Transgenerational effects of gestational exposure to a real-life environmental chemical mixture on adult cardiac function

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I would like to dedicate this work to my beloved parents and my grandmother (recently passed away), whose prayers have always been a driving force behind my every success. This piece of work is also dedicated to my wife for her moral support and my little one for bringing joy to our lives.

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Abstract

Globally, cardiovascular diseases (CVD) persist to be the leading cause of death with an increased incidence of CVD related mortality and morbidity in men compared to women. It is increasingly recognized that adverse exposures during early development can result in adult onset of diseases including CVD. While traditional CVD risk factors including diabetes, obesity, hypertension and lack of physical activity are well-known, exposure to anthropogenic environmental chemicals (ECs) during critical period of development may also present a risk factor in the emergence of CVD in later life. Using a sheep model of translational value, the current research investigated sex-specific cardiac effects of developmental exposure to mixture of low-level chemicals by allowing pregnant sheep to graze on either inorganic fertilizer (C) or pastures treated with biosolids (B). At adult age, in-vivo cardiovascular studies followed by histological and molecular investigations were conducted in male and female offspring in the first (F1) and second generation (F2). Findings revealed sexually dimorphic changes in adult cardiovascular functioning. EC-exposed males in F1 exhibited increased left ventricular dimensions, higher diastolic, systolic and stroke volumes, and a higher cardiac output, suggestive of an eccentric left ventricular hypertrophy. In contrast to males, EC-exposed females in F1 displayed autonomic imbalance in the form of higher sympathetic dominance as indicated by an apparent pattern of lower heart rate variability metrics, including root mean square of successive differences (RMSSD) and standard deviation of normal-to-normal intervals (SDNN). Subsequent histological and molecular investigations identified an increased level of interstitial, perivascular and replacement fibrosis complimented by an increased mRNA expression of apoptosis (CASP3), inflammation (DYA, DRB1) and insulin signalling (IGF1, IGF1-R) in EC-exposed males but not in females. Males in the F2 presented increased wall thicknesses without an enlargement in ventricular dimensions and had increased mRNA expression of markers related to fibrosis, inflammation, and hypertrophy, indicative of concentric remodelling, while females exhibited an increase in COL1A1, a marker for fibrosis. Taken together, these findings suggest sexually dimorphic impacts of gestational EC mixture exposure on adult cardiac functioning with intergenerational consequences.

List of Publications

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List of Abbreviations

4-C	Four-Chamber View
AKT1	AKT Serine/Threonine Kinase 1
ANP	Atrial Natriuretic Peptide
ANS	Autonomic Nervous System
Ao	Aorta
AoS	Aortic Sinus
AR	Androgen Receptor
ASPA	Animals (Scientific Procedures) Act 1986
BAX	BCL2 Associated X Protein
BNP	B-type Natriuretic Peptide
BP	Blood Pressure
BPA	Bisphenol A
BPF	Bisphenol F
BPS	Bisphenol S
BTP	Biosolid treated pasture sheep model
CAD	Coronary Artery Disease
CASP3	Caspase-3
Cd	Cadmium
CDC	Center for Disease Control and Prevention
CHDs	Congenital Heart Diseases
CM	Cardiomyocyte
CO	Cardiac Output
COL1A1	Collagen Type I Alpha 1
COL3A1	Collagen Type III Alpha 1
CV	Cardiovascular
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di-(2-ethylhexyl) Phthalate
DES	Diethylstilbestrol
DOHaD	Developmental Origins of Health and Disease
EC	Environmental Chemical
ECG	Echocardiography
EDC	Endocrine-Disrupting Chemical
EDV	End-Diastolic Volume
EF / LVEF	Left Ventricular Ejection Fraction
ER	Estrogen Receptor
ERα	Estrogen Receptor Alpha
ERß	Estrogen Receptor Beta
ESV	End-Systolic Volume
F1	First Generation
F2	Second Generation
F3	Third Generation
FS	Fractional Shortening
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GPR30	G-Protein Coupled Estrogen Receptor 30
HBR	Heart-to-Body Weight Ratio

HF	Heart Failure
HF (HRV)	High-Frequency Band (Heart Rate Variability)
HPG	Hypothalamic-Pituitary-Gonadal Axis
HR	Heart Rate
HRV	Heart Rate Variability
iPSC-CM	Induced Pluripotent Stem Cell-Derived Cardiomyocyte
IGF1	Insulin-Like Growth Factor 1
IGF1-R	Insulin-Like Growth Factor 1 Receptor
IVS	Interventricular Septum
LA	Left Atrium
LAD	Left Atrial Diameter
LF (HRV)	Low-Frequency Band (Heart Rate Variability)
LF/HF	Low-Frequency to High-Frequency Ratio
LFWd	Left Ventricular Free Wall (Diastole)
LFWs	Left Ventricular Free Wall (Systole)
LV	Left Ventricle
LVH	Left Ventricular Hypertrophy
LVID	Left Ventricular Internal Diameter
LVDd	Left Ventricular Diameter in Diastole
LVDs	Left Ventricular Diameter in Systole
LVOT	Left Ventricular Outflow Tract
MABP	Mean Arterial Blood Pressure
MI	Myocardial Infarction
mTOR	Mechanistic Target of Rapamycin
MV	Mitral Valve
MVA	Mitral Valve Annulus
NHANES	National Health and Nutrition Examination Survey
NHBCS	New Hampshire Birth Cohort Study
NPPA	Natriuretic Peptide A
NPPB	Natriuretic Peptide B
PA	Pulmonary Artery
PAHs	Polycyclic Aromatic Hydrocarbons
PBDE	Polybrominated Diphenyl Ether
PCM	Pathological Cardiac Remodelling
PCB	Polychlorinated Biphenyl
PFAS	Per-and polyfluoroalkyl substances
PFOS	Perfluorooctane sulfonate
PFOA	Perfluorooctanoic Acid
PNS	Parasympathetic Nervous System
qPCR	Quantitative Polymerase Chain Reaction
RMSSD	Root Mean Square of Successive Differences
RVOT	Right Ventricular Outflow Tract
SA	Short-Axis View
SBP	Systolic Blood Pressure
SDNN	Standard Deviation of NN Intervals
SLC2A4	Solute Carrier Family 2 Member 4
SV	Stroke Volume
TC	Total Cholesterol
TGF-β	Transforming Growth Factor-Beta

TG	Triglycerides
TR	Thyroid receptor

Authors Declaration

I declare that, except where reference is made to the contribution of others, this represents my own work. I was responsible for collecting and analysing data, the interpretation of results, and writing-up this thesis.

The work contained herein has not been previously submitted to the University of Glasgow, or any other institution, for the award of any qualification.

_____ 13th January 2026

Noor Muhammad Khan

Chapter 1 Introduction

1.1 Cardiovascular diseases-a leading cause of death

Cardiovascular diseases (CVD), which comprises a group of related disorders including heart failure, stroke, ischemic heart disease, cardiomyopathy, peripheral arterial disease, and a number of associated cardiac and vascular conditions, is one of the leading causes of mortality worldwide and a major contributor towards reduced quality of life (Cooper, 2018; Roth et al., 2018). According to estimates from global burden of disease published in 2019, the prevalence of CVD has almost doubled (523 million in 2019) compared to 271 million in 1990, while CVD related mortality has increased from 12.1 million (1990) to 18.6 million (2019). Based on prediction of future trends in CVD, it is estimated that there will be a 90% increase in the prevalence of CVD between 2025-2050 as well as an increase 73.4% is predicted in crude mortality rate and a total of 35.6 million deaths anticipated in 2050 (Chong et al., 2025). These CVD related figures are worrisome and carry a huge financial and psychological impact both at global and household levels. Importantly, out of total CVD related deaths in 2019, 9.6 million deaths were reported in men while 8.9 million deaths were reported in women, indicating that there is a sexually dimorphic nature of CVD. While there has been an increase in deaths pertaining to CVD in women taking place per year, it should not be confused with age adjusted CVD mortality, which is still higher in men compared to women.(Mosca et al., 2011). The gender differences in CVD are widely discussed (Connelly et al., 2021; Gao et al., 2019; Zhou and Bei, 2020) and it is generally believed that these differences might originate from differences in endogenous steroid sex hormones (Barrett-Connor, 1997; Vitale et al., 2010). While women are considered to be protected by oestrogens in CVD related risk, this remains largely debated since hormone replacement therapies in postmenopausal women did not significantly improve the CVD outcome (Grady et al., 2002; Herrington, 1999; Reckelhoff, 2005). This highlights the importance of recognizing sex as a biological variable (SABV) and underscores the need to gain an understanding of the sex-specific mechanisms involved in the CVD risk pattern.

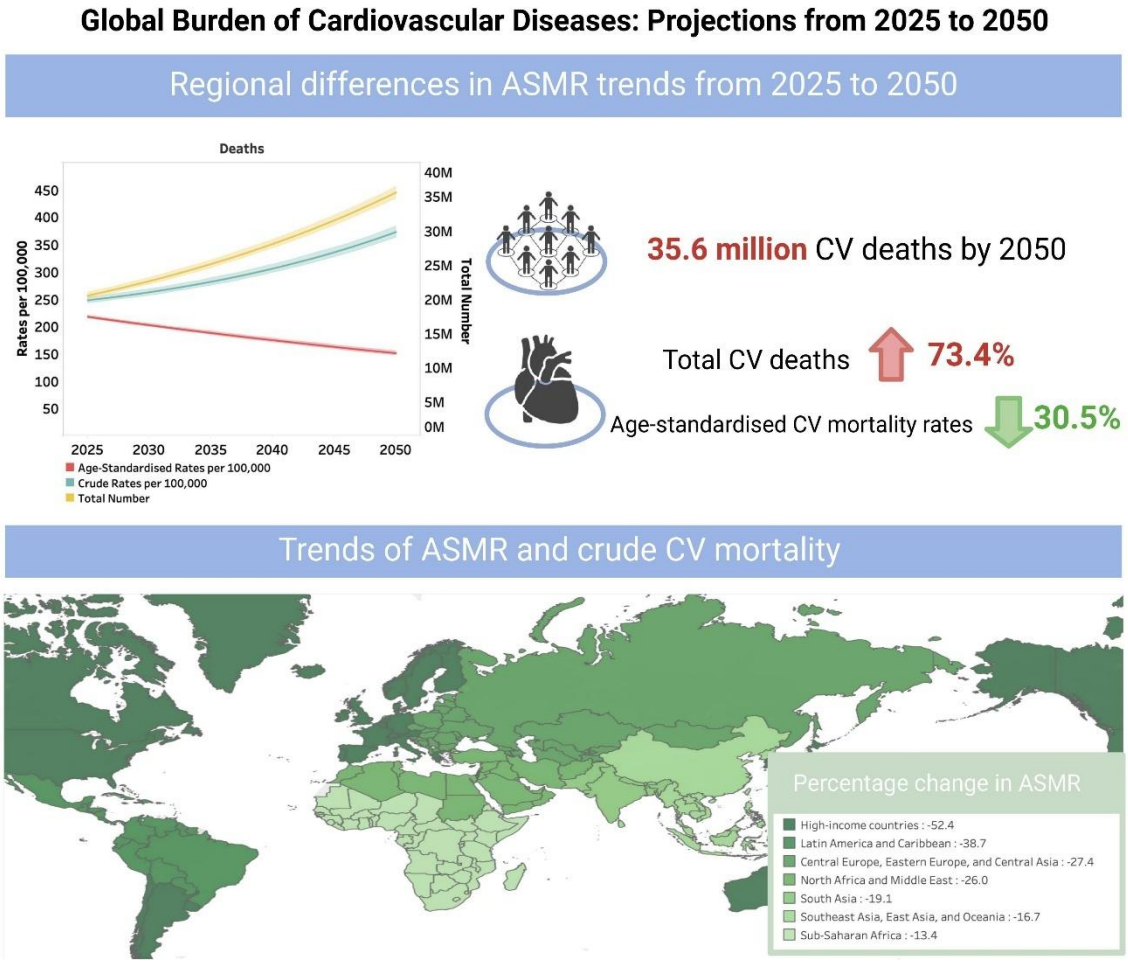

Figure 1-1. Global trend in cardiovascular disease projection showing crude and age standardized mortality rates (ASMR) (2025-2050). Taken from (Chong et al., 2025).

1.2 Environmental chemical exposure and CVD

There are multiple risk factors involved in the development of CVD including genetics, age, diet, obesity, diabetes, physical activity, and lifestyle factors such as smoking and alcohol consumption (Li et al., 2022). While it is difficult or impossible to influence the non-modifiable factors such as age and genetics, a significant proportion of CVD risks (about 80%) is derived from modifiable risk factors including diet, physical activity, smoking, alcohol consumption and environmental factors (Kirkley and Sargis, 2014b). Since prevention provides the highest gain against severe and unpredictable CVD outcomes, we need to better identify these modifiable risk factors of CVD. One such modifiable CVD risk factor is exposure to anthropogenic environmental chemicals (ECs). As a result of human activity and rapid industrialization, there has been a significant rise in the global production of ECs over the past few decades, with a predicted 3.4% annual increase until 2030 (Börjeson, 2017; Janković Šoja et al., 2016). These ECs include bisphenols such as bisphenol A (BPA), perfluoroalkyl substances (PFAS) polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), poly aromatic hydrocarbons (PAHs), pesticides, heavy metals, tobacco smoke and bacterial toxins (Bodin et al., 2015b; Organization, 2015). Many of these ECs are suspected of endocrine disrupting actions and capable of bioaccumulation and resistant to environmental degradation (Bodin et al., 2015b).

