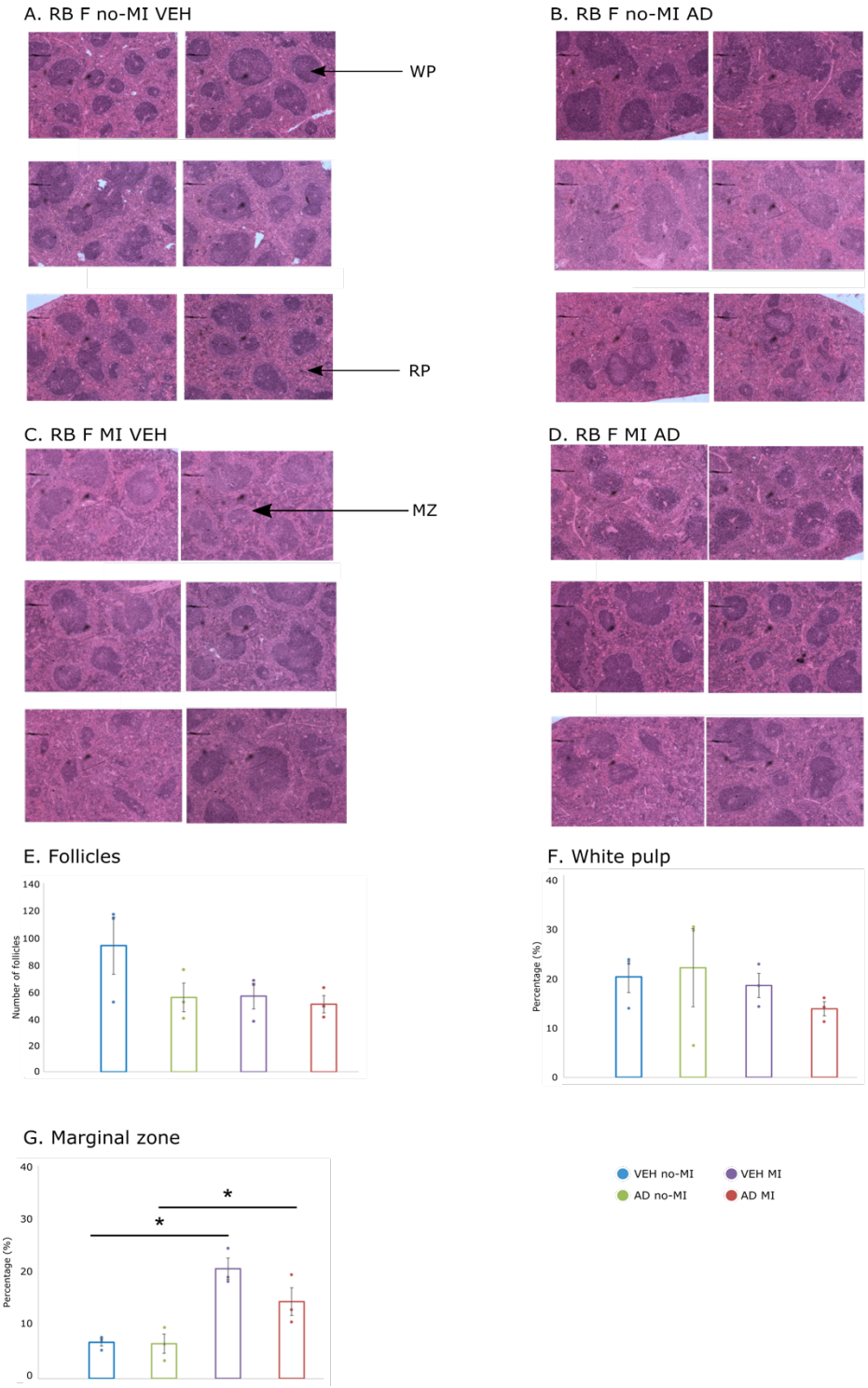


Figure 32 - Spleen histology for retired breeder females.