1.2.1 Endocrine disrupting chemicals (EDCs)

As a subclass of ECs, endocrine disrupting chemicals (EDCs) represent a heterogenous group of structurally diverse compounds capable of changes in the endogenous hormone signalling. EDCs primarily include industrial pollutants, waste materials, pesticides, pharmaceuticals, plastics and other consumer-derived chemicals, each exhibiting diverse chemical properties and mode of action (Kirkley and Sargis, 2014b). EDCs are chemicals that were originally produced for a specific purpose such as a pesticide, plasticizer, or solvent, but now have been found to have side effects when absorbed into the body which can either mimic or block hormones and disrupt the body's normal endocrine function. This disruption can occur by changing normal hormone levels, inhibiting or stimulating the production and metabolism of hormones, or changing the distribution of hormones through the body, thus interfering with the

functions controlled by these hormones. Historically, EDCs were believed to act primarily through nuclear hormone receptors, including estrogen (ER), androgen (AR), progesterone (PR), thyroid (TR), and retinoid receptors (Diamanti-Kandarakis et al., 2009) however, emerging evidence suggest that their mechanisms of action are far broader than originally understood. In addition to modulating nuclear receptor pathways, EDCs can exert their effects through non-steroid receptors, transcriptional co-regulators, and enzymes involved in steroid biosynthesis and metabolism, as well as other pathways involved in modulating endocrine and reproductive function (Diamanti-Kandarakis et al., 2009; Diamanti-Kandarakis et al., 2010). EDCs may also directly affect gene expression and induce epigenetic modifications during early life which can lead to transgenerational inheritance of disease risk in adulthood (Anway and Skinner, 2008; Moral et al., 2008).

1.2.2 Transgenerational cardiac consequences of EC exposure

Separate to the EC exposure effects on CV system in the exposed individuals, it is also hypothesized that the CV impacts resulting from in utero EC exposure could be transmitted to the subsequent generations through epigenetic transgenerational inheritance of phenotypes. This transgenerational inheritance refers to the germline transmission of epigenetic marks and phenotypes across generations without any evidence of direct environmental exposures (Skinner et al., 2010). The germline epigenetic inheritance can result from alteration in the embryonic stem cells and all cell types derived from these cells will carry an altered epigenome and transcriptome susceptible to adult onset of diseases across multiple generations (Skinner, 2011). Animal exposure models investigating certain ECs such as BPA, Phthalates, parabens, pesticides and fungicides have been associated with obesity, tumours, altered immune system, kidney and reproductive diseases in future generations (Anway et al., 2006; Manikkam et al., 2014; Manikkam et al., 2013; Nilsson et al., 2008). The transgenerational evidence on ECs associated CV impacts is however limited to few chemical studies. In mice, gestational BPA exposure was shown to induce myocardial hypertrophy and increased blood pressure in the F2 offsprings, however these observations were based on synergistic effects of high fat diet and BPA exposure (Liu et al., 2022). In a separate study, BPA exposure altered cardiac development in F1 and F2 offspring (Lombó et al., 2015). Another study investigating the transgenerational

effects of caffeine exposure revealed that caffeine exposure can result in hypertrophic cardiomyopathy like phenotype in the F2 and increased cardiac mass in the F3 adult offsprings of mice (Fang et al., 2016). These individual chemical exposure studies hints on potential transgenerational cardiac effects of EC exposures, however, the transgenerational effects of real-life EC mixtures on cardiac function and whether these are sex-specific have not been investigated and yet to be determined.

1.2.3 Developmental programming of CVD

Exposure to ECs during critical window of development such as the prenatal period is of particular concern as this is the period where the formation of essential organs and systems takes place. Exposure to ECs during this "sensitive" period of development can heighten their impact, resulting in health consequences across lifetime and risk of potential transmission to the following generations (Wang et al., 2016a; Zota et al., 2013). This concept of developmental programming which is now formally recognized as developmental origins of health and disease (DOHaD) was originally proposed by Barker and his colleagues (Barker and Osmond, 1988; Barker, 1995; Barker et al., 1989a; Barker et al., 1989b) which states that the emergence of later life diseases such as CVD may originate during early period of development. By the time of mid-gestation, the components of CV system i.e. heart and vascular system are already formed and particularly vulnerable to adverse intrauterine conditions and developmental cues (Mone et al., 2004). Any structural and functional changes such as endothelial dysfunction, smaller coronary arteries and fewer cardiomyocytes endowment during cardiogenesis may result in adverse CV "programming", which can lead to increased susceptibility of CVD in adulthood (Blackmore and Ozanne, 2015). Several studies have now documented that developmental exposure to certain ECs during critical periods of differentiation can lead to adverse effects, some of which may not be apparent until adult life. Epidemiological studies in human and experimental studies in animal models provides evidence on an association between EC exposure and CVD, discussed in the following sections.

1.2.4 Human studies

In humans, The National Health and Nutrition Examination Survey (NHANES) data set has been a substantial source of information on the association between ECs exposures

and CVD. Studies analysing NHANES data have consistently shown a positive association between several EC exposure and CVD associated risk factors. For example; exposure to PCBs has been positively associated with hypertension, an independent risk factor for CVD (Peters et al., 2014; Yorita Christensen and White, 2011). Similarly, BPA exposure is not only linked with increased risk of high blood pressure (Aekplakorn et al., 2015; Bae et al., 2012; Shankar and Teppala, 2012), but also low heart rate variability (Bae et al., 2012), and coronary and peripheral artery diseases (Melzer et al., 2012a; Shankar et al., 2012a) indicating that BPA exposure can impact different end points of CVD development. A positive association between perfluoroalkyl substances (PFAS) and perfluorooctanoic acid (PFOA) exposure and adult CVD has also been established (Lindstrom et al., 2011; Shankar et al., 2012c). Exposure to heavy metals also contribute to this evidence such as arsenic, lead and mercury is associated with high blood pressure and carotid artery diseases (Farzan et al., 2018; Gambelunghe et al., 2016; Mateen et al., 2017; Valera et al., 2009; Xu et al., 2020). In addition to adult EC exposures, there are only few studies examining the association between prenatal EC exposure and adult CVD. This include exposure to PAHs which has been associated with high blood pressure in childhood, an important risk factor for adult CVD (Chen et al., 2025a). Likewise, maternal PFAS exposure is reported to be associated with an increased risk of congenital heart diseases (CHDs) in offsprings (Ou et al., 2021). In a prospective follow-up study on women prenatally exposed to diethylstilbesterol (DES), it was revealed that prenatal DES exposure was associated with coronary artery disease (CAD) and myocardial infarction (MI) (Troisi et al., 2018). The New Hampshire Birth Cohort (NHBCS) study found an association between in utero Pb exposure and increased blood pressure in children with the effects being stronger among males (Farzan et al., 2018). Findings from NHBCS cohort held an immense significance as these highlighted sex-specific EC exposure susceptibilities of adult CVD outcomes. It has been suggested that fetuses exposed to aluminium and Pb might be at a higher risk of oxidative stress and CHD (Liu et al., 2018). Moreover, pregnant women exposed to cigarette smoke which contains a mixture of EDCs including dioxins, PAHs, heavy metals and other chemicals is reported to disrupt endothelial and vascular function in the offspring (Barbagallo et al., 2024). While the human observational studies provide valuable insights into potential link between EC exposure and CVD, they are limited by the fact that these studies are associative in nature and does not determine the causal relationship, or mechanistic understanding of EC exposure and CVD. In addition,

majority of human cohort studies examine single EC exposures at a particular timepoint and lack sex-specific interrogation as well as confounded by factors such as genetic background, diet, physical activity and socioeconomic status etc. Human EC exposure characterization is also complicated by an inconsistency in the duration and level of exposure to a particular EC which is occurring at intermittent and inconsistent doses (Nachman et al., 2011) and therefore warrants controlled EC mixture testing in animal models.

1.2.5 Animal studies

Experimental studies conducted in animal models of different species have also demonstrated adverse effects of several ECs on CV system via specific mechanisms. Studies conducted in rodents, non-human primates and zebrafish have demonstrated that prenatal BPA exposure alters cardiac development and function including changes in cardiac miRNA and fibrosis, altered cardiac transcriptome and disrupted morphogenesis (Chapalamadugu et al., 2014; Lombó et al., 2015; Rasdi et al., 2020). Prenatal BPA exposure can also disrupt cardiometabolic and cardiac stress markers as shown by increased total serum cholesterol levels in mice (Miyawaki et al., 2007) and an increase in natriuretic peptide gene expression in cardiac tissue of offsprings in sheep (MohanKumar et al., 2017) respectively. In rat, prenatal BPA exposure demonstrated an increase in the expression of hypoxia induced factor alpha-1 (HIF-1α) in fetal cardiac tissue (Rasdi et al., 2023), a transcriptional factor involved in regulating cellular hypoxic response. Likewise, prenatal exposure to mixture of BPA and PFOS during prenatal window led to an increased collagen levels and septal thickness, mitochondrial damage and enlargement of cardiomyocytes (Zhou et al., 2020). ECs can also induce oxidative stress in the developing fetal heart as shown by increased reactive oxygen species (ROS) generation in sheep exposed to dexamethasone (Roghair et al., 2007). In rat, maternal exposure to PAHs demonstrated placental toxicity as well as affected fetal blood vascular system while perinatal exposure to insecticide Dichlorodiphenyltrichloroethane (DDT) indicated hypertension and cardiac hypertrophy in adult mice (La Merrill et al., 2016). Gestational exposure to di(2-ethylhexyl) phthalate (DEHP) in mice promote myocardial cell toxicity (Yu et al., 2022) and disrupts the mRNA levels of important genes implicated in heart development thereby increasing the risk of congenital heart disease (CHD) in the offspring (Shi et al., 2025).

Developmental lead exposure in mice compromises cardiac development in the resulting offspring, the pathology of which can become worse in adulthood (Liu et al., 2023). Similarly, maternal exposure to cadmium (Cd) in mice resulted in increased heart weights in the offsprings along with an increased susceptibility to hypertension in adult life (Hudson et al., 2019). The CV evidence gained from animal studies is compelling; however, they carry certain limitations. Most studies have considered single EC exposures at specific doses, which does not reflect the complexity of ECs individuals are exposed to. This includes the risk posed by complex interactions between individual EC components when presented in a mixture form which would otherwise not exert any adverse effects. Moreover, due to the use of short-lived animal species in the above studies, the CV impacts of EC exposure were examined under acute conditions, which does not replicate the chronic low-level human exposure. An important limiting factor is that most studies have tested the effects of single EC in a single sex, and findings from those investigating sex-specific effects on cardiac function where both sexes were examined are conflicting as either sex is affected differently in different studies.

1.2.6 In vitro cardiomyocyte studies on EC exposure

To validate the cardiotoxic effects of ECs in an in vitro setup, adverse CV effects of EC exposure have also been assessed in the isolated cardiomyocytes where some recent studies have shown that ECs exposure altered myocyte functioning. Induced pluripotent stem cells-derived cardiomyocytes (iPSC-CM) were shown to exhibit proarrhythmic effect following exposure to BPA (Hyun et al., 2021; Ma et al., 2023) and phthalates (Lee et al., 2025). Exposure to low doses of mixture of bisphenols including bisphenol A and its analogues bisphenol S (BPS) and bisphenol F (BPF) had an additive effect on cardiomyocyte differentiation along with increased type 1 and 3 collagens (Zhou et al., 2021). Exposure of embryonic stem cells to low dose Cd suppressed cardiomyocyte differentiation and cardio-genesis(Wu et al., 2022). These findings provide mechanistic understanding of the cardiotoxic effects of ECs and cardiac responses in an isolated environment.

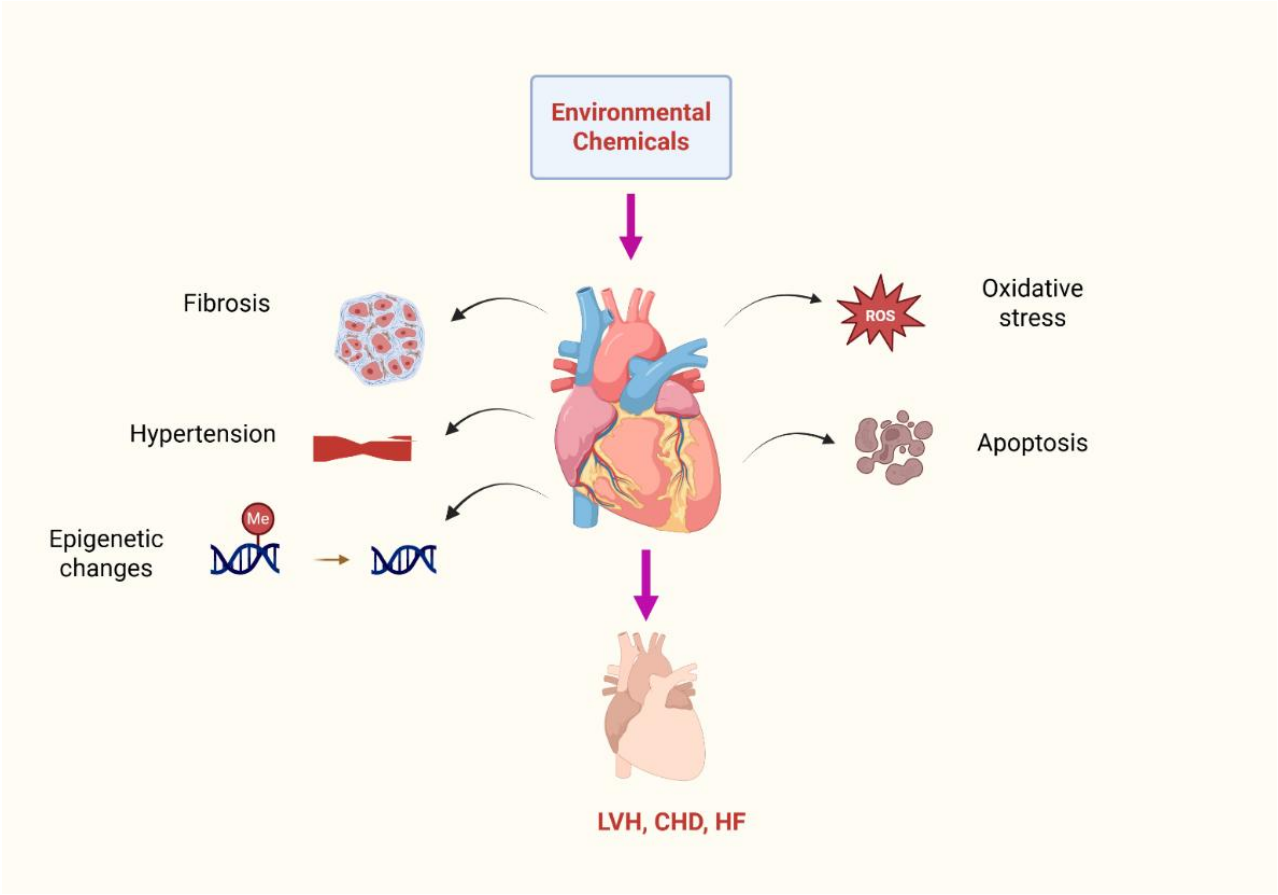

Figure 1-2. Environmental chemical exposure and their possible cardiovascular effects which can lead to heart disorders such as left ventricular hypertrophy, congenital heart diseases and heart failure. Figure generated on BioRender.com.

1.2.7 Sex-specific cardiac effects of EC exposure

Sex is considered as an important predictor of cardiac pathology. Sex steroids hormones i.e. estrogen in females regulate the gonadal maturation and growth of tissue/organs and the same is carried out by testosterone in males. However, these functions are regulated differently due to differences in the levels of circulating hormones. There is however limited understanding of the role of estrogen and testosterone in CVD susceptibility. When adjusted for age, estrogen is generally considered to exert cardioprotective effect in premenopausal women compared to men and after menopause there is a catchup in female specific CVD related morbidity and mortality

(Rappelli, 2002; Yang and Reckelhoff, 2011). In comparison to estrogen, testosterone in males is suggested to exert opposite effects on cardiac function. While it has been shown by studies that estrogens play a role in cardio-protection and testosterone worsens cardiac dysfunction (Cavasin et al., 2003; Lagranha et al., 2010; Mendelsohn and Karas, 1999), this is still controversial as hormone replacement therapies in post-menopausal females did not have any beneficial effects on cardiac outcomes (Grady et al., 2002; Herrington, 1999; Reckelhoff, 2005) and some studies reporting improved cardiac function following testosterone therapy (Bianchi, 2018; Mirdamadi et al., 2014). In addition to hormonal differences, heart also exhibit differential expression of sex steroid receptors. These receptors include estrogen receptor alpha (Erα), estrogen receptor beta (Erβ), androgen receptor (AR) and G protein-coupled receptor (GPR30) which influences cardiac function through genomic and non-genomic pathways in a sex-specific manner (Regitz-Zagrosek and Kararigas, 2017). It is therefore expected that CVD progression maybe sex-specific and it is important to gain an understanding of such sex-specific mechanisms in ECs induced cardiac disorders for targeted interventions. While not extensively investigated, studies in animal models particularly rodents have yielded some interesting results on the sex-specific nature of EC susceptibility in cardiac effects. Recently, a review paper has been published recognizing the sexually dimorphic nature of ECs, particularly EDCs in CV disorders. This review has combined evidence from invitro, in vivo and epidemiological studies to reflect on the sexually dimorphic effects and mechanisms implicated in ECs induced CV changes. However, it does recognize the limitation of substantial lack of sex-specific studies and emphasizes the need to include sex-specific investigations of ECs associated CV effects in future studies (Ma et al., 2025a). Studies conducted mainly in rodent species have reported sex-specific CV effects of single ECs exposure. However, the findings are conflicting in that either sex is affected differently in different studies. For example, gestational exposure to PAHs in mice induced heart mass reduction in males but not in females (Zhang et al., 2021). Female but not male mice chronically exposed to BPA developed an increase in pericardial fat and cardiac hypertrophy (Patel et al., 2015). Gestational exposure to particulate matter resulted in fibrosis, inflammation and left ventricular remodeling in male mice while females were unaffected (Tanwar et al., 2017). In another study, in utero nicotine exposed female mice offspring exhibited high mean blood pressure while mean heart rate was increased in males (Fox et al., 2012). Maternal exposure to nano plastics in mice model led to a significant increase in

fibrosis and reduction of body and heart weights in females compared to males (Chen et al., 2024). In sheep, excess of developmental testosterone exposure elevated markers of cardiac stress and hypertrophy in both sexes however, myocyte hyperplasia was only seen in females (Ghnenis et al., 2022). The sex-specific effects in above mentioned studies were tested against single EC exposure during early development and lacks the translational paradigm of adult onset of CVD related to gestational EC mixture exposure.

1.3 Biosolids treated pasture sheep model of mixed EC exposure

As discussed in the previous section, studies investigating the CV effects of EC exposure have been predominantly conducted in rodent species which have a short lifespan and therefore a limited significance for cardiotoxicity studies of chronic nature. Moreover, majority of studies have tested single ECs in a single sex. Given that human beings are exposed to complex mixture of ECs in daily lives present at low levels, there is a need to address the sex-specific CVD risk associated with mixture of ECs in a large animal model of translational relevance. The presence of chemicals in mixture is particularly important as they exhibit a non-monotonic dose response and can lead to complex interactions. Moreover, when presented in a mixture form, ECs can exhibit additive, synergistic and antagonistic effects (Delfosse et al., 2015; Faust et al., 2003; Rider et al., 2010). Biosolid treated pasture sheep (BTP) model is one model of real-life EC exposure. Biosolids (also known as sewage sludge) is derived from human waste treatment and commonly applied as fertilizer which contains a mixture of anthropogenic ECs at low concentrations and reflect human exposome (Rhind et al., 2010; Rhind et al., 2002; Venkatesan and Halden, 2014). Following grazing of pregnant sheep on BTP, measurable levels of prominent EDCs were detected in tissue samples collected from offsprings including PAHs, Phthalates, PCBs and Phenols (Rhind, 2005; Rhind et al., 2011). Previous work on BTP has shown adverse impacts on reproductive system (Bellingham et al., 2012; Elcombe et al., 2022; Elcombe et al., 2021), metabolic pathways (Ghasemzadeh-Hasankolaei et al., 2024), bone development (Lind et al., 2009), inflammation and oxidative stress (Thangaraj et al., 2023), liver function (Thangaraj et al., 2025), growth and body weight (Evans et al., 2023) and lipid profile (Thangaraj et al., 2025) of the resulting offspring. It is important to note that the

adverse effects mentioned in above studies were observed across different developmental timepoints i.e. fetal, prepubertal and adults which shows that individuals across all ages are at risk of EC mixture exposure effects. Given a variety of adverse impacts on other systems in BTP studies, it is anticipated that the developing CV system may also be at risk of developmental programming and insult which is worth investigating using this model.

The BTP model (Fig. 1-3) has high potential as a translational model. Human beings are exposed to a cocktail of low-level ECs every day in real-life and effects these chemicals exert on CV system are unknown. The low-level EC exposure in BTP can therefore be extrapolated into humans. Compared to rodents, sheep is an excellent model for developmental studies due to its high translational relevance to human beings. As an outbred species with longer lifespan and extended gestational period, it is well suited animal model for chronic exposure testing. Of relevance is the anatomical and functional features of sheep heart which are nearly similar to human beings (Rusakova and Zhuravleva, 2025). Gross features and physiology of sheep heart such as heart weights, heart rate, electrophysiology and coronary circulation is also comparable to human beings. Sheep model has been extensively utilized in CV research related to myocardial remodelling, heart valve functioning, atrial fibrillation, myocardial infarction and as translational models for cardiac surgeries such as coronary artery and cardiopulmonary bypass, valve replacement and implantable devices (Ali et al., 1996; DiVincenti Jr et al., 2014; Duchenne et al., 2019; Rabbani et al., 2008; Shofti et al., 2004). Compared to rodents, their large body size facilitates better manipulation of heart scanning such as cardiac MRI and echocardiography.

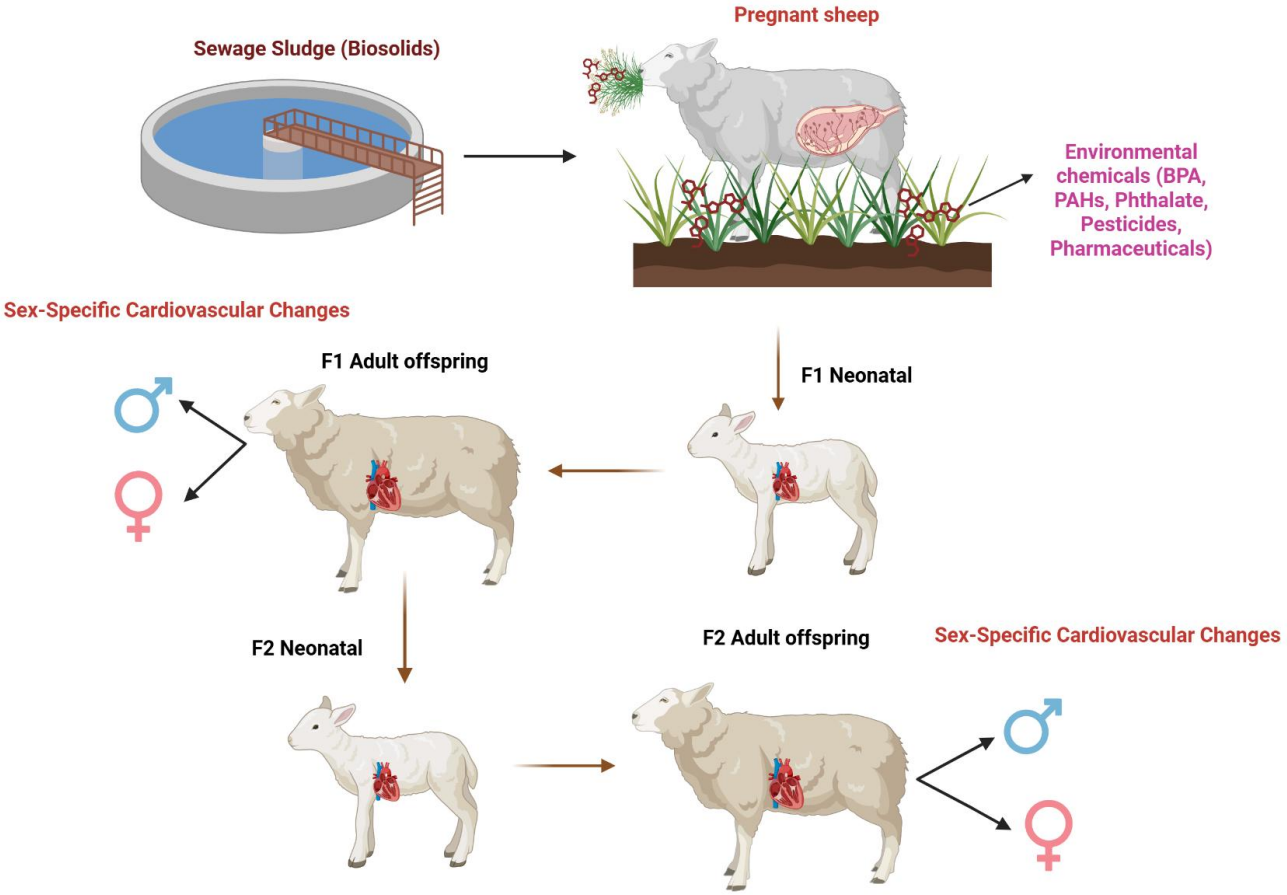

Figure 1-3. Biosolids treated pasture sheep model of real-life EC exposure. Pregnant sheep exposed to chemicals present in biosolids may lead to sex-specific developmental programming of CVD in F1 and F2 generations. Figure generated on BioRender.com.

1.4 Aims

The CV evidence gained from single EC exposure in unisex models of rodents indicates that ECs can exert adverse CV effects. However, sex-specific investigation on the mechanistic insights into developmental programming of CVD by mixed EC exposure such as those occurring in real-life has not been conducted. By using BTP sheep model of gestational exposure, the current thesis attempted to:

A. Investigate the sexually dimorphic effects of in utero exposure to a real-life mixture of ECs in the resulting male and female adult offspring in sheep.

B. Investigate the sexually differentiated and transgenerational CV impacts of real-life EC mixture exposure in the adult (male and female) offspring of second generation (F2) sheep.

To address these aims, pregnant sheep were exposed to mixture of ECs via grazing on BTP, and cardiac function was assessed in adult F1 and F2 (male and female) offsprings via in vivo and molecular studies on cardiac tissue samples. What follows is an in vivo assessment of CV structure and function in the F1 adult offspring following in utero exposure to EC mixture (Chapter: 2 Published), histological and molecular studies on cardiac tissues from F1 (Chapter: 3 Published), in vivo CV studies in the F2 adult offsprings (Chapter: 4) and histological and molecular findings from the F2 adult offspring (Chapter:5).