Histology slides and data for RB females. A, Spleen of 3 mice (RB F no-MI VEH) stained with H/E at 8x magnification. B, Spleen of 3 mice (RB F no-MI AD) stained with H/E at 8x magnification. C, Spleen of 3 mice (RB F MI VEH) stained with H/E at 8x magnification. D, Spleen of 3 mice (RB F MI AD) stained with H/E at 8x magnification. E, Total number of follicles per spleen for each of the 4 groups of RB females. F, Percentage of white pulp per spleen for each of the 4 groups of RB females. G, Percentage of marginal zone per spleen for each of the 4 groups of RB females. A p value <0.05 was considered significant.

I. Echocardiography

Finally, the treatment's effect on heart function was assessed by performing echocardiography on VEH and AD mice which underwent surgery. Echocardiography was performed for 51 mice which previously underwent cardiac surgery. 25 of them were treated with VEH oil and 26 were treated with AD. 23 were males and 28 were females.

Data was analyzed using the short and long axes. The measurements of interest for the long axis are the LV volume in systole and diastole, the LV area in systole and diastole, the stroke volume, the ejection fraction and the cardiac output. As for the short axis, the only parameter of interest is the FAC.

Table 11 shows the average values obtained for each of the parameters, in each of the 4 groups (M VEH, M AD, F VEH, F AD). Statistical analysis shows that there is no significant difference between any of the groups for the stroke volume, the ejection fraction or the cardiac output. The LV volume in systole was significantly smaller in females compared to males within VEH groups and the AD treatment significantly increased it within the females. The LV volume in diastole, as well as the area, were significantly smaller in the F VEH compared to M VEH, and smaller in the AD males compared to VEH males. Finally, the FAC was only significantly different between treatment groups and not between the sexes. Specifically, it was bigger in AD males compared to VEH males, and smaller in AD females compared to VEH females.

Table 11 - Average values for different parameters obtained by echocardiography.

Average values for cardiac measurements obtained by analysis of echocardiography data for mice which underwent surgery. A two-way ANOVA, followed by the Student Neuman-Keuls method, were performed to determine significant differences between sex and/or treatment groups. A p value < 0.05 was considered significant. * indicates a significant difference with M VEH. † indicates a significant difference with M AD. ‡ indicates a significant difference with F VEH.

	M VEH	M AD	F VEH	F AD
LV volume (systole) (µL)	88.54 ± 6.51	71.86 ± 6.52	53.91 ± 4.65 *	70.48 ± 5.01 ‡
LV volume (diastole) (µL)	114.31 ± 8.56	92.41 ± 5.80 *	74.33 ± 5.33 *	84.11 ± 5.03
Area (mm^2)	28.73 ± 1.70	24.25 ± 1.37 *	22.21 ± 1.61 *	24.99 ± 1.11
Stroke volume (µL)	25.77 ± 6.53	20.55 ± 4.10	20.42 ± 3.69	13.63 ± 2.78
Ejection fraction (%)	20.94 ± 4.75	22.74 ± 4.73	27.20 ± 4.84	16.25 ± 3.00
Cardiac output (mL/min)	13.38 ± 3.40	10.38 ± 2.06	10.47 ± 1.88	7.15 ± 1.49
FAC (%)	12.63 ± 1.97	20.11 ± 2.94 *	16.02 ± 1.30	13.44 ± 1.89 †

IX. Discussion

A. DEHP and BPA induce sex-specific alterations in the gut microbiome

The results of the bacterial 16S rRNA sequencing indicate that the MI surgery and treatment with AD induce gut dysbiosis in RB males and RB females. Notably, a sex-specific effect was detected whereby females seem to be less impacted by the MI and AD in terms of gut microbiota.

The *F/B* ratio is an established indicator of GI and even systemic health (110). This ratio is highly conserved and an imbalance towards any of the two phyla can lead to different diseases and pathologies (113). In RB males, we observed an increase in the *F/B* ratio following the treatment in the absence of an MI. This is due to the significant decrease in the abundance of *Bacteroides vulgatus* detected in the mice which were exposed to AD. The increase of the *F/B* ratio indicates that the exposure to AD alone causes dysbiosis (105).

In VEH RB mice which had undergone an MI, we detected a decrease in the *F/B* ratio due to a significant colonization of the microbiome by *Akkermansia muciniphila* (a *Verrucomicrobium*) at the expense of *Firmicutes*. Overall, our findings indicate surgery causes dysbiosis in the guts of male mice by decreasing the *F/B* ratio and promotes the colonization of the microbiome by *Akkermansia*. These mouse data mirror findings in a recent study on patients who experienced acute MI and showed a decrease in the *F/B* ratio and an increase of *Akkermansia* abundance (153).

In addition, our data suggest that exposure to AD also influences the microbiome by increasing the *F/B* ratio and decreases the abundance of *Bacteroides vulgatus*. To date no studies have assessed concomitant exposure and have only reported dose-dependent results for BPA-only or DEHP-only exposures in regard to microbiome alterations. In contrast to our findings which use a BPA dose of 0.4 µg/kg/day, BPA exposure at higher doses (up to 25 µg/kg/day) was linked to increased abundance of pro-inflammatory bacteria such as *Bacteroides* and an increase in the *F/B* (116, 154, 155). However, these findings have been reported for high doses of BPA, as

opposed to the low-dose setting that was used for our purposes. Others found that exposure to lower doses of BPA caused a decrease in *Bacteroides* spp. and is consistent with our own results (156). On another hand, DEHP induced an increase in the *F/B* ratio in studies on rats and mice (118, 157). This is consistent with our own findings on the treatment effects suggesting that this is a uniform consequence with DEHP exposure. Another study found that DEHP caused an increase in *Akkermansia*'s abundance (158). This finding is also consistent with our results obtained from both DNA sequencing and metabolome analyses. The DNA sequencing showed a considerable colonization of the gut by *Akkermansia muciniphila* following surgery, and that seems to be an important element in the dysbiosis caused by the MI. Further, our microbial pathway analysis determined that mucin-o-glycan degradation is significantly upregulated following AD treatment, which directly correlates with the increase in *Akkermansia*'s abundance (159).

In RB females, *Akkermansia* was abundant in all groups regardless of the treatment or surgery. Uniquely, *Klebsiella* (*Proteobacteria*) was found in RB females which had been exposed to AD. The *F/B* ratio was similar in all groups except for the "AD MI" group in which *Firmicutes* was reduced in abundance relative to *Klebsiella* and *Akkermansia*. Studies have linked an increase in *Proteobacteria* abundance to BPA exposure (116, 160). The apparent resistance to endocrine disruptors and MI in the female gut, mirrored in the stability of the *F/B* ratio, has also been reported in several studies (158, 161). It is speculated that reduced androgen levels can alleviate gut alterations and vulnerability. This further confirms the sex-specific responses to treatment and myocardial infarction, and the increased vulnerability of males compared to their female counterparts.

B. **DEHP+BPA treatment causes reduced body weight loss in RB males**

All the mice lost body weight throughout the experiments, regardless of surgery and treatment group. This is expected as a result of social isolation one week before the start of the treatment (162, 163). In addition, all mice which had a MI lost more BW than the corresponding non-surgery group, which is also expected as a result of the cardiac surgery (164). However, a significant BW change due to treatment was seen in RB males. Here, the AD treatment prevented a decrease in BW compared to the VEH group. This is consistent with studies that reported increased BW in mice and humans exposed to both BPA and DEHP (165, 166). The reduced BW loss that was reported in those studies was relevant for low-dose BPA and high-dose DEHP exposures, which is similar to our experimental model. Interestingly, exposure to high-dose BPA did not increase the BW, indicating that BPA might have non-monotonic properties (167).

BW and gut microbiota changes are linked. An increased abundance of *Lachnoclostridium* spp. is linked to reduced body weight (168). Consistent with this finding, we found increased *Lachnoclostridium* spp. in “RB M MI VEH” group which lost more BW than the AD cohort mice. A corollary to this suggests that exposure to AD decreases the abundance of *Lachnoclostiridum* spp. Then again, *Akkermansia* and *Prevotella*, which were also upregulated in AD mice, were associated with body weight decrease in obese mice, which contradicts our data (169-171). However, these findings focused on gut bacterial presence in obese mice, and did not involve surgery or AD exposure. Therefore, more research is needed to fully characterize the role of the microbiome in both weight loss following surgery and weight conservation following BPA or DEHP exposure. The upregulated metabolites identified through metabolomics analyses did not relate in any way to body weight, so further studies need to be carried out to determine how these metabolites can affect body weight fluctuations.