1.5 Hypothesis

In view of the above discussion, it is hypothesized that developmental exposure to a real-life EC mixture may result in sex-specific cardiac changes which can lead to adverse cardiac function in adult life.

Chapter 2 Sexually dimorphic cardiovascular impacts of prenatal exposure to real-life environmental chemical mixture in adult offspring

2.1 Abstract

Cardiovascular disease (CVD) is a leading cause of death that is sexually dimorphic. This study used an ovine model to investigate whether maternal exposure to an environmental chemical (EC) mixture (biosolids) prior to and throughout pregnancy, affected offspring cardiovascular (CV) structure and function in adulthood. CV function of male and female offspring from ewes grazed on either conventionally fertilized (control, C) or biosolids-treated pasture (B) was assessed. Males exhibited higher blood pressure compared to females with no significant effect of EC exposure. Heart rate variability in females suggested reduced autonomic regulation in the B group. EC-exposed males, but not females, showed significantly increased left ventricular dimensions, end-diastolic and systolic volumes, and cardiac output. The findings indicate sexually dimorphic effects of maternal EC mixture exposure on adult CV structure and function. Further studies are needed to explore the mechanisms and long-term implications of prenatal exposure to ECs on CV health.

Keywords; Cardiovascular diseases, Environmental Chemicals, Biosolids, HRV, Echocardiography

2.2 Introduction

Globally, cardiovascular disease (CVD) is a leading cause of death (Cdc, 1999) with prevalence nearly doubling between 1990 and 2019 (Roth et al., 2020). CVD encompasses a variety of conditions including heart failure, hypertensive heart disease, and coronary artery disease (Thomas et al., 2018). While non-modifiable risk factors such as age and genetics and modifiable risk factors such as obesity and diet are well established contributors to CVD, exposure to

environmental chemicals (ECs) has more recently been proposed as a risk for the development of CVD (Fu et al., 2020). For instance, ECs contributing to air pollution have been identified as the world's single largest environmental-health risk by the WHO with 80% of the fatalities related to air pollution occurring as a result of ischemic heart disease and stroke (Cosselman et al., 2015). Airborne ECs can include particulate matter, metals like arsenic and organic tin derivatives, N-nitroso compounds, bacterial toxins, ozone, polycyclic aromatic hydrocarbons, persistent organic pollutants like polychlorinated biphenyls (PCBs, dioxins, pesticides, and flame retardants), bisphenol A, phthalates, and triclosan, and many of them are capable of bioaccumulation due to their resistance to biodegradation (Bodin et al., 2015a).

A subclass of ECs, are the endocrine disrupting chemicals (EDCs) which are substances known to modify the actions and effects of the endocrine system in ways that adversely affect the organism itself or its offspring (Marcoccia et al., 2017). EDCs that include industrial pollutants, phytochemicals, pharmaceuticals, pesticides, and plastics vary widely in both structure and mode of action (Kirkley and Sargis, 2014a). While adult exposure to EDCs has been proven to lead to adverse health effects, the developing fetus is even more susceptible to the effects of EDCs at concentrations that are much lower than those deemed “safe” by current risk assessments (Singh et al., 2021). Indeed, maternal exposure to EDCs is one of the well-known ‘insults’ that can lead to intrauterine growth restriction (IUGR) (Diamanti-Kandarakis et al., 2009), which is linked with increased susceptibility to CVDs later in life (Barker et al., 1989a), (Vijayakumar et al., 1995), (Newbold et al., 2008).

Several epidemiological studies in humans have indicated that exposure to EDCs can adversely affect the cardiovascular system both in adults and in developing fetuses. For example, higher urinary bisphenol-A (BPA) concentrations have been associated with an increased risk of hypertension, coronary artery disease, carotid atherosclerosis, angina, myocardial infarction, and decreased heart rate variability (HRV) and elevated cholesterol (Bae et al., 2012). In preclinical studies, developmental exposure to BPA resulted in elevated cholesterol levels

(Kirkley and Sargis, 2014a), cardiomyopathy (Gear et al., 2017), and fetal and postnatal cardiac fibrosis in rodents (Belcher et al., 2015). Maternal BPA exposure also results in disruption in the fetal cardiac transcriptome in primates (Gao and Wang, 2014) and in adult offspring in sheep (Koneva et al., 2017). Other known EDCs, such as phthalates, have been reported to interfere with normal cardiac functioning and impair the regulation of calcium within human cardiomyocytes (Posnack et al., 2015). Phthalates have also been reported to result in modified autonomic system activity, leading to elevated sympathetic and lowered parasympathetic tone, with resultant lowered heart rate variability, and heightened cardiovascular reactivity and recovery (Jaimes III et al., 2017). Prenatal exposure to BPA and phthalates can disrupt the developmental trajectory and induce epigenetic modifications that are linked to increased risk of CVD later in life (Bhatnagar, 2006), (Philips et al., 2017), (Svoboda et al., 2020). It is also important to note that consequences of prenatal EC exposure on the cardiovascular (CV) system are not limited to exposed individuals but can be trans/multigenerational, with effects of EC exposure of adult-onset disease being epigenetically passed to subsequent generations (Manikkam et al., 2013).

It is well documented that substantial sex differences exist in both the prevalence and burden of CVD (Mosca et al., 2011) with age adjusted CVD mortality, pathological cardiac remodelling (PCM), and cardiac outcomes being worse in men compared to women (Ghali et al., 2003). Sex-specific physiological changes have been reported with EC exposure in human and animal studies (McCabe et al., 2017). While sex-specific effects of developmental exposure to ECs on CV function have been less extensively investigated, ECs have also been reported to have sex-specific effects on the myocardium and vasculature (Svoboda et al., 2020), (Belcher et al., 2015), (Sol et al., 2020). In rodent studies, BPA exposure induces concentric hypertrophy in males, but increased systolic and diastolic blood pressure are seen in females (Patel et al., 2013). Heart weights were also found to be increased in BPA-exposed female sheep fetuses (Vyas et al., 2019).

Our understanding of the real-life risk of EC exposure on CVD is currently limited by the fact that most studies have focused on the impact of single chemical, predominantly in altricial rodents of a single sex that are not reflective of the multitude of ECs humans are exposed to. Biosolids are solid/semisolid residues produced from wastewater treatment and are a source of ECs, which reflects real-life human EC exposure. It contains the range of chemicals which humans are exposed to through their daily activities and have passed through their bodies. These include personal-care products, pharmaceuticals, and chemicals used in agriculture and in industrialized manufacturing processes (Venkatesan and Halden, 2014). Analysis of the content of biosolids has confirmed the presence of a wide range of ECs including phthalates, BPA, PCBs, Polybrominated diphenyl ethers (PBDE), polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides and pharmaceuticals are detectable in biosolids (Lind et al., 2009). Biosolids are routinely used as an agricultural fertilizer and in land remediation. Following their use as a fertilizer, concentrations of ECs in the soil from treated pastures (Rhind et al., 2013) as well as tissue and blood samples collected from sheep maintained on biosolids-treated pastures are increased (Rhind et al., 2005b), (Rhind et al., 2010). Sheep are an excellent model of high translational relevance for human medicine as they are precocial and exhibit a cardiac developmental profile similar to humans. Previous studies utilizing this model have reported adverse effects of biosolids on reproductive (Elcombe et al., 2022), (Elcombe et al., 2023), (Bellingham and Evans, 2024), and metabolic systems (Ghasemzadeh-Hasankolaei et al., 2024) and sex specific effects on growth dynamics (Evans et al., 2023). Considering the adverse impact of biosolids exposure on metabolic system, a risk factor for the development of CVD, we hypothesized that exposure of pregnant sheep to a real-life ECs mixture, by grazing on pasture treated with biosolids, will adversely impact the cardiac structure and function of their adult offspring, in a sexually dimorphic manner.

2.3 Material and methods

2.3.1 Ethical statement

The experiments were conducted under the United Kingdom's Animals (Scientific Procedures) Act 1986, under the specific authority of Project License PF10145DF. All animals were humanely treated throughout the study, with due consideration to the alleviation of pain, suffering, distress, and lasting harm.

2.3.2 Experimental design and animal work

Approximately one month prior to mating, 320 Easy-care ewes were randomly allocated to two groups. The first group (n=160), was grazed on pasture treated with inorganic fertilizer, designated as controls (C), while the second group, was grazed on pasture fertilized with biosolids at conventional rates (4 tonnes/ha, twice a year, in April and September), designated as biosolids (B). The biosolids application regime provided the pasture with equivalent amounts of nitrogen as the inorganic fertilizer to avoid nutritional differences between the two pastures. The ewes from both groups were balanced for parity and body condition prior to conception and body condition at parturition was also not different between ewes in control and biosolids groups. To control for parental effects and ensure that there were an equal number of females mated to each male, ewes were artificially inseminated using semen from four unrelated rams that had been raised on C pasture. As a result, the progeny consisted of four different genetic backgrounds or sire families. The pregnant ewes were kept on their respective pastures until approximately two weeks before lambing, at which point they were brought inside and fed according to normal husbandry practices; however, the feed for B ewes was derived from pastures treated with biosolids. Following parturition, both C and B ewes and their lambs were maintained outdoors on C pasture until weaning when male and female lambs were removed to prevent mating. Birth weights were not different between biosolids and control groups in either male or female lambs as reported previously (Evans et al., 2023). Subgroups of male and female offspring (n=20 per group) were kept until 2.5 years of age (early adulthood) maintained on C

pasture, for conduct of *in-vivo* cardiovascular studies. This age was selected due to established reliability of echocardiography techniques performed in sheep at 2-4 (Hallowell et al., 2012) and 2-5 (Moses and Ross, 1987) years of age.

2.3.3 In-vivo cardiovascular studies

2.3.3.1 Blood pressure

Non-invasive blood pressure measurements were obtained using a standard veterinary blood pressure cuff (Midmark Inc. USA) (size = L) and a digital blood pressure monitor (Cardel 9401 Midmark Inc. USA). Measurements were acquired at same time point each day, for 15 consecutive days, with the animal in a standing position as described previously (MohanKumar et al., 2017). At same time point on each day (07:00 am-09:00 am), three consecutive measurements were obtained from individual animals for the systolic, diastolic, mean arterial pressure and heart rate as shown in **Fig. 2-1(A)** and the mean of each parameter was calculated to give one daily BP value.

2.3.3.2 Heart rate variability (HRV)

HRV was measured using a two lead ACTiheart 5 (CamNtech Inc. USA*)* heart rate monitor (HRM) as shown in **Fig. 2-1(B).** The abdominal region just behind the elbow was clipped and Polar® wearable belts fitted with the ACTiheart 5 monitors were secured as close to the elbow region as possible. To ensure uniform contact, ECG conductive gel (Henry Schein Medical, Gillingham, UK) was liberally applied to the electrodes. To ensure synchronous initiation of the recordings for all animals, the starting time was set few minutes after the ACTiheart was secured. Each animal was recorded for 30 minutes and data saved on laptop for further analysis. Following the acquisition of data, the inter beat intervals (IBIs) were manually corrected by running full waveform analysis using the ACTiheart software. Any artifacts such as missing or misplaced beats were identified along the waveform and corrected accordingly. A five-minute period of steady data along the waveform was selected to avoid any moving artifacts from each animal, loaded into a txt file and analyzed by Kubios software (Kubios HRV Standard 3.4.1, Kubios Oy, Finland), validated for HRV analysis in sheep

(Wojniusz et al., 2011). HRV metrics analyzed included the time domain parameters, root mean square of successive differences (RMSSD), and the standard deviation of the IBI of normal sinus beats (SDNN), the frequency domain parameters high frequency power (HF), low frequency power (LF) and the LF/HF ratio. The frequency bands used in frequency domain analysis were (VLF): 0.0033–0.04 Hz, low-frequency (LF): 0.04–0.15 Hz, and high-frequency (HF): 0.15–0.4 Hz as reported for sheep (Magawa et al., 2022).

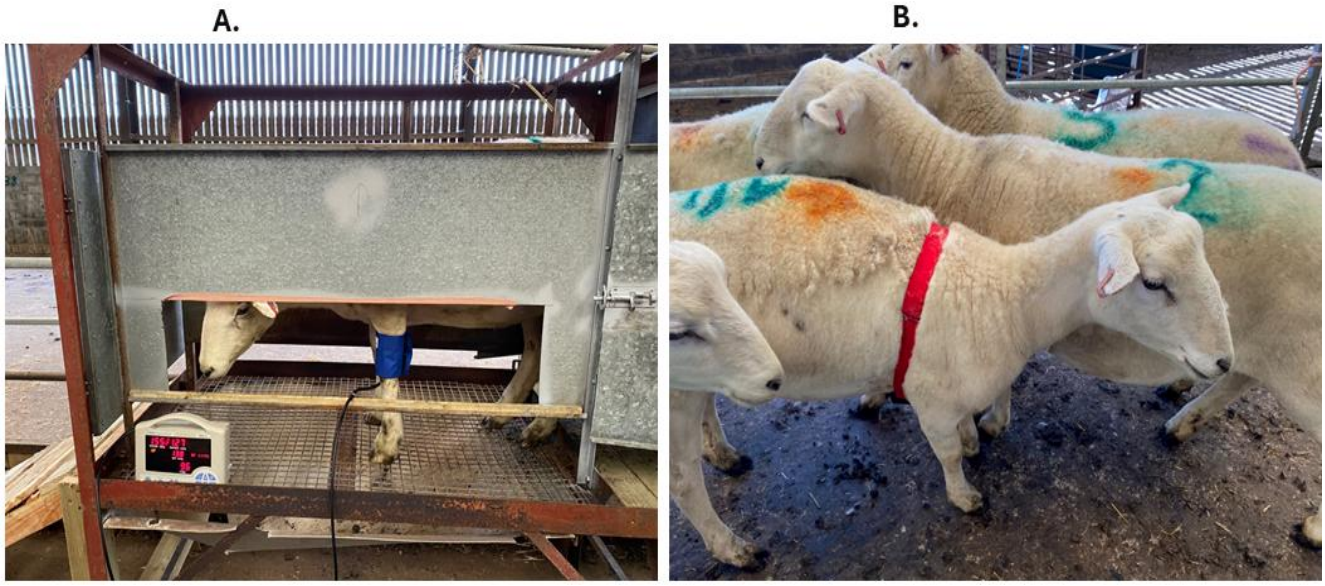

Figure 2-1. Non-invasive blood pressure measurement (A) and HRV recording using Polar ACTiheart monitor (B).

2.3.3.3 Echocardiography

Animals were prepared for scanning by clipping wool from the right hemithorax from the 3rd to the 5th intercostal space as described previously (Hallowell et al., 2012). Alcohol (70%) was then applied to the scanning site before a coupling gel was applied to the transducer and skin. All the animals were examined by the same team of individuals in a standing position. To obtain good quality images, the right front leg was pulled slightly forward and abducted as recommended (Boon, 2011). The right parasternal 2-D long axis and short axis images were obtained along with 1-Dimensional M-mode (Fig 2-2) according to the protocol described previously (Vloumidi and Fthenakis, 2017). The different

views obtained included long axis 4-chambered (4-C), left ventricular outflow tract (LVOT), right ventricular outflow tract (RVOT), Short axis left ventricle at mitral valve and chordae tendineae (SA-LV), Short axis aorta (SA-Ao) and 1-D M-mode. The parameters measured included left atrial diameter in end systole (LAD), Mitral valve annulus at end systole (MVA), pulmonary artery (PA) and Aortic (Ao) diameter at end diastole, left ventricular diameter in diastole (LVDd) and systole (LVDs), Interventricular septum thickness in diastole (IVSd) and systole (IVSs), Left ventricular free wall thickness in diastole (LFWd) and systole (LFWs), Left ventricular ejection fraction (LVEF), Fractional shortening (FS), End-diastolic (EDV) and end-systolic (ESV) volumes, Stroke volume (SV), Cardiac output (CO) and resting heart rate (HR). Each parameter was measured for three consecutive cycles and values averaged. The structural and functional cardiac parameters measured along with their corresponding image types, phase cycles and views used are outlined in table 2-1.

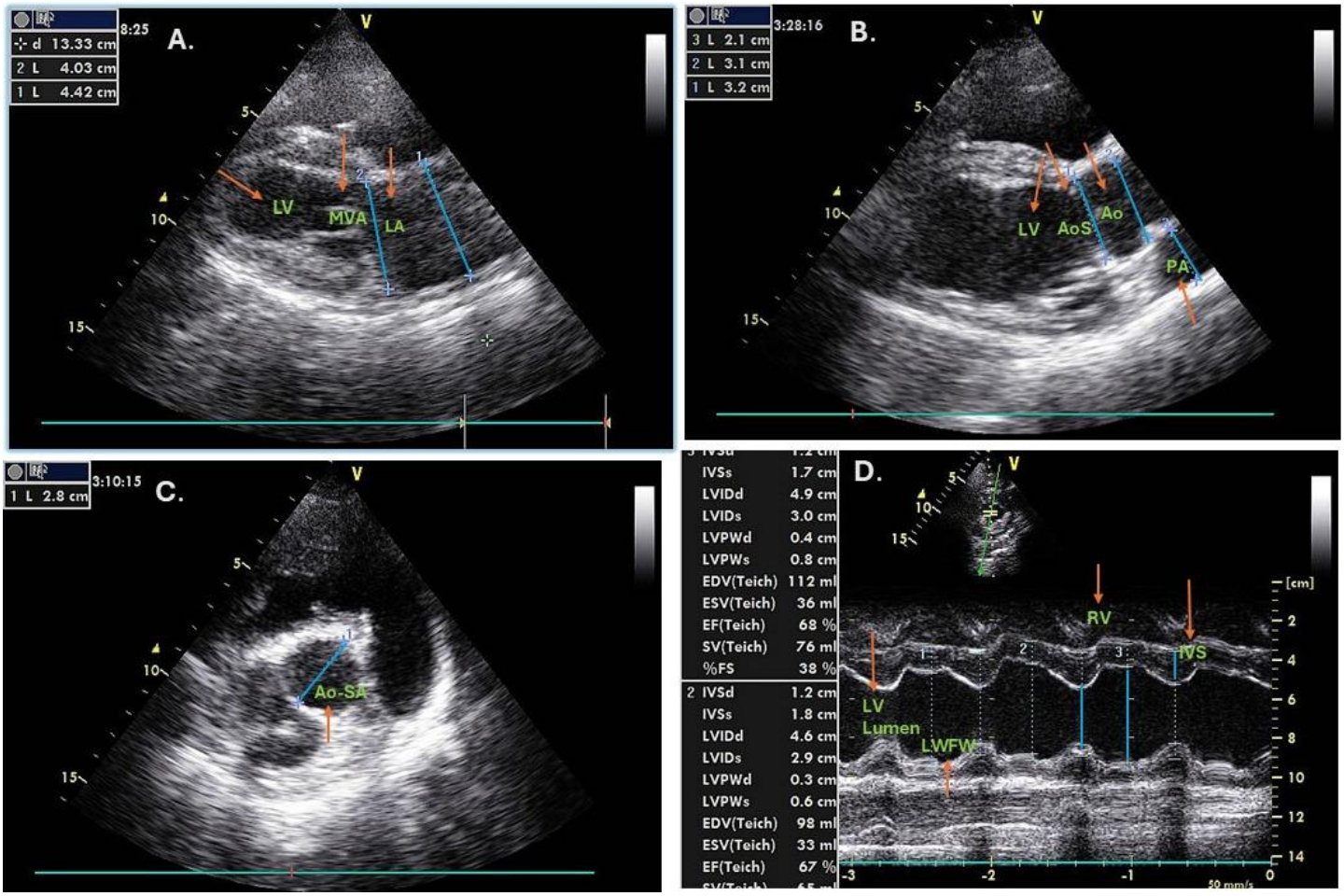

Figure 2-2. Standard right parasternal echocardiographic views. A) 4-Chambered view showing left atrium (LA), mitral valve annulus (MVA) and left ventricle (LV) in cross section. Left atrial diameter was measured parallel to the MVA at the end-systole (Schwarzwald, 2019). B) Long axis left ventricular outflow tract (LVOT) showing Aorta (Ao), Aortic sinus (AoS) and pulmonary artery (PA) in cross section. Ao, AoS and PA were measured at end-diastole (Hallowell et al., 2012). C) Short axis aorta (Ao-SA) in cross section at end-diastole. D) M-mode view showing right ventricle (RV), interventricular septum (IVS), left ventricular lumen and left ventricular free walls in cross section.

Table 2-1. Echocardiography parameters and their associated units, image types and different views used for measurements. Abbreviations: LAD = Left atrial diameter, MVA = Mitral valve annulus, Ao=Aorta, PA = Pulmonary artery, AoS = Aortic sinus, LVDd = Left ventricular diameter in diastole, LVDs = Left ventricular diameter in systole, IVSd = Interventricular septum thickness in diastole, IVSs = Interventricular septum thickness in systole, LFWd = Left ventricular free wall thickness in diastole, LFWs = Left ventricular free wall thickness in systole, FS=Fractional shortening, LVEF = Left ventricular ejection fraction, SV = Stroke volume, CO = Cardiac output, HR = Heart rate. SA = Short Axis, LA = Long axis, LV = Left ventricle.

Structural Parameters				
	Unit	Image Type	Phase Cycle	View
LAD	cm	2D	End-Systole	LA-4C
MVA	cm	2D	End-Systole	LA-4C
Ao	cm	2D	End-Diastole	LA-LVOT
PA	cm	2D	End-Diastole	LA-LVOT
AoS	cm	2D	End-Diastole	LA-LVOT
LVDd	cm	M-Mode	End-Diastole	SA-LV
LVDs	cm	M-Mode	Peak Systole	SA-LV
IVSd	cm	M-Mode	End-Diastole	SA-LV
IVSs	cm	M-Mode	Peak Systole	SA-LV
LFWd	cm	M-Mode	End-Diastole	SA-LV
LFWs	cm	M-Mode	Peak Systole	SA-LV
Functional Parameters				
FS	%	M-Mode	-	SA-LV
LVEF	%	M-Mode	-	SA-LV
SV	ml	M-Mode	-	SA-LV
CO	ml	M-Mode	-	SA-LV
HR	bpm	M-Mode	-	SA-LV

2.3.4 Statistical analysis

Data were analyzed by multifactorial analysis of variance (ANOVA) with statistical software R (RStudio version 2022.02.2+485), considering treatment, sex, litter size, birth weight, and sire as the explanatory variables. Litter size and birth weight had no effect in the model. Multiple comparisons of means were conducted using the Tukey-honestly significant difference (HSD) test. Differences were considered statistically significant at $p < 0.05$. In addition to statistical significance, physiological significance was assessed by categorized effect sizes (d), as small (d=0.2), medium (d=0.5), and large (d=0.8) (Cohen, 1992). All graphs were generated in GraphPad (Prism Windows 5.04) and presented as mean ± SEM unless otherwise stated.

2.4 Results

2.4.1 Blood pressure

Significant sex differences in systolic (p = 0.0002), diastolic (p = 0.03), and mean arterial blood pressures (p = 0.0007*),* all of which were higher in males compared to females, were evident (**Fig. 2-3 A-C**). There were no significant (p > 0.05) biosolid exposure and sex-specific exposure effects in the blood pressure measurements. In contrast, heart rate observed during the collection of blood pressure showed a significant (p = 0.01) exposure-sex interaction, being significantly lower in the B males compare to the controls, but not different between B and C females **(Fig 2-3 D)**.

2.4.2 Heart rate variability

Time-domain and frequency domain metrics RMSSD, SDNN, HF, LF were not significantly different (p > 0.05) between the C and B groups in either the males or females (**Fig.2-4 A-D**)., however, there was an apparent pattern (p = 0.06) for exposure-sex interaction where B females had lower RMSSD and SDNN compared to C females. In contrast, RMSSD and SDNN were lower in the C compared to B males. LF/HF ratio was overall significantly (p = 0.02) greater in the females relative to the males (**Fig.2-4 E**). In the HRs observed during the HRV recordings, a significant (p = 0.003) exposure effect was seen in males, and a significant exposure-sex interaction was evident; this was because the B males had significantly lower HRs compared to C males and there was no difference between B and C females, as shown in **Fig. 2- 4(F)**.

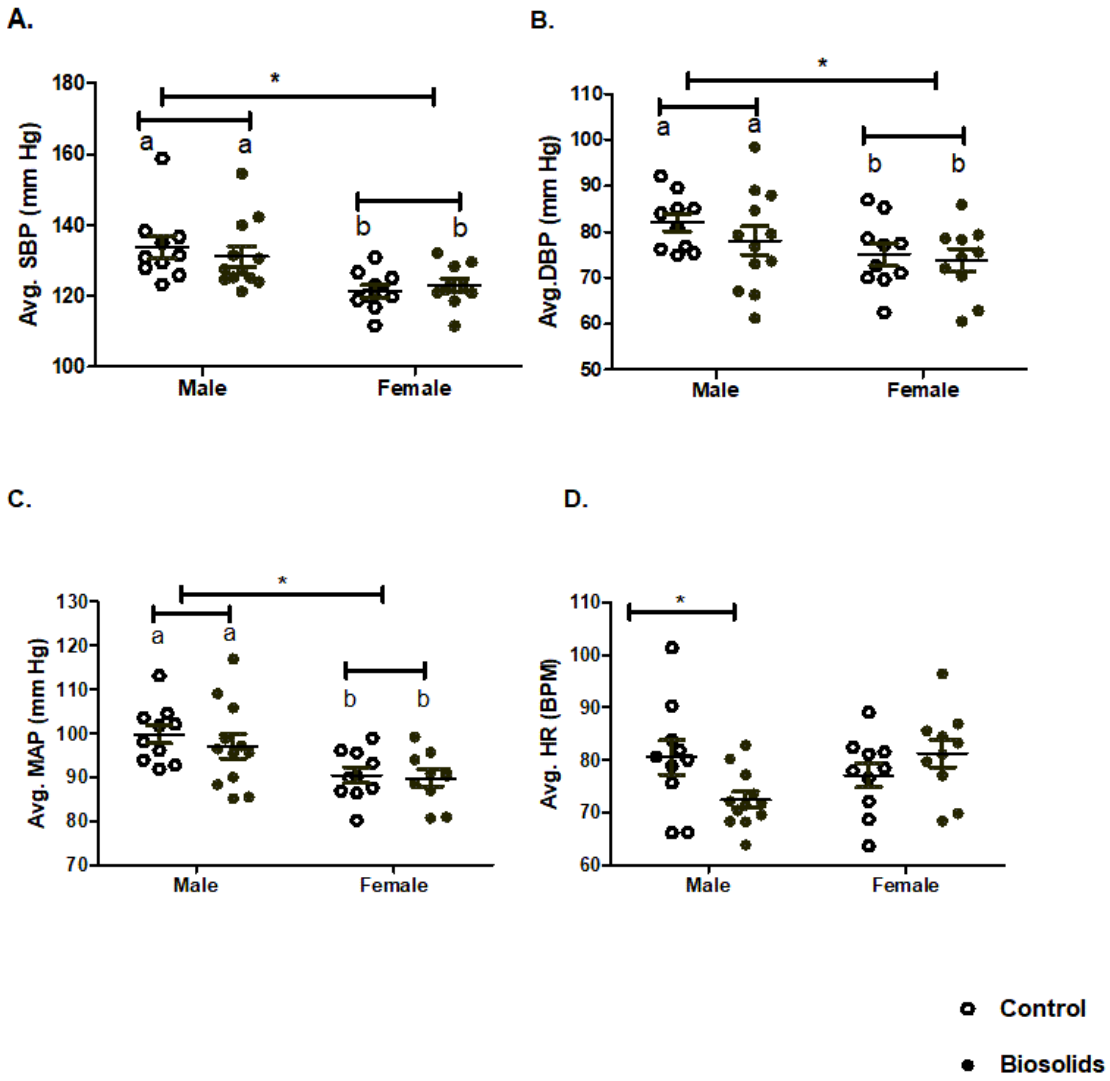

Figure 2-3. Scattered plots showing representative data from blood pressure measurements collected over 15 days (A) Average systolic blood pressure (SBP) shows no significant effect of B exposure ($p = 0.8$), while a significant sex effect was observed (aa~bb) ($p = 0.0002$) B) Average diastolic blood pressure (DBP) exposure effect ($p = 0.8$), sex effect (aa~bb) ($p = 0.03$) (C) Mean arterial blood pressure (MABP) exposure effect ($p = 0.4$), sex effect (aa~bb) ($p = 0.0007$) D) Average Heart Rate (HR) shows significant exposure effect in males ($p = 0.03$), sex effect ($p = 0.2$) and exposure~sex interaction ($p = 0.01$, $d = 1.27$). Data shown as Mean ± SEM. * indicate significant ($p < 0.05$) differences between groups (Control vs Biosolids) and sex (Male vs Female).