Aside from BW, cecum weight was noted upon euthanasia as an indicator of GI function as well as higher microbial content (172). No significant difference was noted between the VEH and

AD mice in any of the groups, so we conclude that cecum weight cannot be used to characterize the effects of acute exposure to low-dose BPA and high-dose DEHP.

Finally, fecal pellets were collected and weighed before and after the treatment and surgery, also as an indicator of GI function. There was a trend in all groups towards smaller and fewer pellets, which suggests reduced food intake as a result of the MI. To date, no studies have reported reduced fecal pellet production and weight as a result of BPA or DEHP exposure.

C. **Spleen analyses suggest anti-inflammatory properties to AD exposure**

Immune cells from the spleen and bone marrow migrate to a wound. Once there, they remove debris and act to begin healing. Here, we analyzed immune cells in the spleen and bone to determine if the exposure to BPA and DEHP initiates an immune reaction and prompts immune cells to proliferate in the spleen and bone marrow.

Most significant differences were due to surgery, which is expected in the presence of the wound. In the absence of a MI, we found that the treatment tends to reduce the number of immune cells in the spleen and bone marrow. Moreover, significant differences in different immune cells' proliferation were also seen following the AD treatment. In young males, macrophages were significantly fewer in the spleen of AD mice compared to VEH mice with and without an induced MI. This correlates with the spleen weight variation for the "YG M MI" group, in which the spleen were significantly smaller following AD treatment. In fact, low doses of BPA similar to ours, were found to cause a decrease in spleen weight (173). This effect was not seen following exposure to high doses, highlighting a non-monotonic property to BPA (173). In addition, a study performed on C57BL/6 male mice reported that acute exposure to high doses of DEHP caused a pronounced atrophy of the spleen, which agrees with our findings (174). DEHP is a highly potent peroxisome proliferator and acts through the peroxisome proliferator-activated receptor α (PPARα). PPARα has anti-inflammatory properties which can explain the reduced inflammation and macrophage

proliferation following the treatment (175). A similar result was obtained with young females, in which spleen monocytes were downregulated following AD treatment.

Histological analyses of spleen provided additional insight. The spleen follicle contains B and T-cells in the white matter and macrophages and dendritic cells in the marginal zone (176). No significant differences were detected due to the treatment in RB mice. In contrast, the size of the marginal zones of young males and females were different. The marginal zone was significantly larger in females and smaller in males following the treatment. This suggests that the spleen was exposed to more antigen, but no literature describes what the area of the marginal zone indicates. It is important to note that the spleen histology was only analyzed for 3 mice per group, and that the results are only preliminary and will require more samples and further analysis.

D. **Cardiac function following treatment**

Echocardiography was performed on mice which had a MI in order to characterize the effect of the treatment on cardiac function following surgery. Several parameters were measured, and statistical analyses showed some differences due to treatment. Data suggest that AD treatment following surgery promotes LV dysfunction in both males and females, although females seem to be less affected.

In the males, the LV volume during diastole, the area and the FAC were significantly affected by the treatment. The LV volume in diastole and area decreased in AD treated mice, while the FAC increased in AD after the treatment in comparison with VEH. These findings are in contrast with previous studies which reported that DEHP and BPA impair cardiovascular functions (177). In fact, DEHP exposure from medical devices was linked to disruptions in heart electrophysiology, autonomic regulation, heart rate variability, and cardiovascular reactivity (178, 179). We have previously reported that DEHP increases cardiac dilation and reduces heart function (95, 180). The differences detected can be attributed to higher levels of DEHP exposure in the current study.

Here, the 5-fold increase in DEHP used was to reflect the maximum rather than average exposure in human patients.