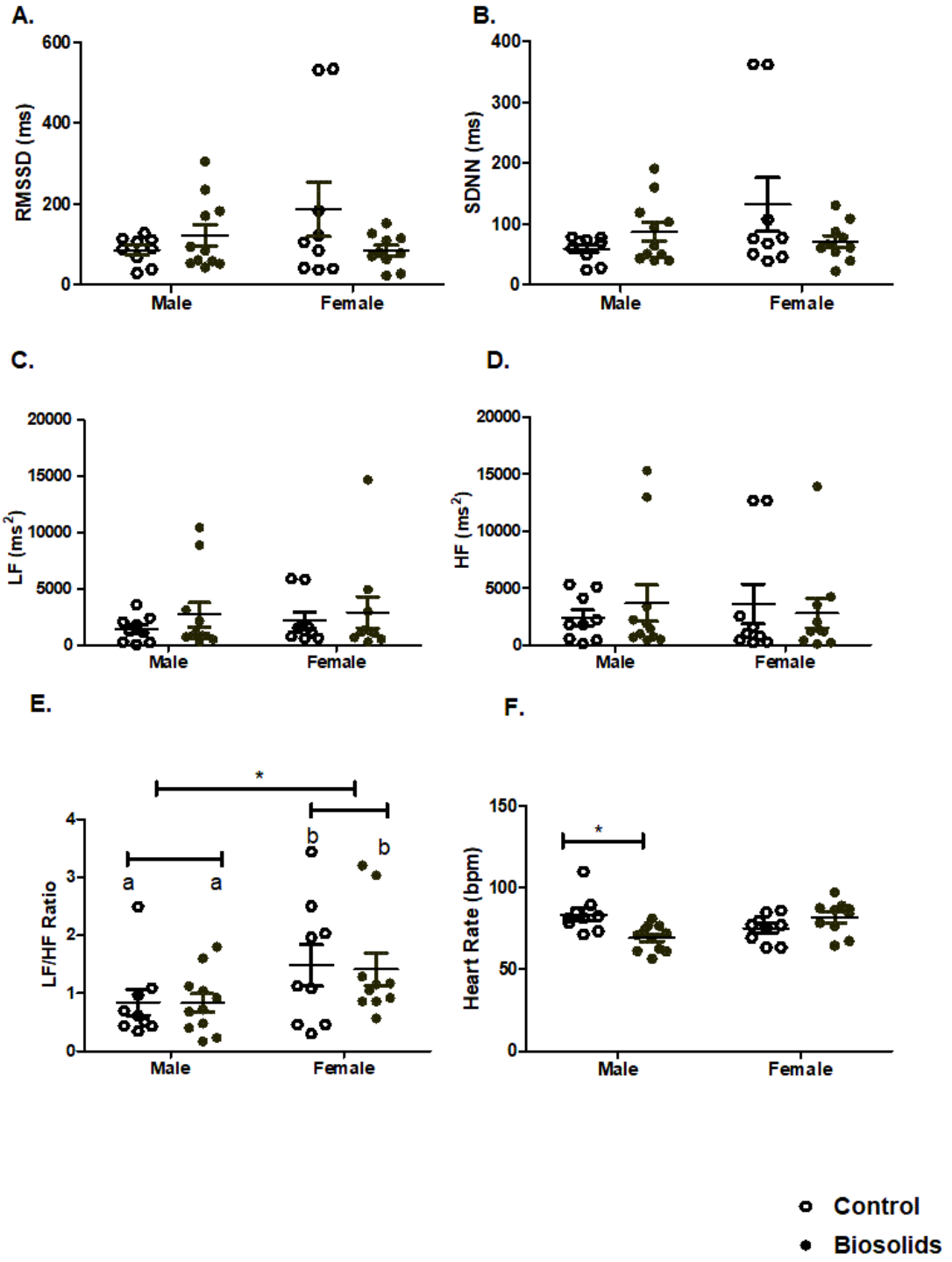

Figure 2-4. Scattered plots showing (A) Root mean square of successive differences (RMSSD) exposure effect ($p = 0.35$), sex effect ($p = 0.37$), Interaction ($p = 0.06$) (B) Standard deviation of the IBI of normal sinus beats (SDNN) exposure effect ($p = 0.46$), sex effect ($p = 0.22$), Interaction ($p = 0.06$) C) Absolute low frequency power (LF) exposure effect ($p = 0.3$), sex effect ($p = 0.6$) (D) Absolute high frequency power (HF) exposure effect ($p = 0.8$), sex effect ($p = 0.9$). (E) LF/HF ratio exposure effect ($p = 0.8$), sex effect ($p = 0.02$). (F) Heart rate (HR) shows significant exposure effect in males, post hoc ($p = 0.003$, $d = 1.45$), sex effect ($p = 0.6$) and a significant exposure~sex interaction ($p = 0.001$, $d = 1.36$). Data shown as Mean ± SEM. * indicate significant ($p < 0.05$) differences between groups (Control vs Biosolids) and sex (Male vs Female).

2.4.3 Echocardiography

Right parasternal 2D and M-mode echocardiography analysis indicated significant ($p < 0.05$) effects of exposure in B males and an exposure-sex interaction for the structural parameters of left ventricular diameter in diastole (LVDd) and systole (LVDs), as well as the functional indices of end-diastolic volume (EDV), and end-systolic volume (ESV) and cardiac output (CO). B males had significantly greater LVDd, LVDs, EDV, ESV and CO compared to C males but there were no effects of exposure in the females. Pulmonary artery (PA) diameter was observed to be significantly smaller in B compared to C females and there was a significant exposure-sex interaction as there was no effects of exposure in the males. An apparent pattern of increase ($p = 0.06$) was noted for exposure-sex interaction regarding mitral valve annulus (MVA) where MVA was greater in B compared to C males while MVA in B females was smaller compared to C females. Finally, a significant overall effect of sex was observed in aortic diameter (Ao), stroke volume (SV) and cardiac output (CO) with males showing greater Ao and higher SV and CO compared to females. No significant exposure ($p > 0.05$) or exposure-sex interaction was observed in the rest of the parameters studied i.e., LAD, IVSD, IVSs, FS, EF and HR **(Fig.2-5 and 2-6).** Results from echocardiography are summarized in table 2-2.

2.4.4 Paternal genotype effects

Analysis of the overall sire (genotype) effect on blood pressure metrics revealed a significant sire-sex interaction. Paternal genotype influenced mean arterial blood pressure ($p = 0.05$), (df=3), observed to be higher in males compared to females, and resting heart rate ($p = 0.02$), (df=3), observed to be lower in B compared to C males. Cardiac output was significantly ($p=0.01$), (df=3) influenced by sire (also affected by B exposure), but this was only observed in males. No significant sire effect was observed in females for blood pressure, heart rate variability, and echocardiography measures. The relevant genotype data for males and females are shown in Fig 2-7 and Fig 2-8 respectively.

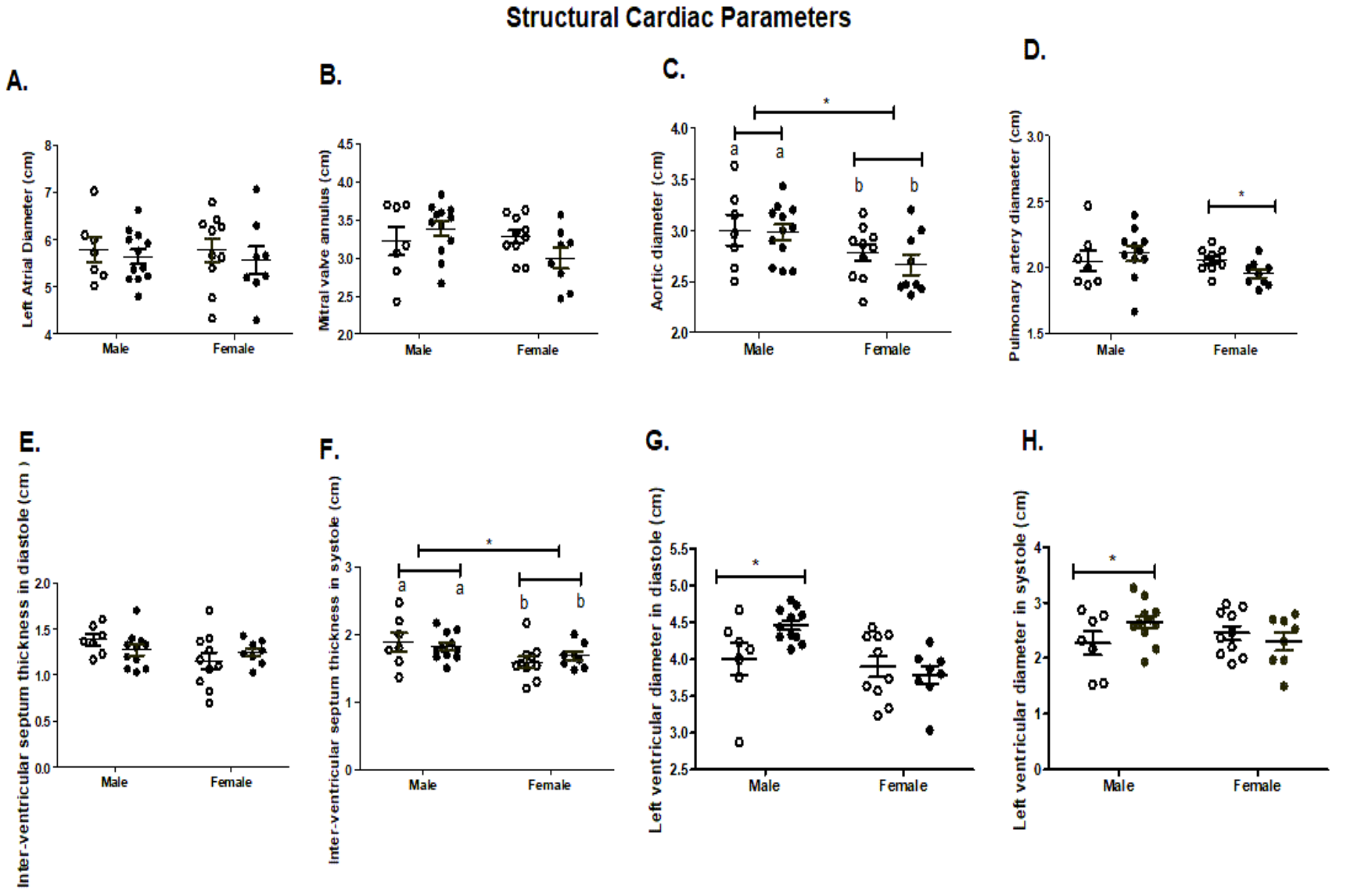

Figure 2-5. Structural cardiac parameters from echocardiography of males and female offsprings in-utero exposed to EC mixture. No significant effects of biosolids exposure and sex were observed in left atrial diameter, mitral valve annulus, and inter-ventricular septum thickness in diastole (A, B, E). Aortic diameter (C) shows significant sex differences between males and females (aa~bb), ($p = 0.01$). Pulmonary artery diameter (D) shows significant exposure effect in females, post hoc ($p = 0.02$) and exposure~sex interaction ($p = 0.03$). Interventricular septum thickness in systole (F) shows significant sex effect (aa~bb) ($p = 0.01$). Left ventricular internal diameter in diastole and systole (G-H) shows significant exposure effect in males ($p = 0.05$) and ($p = 0.03$) respectively. * Indicate significant ($p < 0.05$) differences between groups (Control vs Biosolids) and sex (Male vs Female). All graphs are presented as Mean ± SEM.

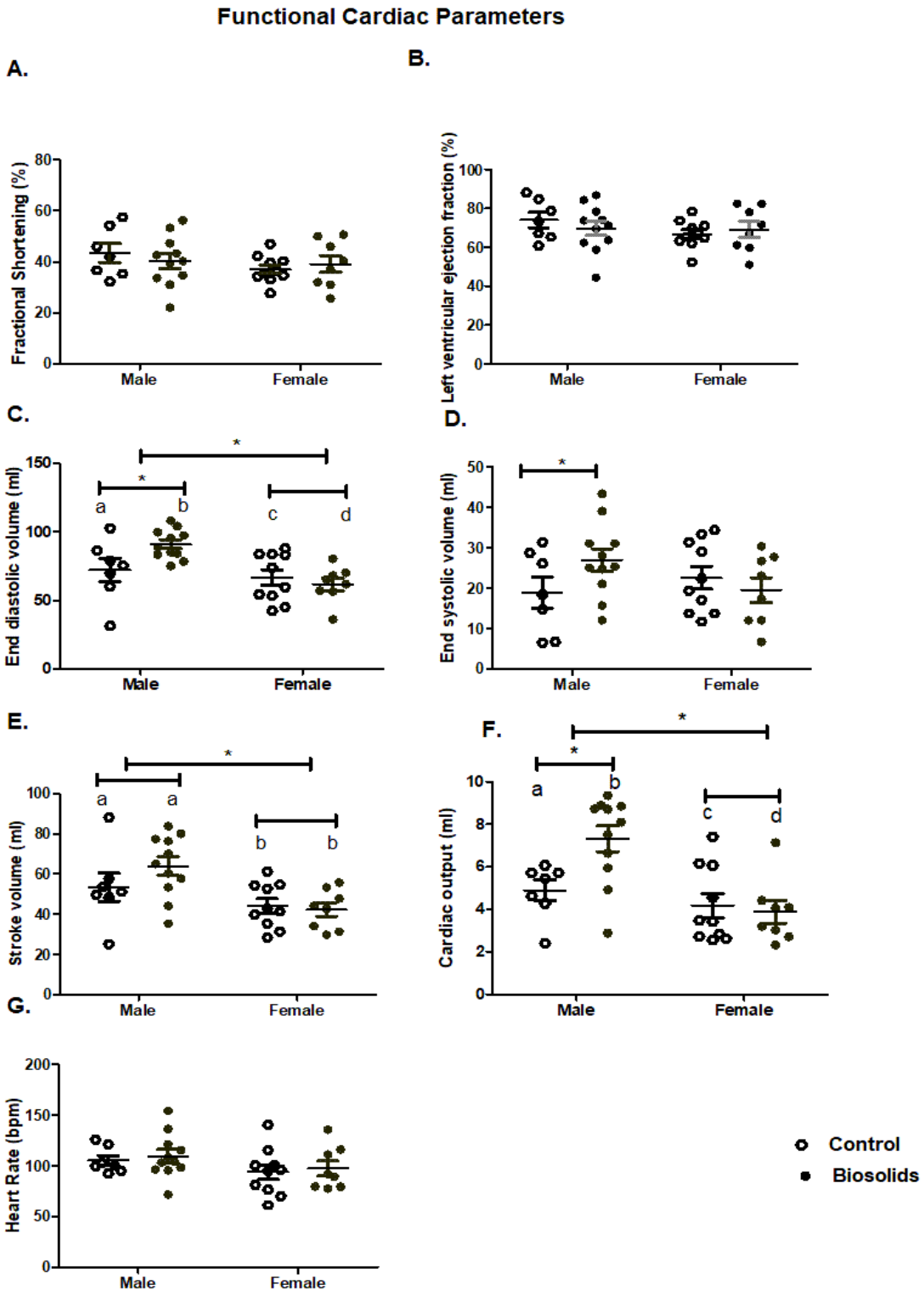

Figure 2-6. Functional cardiac parameters from echocardiography of males and female offsprings in-utero exposed to EC mixture. (A). No significant exposure or sex effects were seen in fractional shortening (p-value exposure = 0.8, p-value sex = 0.2). (B). Left ventricular ejection fraction (p-value exposure = 0.7, p-value sex = 0.2). (C). A Significant effect of exposure ($p = 0.04$), sex ($p = 0.002$) and exposure~sex interaction ($p = 0.03$) was observed in males in end-diastolic volume. (D). Shows significant effect of exposure in end-systolic volume in males ($p = 0.03$) and exposure~sex interaction. (E). Stroke volume shows significant effect of sex ($p = 0.002$). (F). Cardiac output was significantly affected by B exposure in males ($p = 0.02$, $d = 1.51$), sex ($p = 0.001$) as well as there was significant exposure~sex interaction ($p = 0.02$). (G). Heart Rate was not affected (p-value exposure = 0.3, p-value sex = 0.1). * Indicate significant differences ($p < 0.05$) between groups (Control vs Biosolids) and sex (Male vs Female). All graphs are presented as Mean ± SEM.

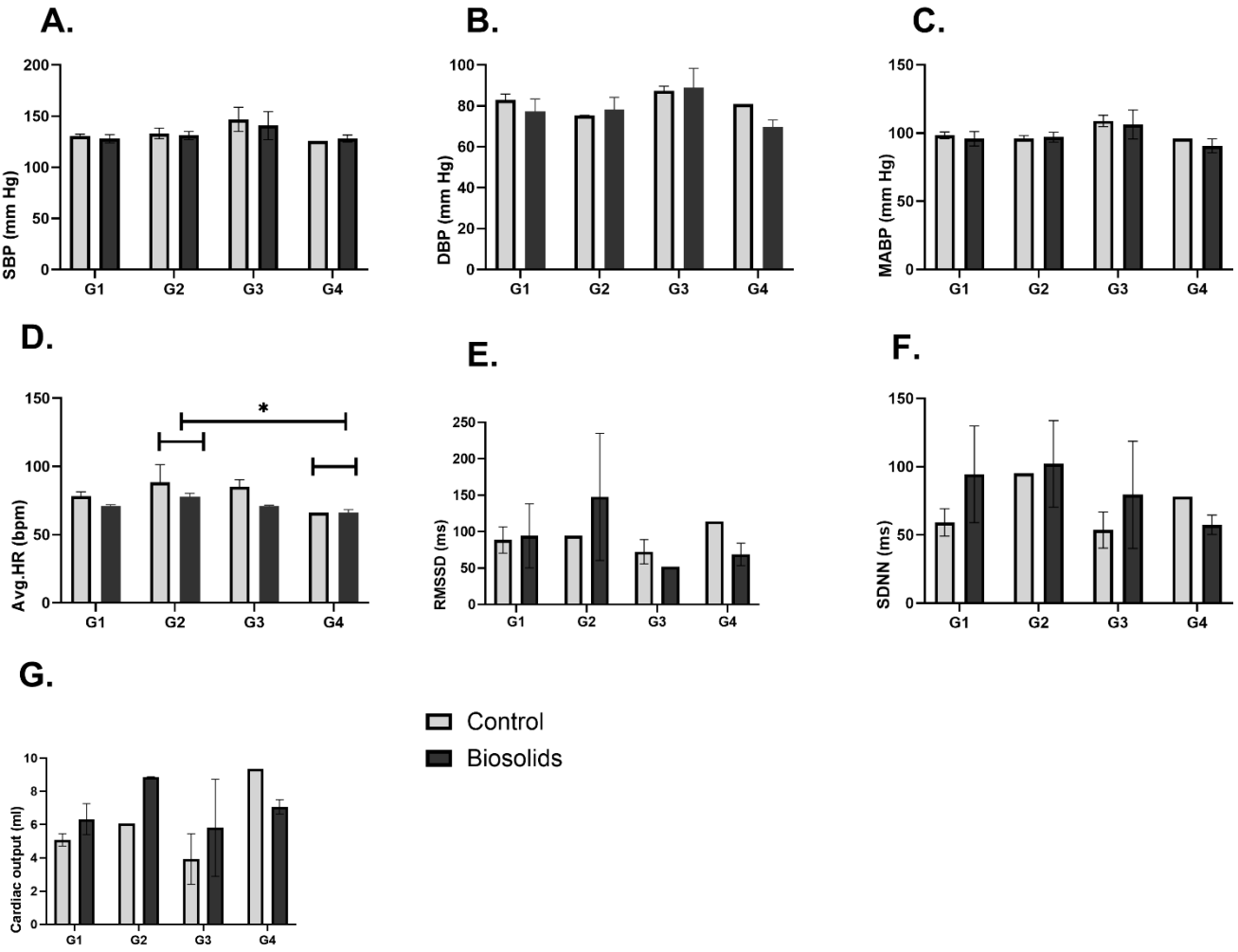

Figure 2-7. Effect of paternal genotype (sire) on markers of cardiovascular function in male offsprings. SBP (A), DBP (B), RMSSD (E) and SDNN (F) were not significantly influenced by paternal genotypes (P-value SBP= 0.08, DBP= 0.1, RMSSD=, 0.82 SDNN= 0.62. MABP (C) was overall significantly influenced by sire (P=0.05). Heart rate (D) was overall significantly influenced by sire (P=0.02) and post-hoc analysis showed significantly higher (P=0.03) HR in G2 compared to G4. Cardiac output (G) was also significantly influenced by sire (P=0.05). Data presented as Mean ± SEM.

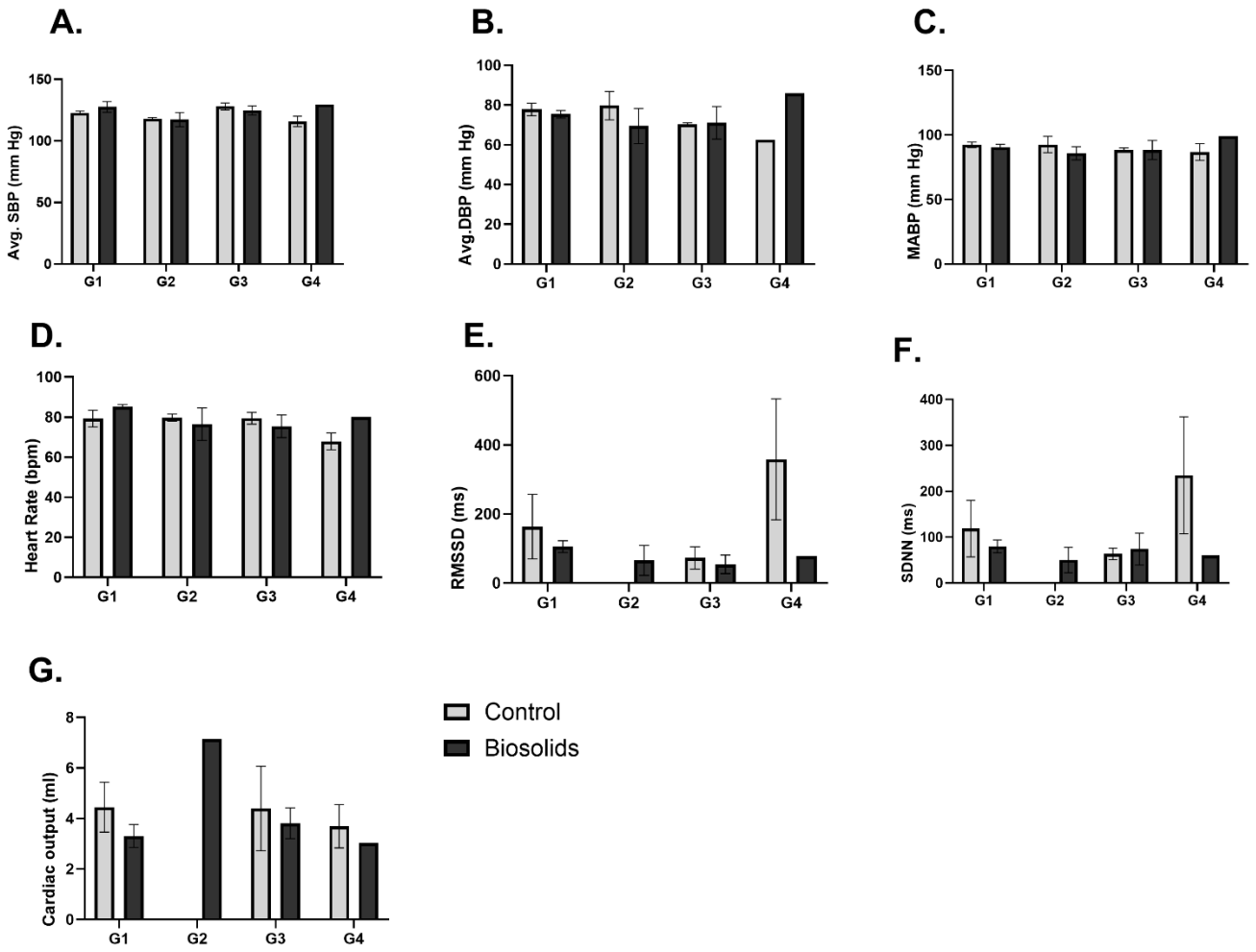

Figure 2-8. Effect of paternal genotype (sire) on markers of cardiovascular function in female offsprings. No significant sire effects or sire-exposure interaction were observed on SBP (A) P= 0.15, DBP (B) P = 0.66, MABP (C) P=0.73, HR (D) P= 0.87, RMSSD (E) P= 0.48, SDNN (F) P= 0.62 and CO (G) P= 0.41. Data presented as Mean ± SEM.