In females, AD treatment only significantly contributed to an increase in LV volume in systole. All other parameters were not significantly affected by the treatment. This increase in LV volume was not seen in females in our previous studies, although the doses of DEHP and BPA were different (180). This suggests that BPA and DEHP have non-monotonic properties and that their effects are dose-specific.

E. **Highly dose-specific effects highlight non-monotonic effects of AD**

Analysis of our data and comparison to existing literature on the effects of BPA and DEHP exposures suggest that they are highly dose-dependent. In fact, higher doses of those endocrine disruptors do not seem to necessarily cause more pronounced adverse effects, and may sometimes be more beneficial than lower, more potent doses. Several studies have investigated endocrine disruptors' non-monotonic properties in order to have a more accurate risk assessment for those substances (138, 181). Our data found improved cardiac function and reduced NLRP3 inflammasome activity in YG male mice exposed to reduced versus higher doses of phthalates (180). Yet, inflammatory cell infiltration into the hearts was augmented in mice exposed to the lower levels. These data indicate not all parameters may be altered similarly and highlight the complexity of dose:phenotype comparisons.

A large study on BPA exposure in rats reported mammary adenocarcinomas only in rats exposed to low doses of BPA (21). This non-monotonic tendency was confirmed by other studies on rats and mice (173, 182, 183). DEHP was also linked to non-monotonic properties in several studies on rodents, and findings suggest that the effects are also sex-specific, meaning that the dose-response curve is different according to sex (136, 138, 184). However, some conflicting results emerged in different studies and the non-monotonic manner in which BPA and DEHP function remains unclear and varies between species and systems within each individual (138).

We observed several instances where our data highlight BPA and DEHP's non-monotonic properties. In fact, it seems that body weight, spleen weight, cardiac function and inflammation are highly dependent of the dose of BPA or DEHP to which they are exposed.

F. **Conclusion**

Our study investigated the effects of BPA and DEHP at doses seen in patients in a hospital setting. We focused on low doses of BPA and high doses of DEHP as seen previously in patients of cardiac surgery.

We observed significant effects of the exposure in gut microbiome composition characterized by the change in *F/B* ratio. This is a concern in that the ratio is an indicator of intestinal and systemic homeostasis and alterations are associated with several pathologies and diseases (113). Both increases and decreases have been linked to metabolic, cardiovascular or digestive impairments (110, 111, 113). On another hand, the body weight measurements indicated that the doses of BPA and DEHP used in our study were beneficial by either promoting weight gain or preventing weight loss. This is conflicting compared to previous studies where we exposed mice to lower doses of DEHP and no BPA, indicating that DEHP might have a non-monotonic effect on body weight. Another indication of BPA and DEHP's non monotonic properties is the reduced inflammation following the exposure, coupled with a reduced spleen weight. These findings were also inconsistent with other studies' results in which the doses used were inferior to our parameters.

In conclusion, our data support our hypothesis that BPA and DEHP at the mentioned doses induce gut dysbiosis in a sex-specific manner, and that males are consistently more affected than females. In addition, our results support the hypothesis that the adverse effects of BPA and DEHP are highly dose-dependent, highlighting their non-monotonic properties. The ambiguity and complex manner in which BPA and DEHP can affect patients make it hard to fully characterize them and is another reason to work towards a safer alternative to produce medical equipment.

In the future, this study could be complemented by additional DNA sequencing and metabolomics analyses of the fecal pellets extracted before and after the treatment, which will allow us to compare the changes in gut microbiota composition and metabolic profile within the same individuals and characterize the effects of the exposure. In addition, the study can also be followed by experiments to determine how the AD-exposed gut microbiome influences the gut's permeability by using FITC dextran assays for instance.

G. **Limitations**

Some limitations should be noted in the study. First, we identified that BPA and DEHP act in a sex-specific manner but can only speculate that this is due to hormonal differences. Tests on mice which underwent gonadectomy might be useful to explain the phenomenon. In addition to that, we only performed metabolome analyses and microbiome sequencing on RB mice, and it will be useful to also analyze the data from YG mice to better characterize the effects of BPA and DEHP exposures.

X. References

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