Table 2-2. Summary of results from structural and functional echocardiography parameters in sheep with corresponding p values for main effects exposure (Control vs Biosolids), Sex (Male vs Female), and exposure-sex interaction. LAD = Left atrial diameter, MVA=Mitral valve annulus, Ao = Aorta, PA = Pulmonary artery, AoS = Aortic sinus, PA = Pulmonary artery, LVDd = Left ventricular diameter in diastole, LVDs = Left ventricular diameter in systole, IVSd = Interventricular septum thickness in diastole, IVSs = Interventricular septum thickness in systole, LFWd = Left ventricular free wall thickness in diastole, LFWs = Left ventricular free wall thickness in systole, FS = Fractional shortening, LVEF = Left ventricular ejection fraction, SV = Stroke volume, CO = Cardiac output, HR = Heart rate. Data presented as Mean ± SEM and analysed by multifactor ANOVA. * Indicates $p < 0.05$.

Response Variable	Males		Females		*p* - value	*p* - value	*p* - value
	Control	Biosolids	Control	Biosolids	Exposure (df=1)	Sex (df=1)	Interaction (df=1)
LAD	5.78 ± 0.25	5.64 ± 0.15	5.78 ± 0.24	5.56 ± 0.29	0.44	0.85	0.86
MVA	3.22 ± 0.18	3.38 ± 0.09	3.28 ± 0.08	3.00 ± 0.13	0.64	0.18	0.06
Ao	3.00 ± 0.14	2.98 ± 0.08	2.78 ± 0.08	2.66 ± 0.09	0.48	0.01*	0.62
PA	2.05 ± 0.08	2.11 ± 0.06	2.06 ± 0.02	1.95 ± 0.03	0.62	0.15	0.03*
AoS	2.96 ± 0.08	2.88 ± 0.06	2.83 ± 0.08	2.83 ± 0.05	0.61	0.61	0.31
LVDd	4.00 ± 0.21	4.46 ± 0.06	3.89 ± 0.14	3.77 ± 0.12	0.05*	0.06	0.04*
LVDs	2.26 ± 0.20	2.64 ± 0.12	2.45 ± 0.12	2.30 ± 0.16	0.03*	0.57	0.06
IVSd	1.38 ± 0.05	1.27 ± 0.06	1.15 ± 0.09	1.24 ± 0.04	0.90	0.08	0.14
IVSs	1.88 ± 0.13	1.81 ± 0.06	1.58 ± 0.08	1.68 ± 0.06	0.89	0.01*	0.36
LFWd	0.76 ± 0.10	0.66 ± 0.10	0.71 ± 0.08	0.72 ± 0.08	0.73	0.99	0.59
LFWs	1.14 ± 0.18	1.06 ± 0.15	1.02 ± 0.12	0.97 ± 0.10	0.68	0.45	0.94
EDV	72.04 ± 8.46	90.78 ± 3.24	66.66 ± 5.55	61.79 ± 4.58	0.05*	0.002*	0.03*
ESV	18.88 ± 3.84	26.90 ± 2.77	22.56 ± 2.77	19.45 ± 3.07	0.03*	0.54	0.05*
FS	43.45 ± 3.65	40.37 ± 2.99	37.06 ± 1.71	39.08 ± 3.28	0.85	0.20	0.39
LVEF	73.95 ± 3.88	69.26 ± 4.01	66.73 ± 2.25	69.12 ± 4.05	0.79	0.27	0.35
SV	53.31 ± 7.01	63.90 ± 4.66	44.16 ± 3.50	42.29 ± 3.48	0.15	0.002*	0.19
CO	4.89 ± 0.48	7.32 ± 0.61	4.18 ± 0.55	3.87 ± 0.53	0.05*	0.003*	0.02*
HR	105.07± 4.91	109.45±6. 66	93.56± 7.33	97.43±7. 47	0.33	0.19	0.90

2.5 Discussion

Findings from the present study evaluating the impacts of maternal EC exposure via grazing on biosolids treated pasture on the CV system of their adult offspring, found significant overall sex differences in blood pressure, with males exhibiting higher systolic, diastolic, and mean arterial blood pressures compared to females. These findings are consistent with several studies conducted in animal models (Maris et al., 2005), (Ganten et al., 1989), and reports in humans (Reckelhoff, 2001). The gender differences in blood pressure can be attributed to differences in sex steroid hormones that are reported to contribute towards blood pressure regulation through their influence of various systems controlling blood pressure, primarily the renin-angiotensin system (Reckelhoff, 2023). EC exposure did not have any effect on blood pressure, in either sex. These findings are in agreement with a previous study which investigated the effects of prenatal single EC exposure (BPA) in sheep (MohanKumar et al., 2017). It should be noted that in the aforementioned study, effects of EC exposure were investigated under different nutritional conditions, and a significant diet-treatment interaction was evident suggesting that exposure to ECs may have a synergistic effect on BP when it is combined with other determinants of cardiovascular health i.e. nutrition and age. This possibility is supported by studies in rats where elevated BP was observed in the male progeny of dams treated with ECs dexamethasone, PFOS, atrazine, nicotine, or arsenic groups at 52 weeks of age, whereas in the female offspring, elevated BP was observed in dexamethasone, PFOS, and atrazine groups at 37 and 65 weeks of age only (Rogers et al., 2014). Several other prenatal exposure studies, however, have reported adverse effects of single ECs on BP. For instance, low level lead exposure in male rats for thirty days resulted in increased BP (Simoes et al., 2017). Similarly, prenatal exposure to dexamethasone increased basal blood pressure in male offspring by ~3mmHg (O'Sullivan et al., 2013) . However, not all EC studies have reported adverse effects, importantly in an experiment where two ECs DEHP and DBP were investigated in male mice, exposure to DEHP induced a sharp increase in blood pressure, while DBP did not (Xie et al., 2019). The lack of an EC exposure effect on BP in our study could potentially be

attributed to several factors. These include differences in sheep compared to the other animal models utilized, and the age at which animals were studied, or the fact that the current study utilized a mixture of low concentrations of ECs which may have had antagonistic actions and led to a lack of a discernible overall effect on BP parameters, whereas in other studies, single EC exposure of acute nature was investigated. Additionally, there is also a possibility that in-utero exposure to EC may have led to some compensatory systemic response to maintain normotension in our animals. Considering EC resistant phenotypes have been reported for instance in testicular disruption (Elcombe et al., 2021) it may be that the vascular system in this model may be resistant to the detrimental effects of the ECs.

Similar to BP, heart rate is under continuous control from autonomic regulation in response to various physiological changes. It has been reported that autonomic regulation can be altered by toxic accumulation of certain ECs such as BPA (Ramadan et al., 2018), lead (Boscolo and Carmignani, 1988), and mercury (Carmignani et al., 1983). In this study HR was assessed alongside both blood pressure (over 15 days) and HRV data. In both instances, the mean HR was lower in B males and tended to be increased in B females, relative to their respective controls. A lower HR, as a result of chronic EC (lead) exposure has been observed previously, in rats, following the combined blockade of cardiac muscarinic and adrenergic receptors (Simoes et al., 2017). That study provided evidence that even at a low dose, chronic administration of an EC modified the sympathovagal control of HR in rats. Similarly, acute EC exposure to lead decreased HR along with prolonging atrioventricular node conduction, reduced coronary flow, and decreased the contractile response to calcium in isolated and perfused rat hearts (Prentice and Kopp, 1985). While the lower HR in the B males in the current study could be a result of direct autonomic regulation by ECs exposure, an alternative explanation is that the observed changes in HR may be a compensatory mechanism in response to the higher EDV, ESV and CO in B males. Although similar trends in lower HR due to increase in vagal tone along with increase EDV, ESV and CO are seen in trained athletes and reported to be associated with increased cardiac efficiency(Hellsten and Nyberg, 2011), these changes however, do not always indicate beneficial effects on cardiovascular

system, and their occurrence in response to EC exposure may reflect different underlying mechanisms. In contrast to B males, HR in B females tended to be increased across both times it was assessed. This increase in HR as a result of B exposure in the females was also associated with a decrease in RMSSD and SDNN, markers for heart rate variability. This would be expected as a high HR reduces the inter-beat intervals and results in lower HRV.

HRV is regarded as a sensitive indicator of the functional regulatory properties of the autonomic nervous system (ANS) on cardiac function (Rajendra Acharya et al., 2006b), (Von Borell et al., 2007). A decrease in HRV is indicative of heightened sympathetic activity and/or withdrawal of parasympathetic activity and is often used as a non-invasive assessment of ANS activity in response to stressors (Kitajima et al., 2021). In the current study, animals were conditioned to experimental methods during 15 days of BP measurements followed by HRV measurements to assess the effects of EC exposure on the ANS regulation of resting cardiac function. An interaction (p = 0.06) between sex and treatment was noted whereby B females had lower RMSSD and SDNN compared to C females and B males. This suggests altered autonomic regulation in the form of decreased parasympathetic tone as a consequence of EC exposure in the B exposed females. This would also agree with the higher HR seen in this group. This specific observation in the females may be related to a sympathetic dominance in females, which was suggested in the current study by the higher LF/HF ratio that was observed in the females relative to the males. These findings are supported by evidence from previous study on healthy individuals (Saleem et al., 2012) which have shown that females tend to have higher sympathetic dominance compared to males. In contrast, another study has suggested that despite higher HR, women can exhibit vagal dominance in the form of lower LF/HF ratio compared to men ((Bigger Jr et al., 1995), (Koenig and Thayer, 2016). Although substantial studies are lacking on effects of exposure to mixture of ECs on HRV, the apparent pattern of lower RMSSD and SDNN observed in B females in our study and evidence on individual ECs exposure such as lead (Poręba et al., 2011) and BPA (Bae et al., 2012) in humans indicate that ECs can modulate autonomic cardiac responses and exposed individuals exhibit reduced HRV which has been associated with increased risk of

development of CVDs (Poręba et al., 2011). The reduced HRV in females could suggest sex-specific alterations in autonomic nervous system regulation and heart function due to EC exposure, potentially increasing their long-term disease risk. One mechanism could be endocrine disrupting actions of EC such as BPA on estrogenic receptors controlling autonomic regulation of heart function (Bruno et al., 2019).

The heart responds to physiological and pathological stimuli. Pressure and volume overload in cardiac chambers is considered one of the main determinants of left ventricular hypertrophy (LVH) (Panidis et al., 1984)). Moreover, changes in cardiac form and mass are mediated by complex mechanical, neurohumoral, inflammatory, and oxidative processes that involve all cell types in the heart and ultimately lead to a disproportionate growth of cardiac chambers and altered cardiac functioning (Pichler et al., 2019). The most significant findings from our echocardiography assessment were significantly greater left ventricular diameters in diastole and systole, end diastolic and systolic volumes along with significantly high CO in EC exposed males. The cardiac phenotype seen in B males corroborates with cardiac findings seen in eccentric left ventricular hypertrophy (Devereux and Roman, 1999). The increase in left ventricular size in these studies was also independent of an increase in BP as observed in the B males in our study. Although the resultant increase in end diastolic and systolic volumes, CO and decreased heart rates in B males could indicate physiological adaptation as seen in human athletics and exercise endurance (Hoogsteen et al., 2003; Messerli, 1983; Mihl et al., 2008), this is unlikely given lack of any postnatal interventions to mimic exercise in our studies. Future studies in these B exposed animals will focus on the cardiac morphology and molecular underpinnings of the cardiac phenotypic findings to distinguish physiological from pathological LVH. Pathological LVH is seen as a significant and independent risk factor for stroke, coronary heart disease, and sudden death (Schillaci et al., 2000), which is also sexually dimorphic as it appears that male hearts tend to develop pathological hypertrophy more easily than females (Regitz-Zagrosek et al., 2010).

It is of interest that these parameters were not affected in females indicating a sexually dimorphic effect of ECs on cardiac function. Similar effects of ECs on the male heart were reported in a rodent study where in-utero exposure to diethylstilbestrol (DES) and a high fed diet led to increased left ventricular internal diameter and increased end diastolic volume (Patel et al., 2015), a result that is similar to our observations in B males. In contrast to our findings, it has been reported that LVH was observed only in female offsprings, in response to gestational exposure to DES and BPA in mice (Dodic et al., 2001), which could be attributed to variability in the EC composition between the studies and differences in impact could be variable depending on the species. Overall, ECs BPA and DES can induce sex-specific changes related to cardiac hypertrophy and pathological cardiac remodeling in male offspring.

Another cardiac parameter affected in B animals in the current study was pulmonary artery diameter. Despite no significant difference in the bodyweights, this was found to be significantly smaller in B compared to control females and B males. A similar thickening of arterial walls, both in coronary and pulmonary circulations, has been reported in rats exposed to air pollution, although in that instance the effect was seen in male but not female animals (Lemos et al., 2006). Pulmonary hypertension which can be a consequence of thickened arterial walls has also been reported in female guinea pigs exposed to cigarette smoke (Wright and Churg, 1991). Following exposure to cigarette smoke, male guinea pigs developed endothelial dysfunction in the pulmonary artery (Ferrer et al., 2009). Furthermore, there is evidence that EC mixtures exposure in the form of cigarette smoking promotes adverse changes in the vasculature of lungs in rodent species (Auerbach et al., 1963),(Liu and Fung, 2012), (Zhao et al., 2014). These findings support the observation that pulmonary vasculature can be affected by EC exposure possibly due to an inflammatory response. Although we did not assess the microvascular architecture of the pulmonary system in this study, changes seen in the pulmonary artery diameter are concerning for adverse pulmonary programming with in-utero biosolid exposure and will be a focus for future studies.

Collectively, our findings relating to the cardiovascular system in a real-life EC exposure model add to the evidence that gestational EC exposure results in sexually differentiated effects on the cardiovascular system/function; males exhibited more pronounced effects of mixed ECs exposure from biosolids in structural and functional markers of CVS, than females as outlined in **Fig 2-9.**

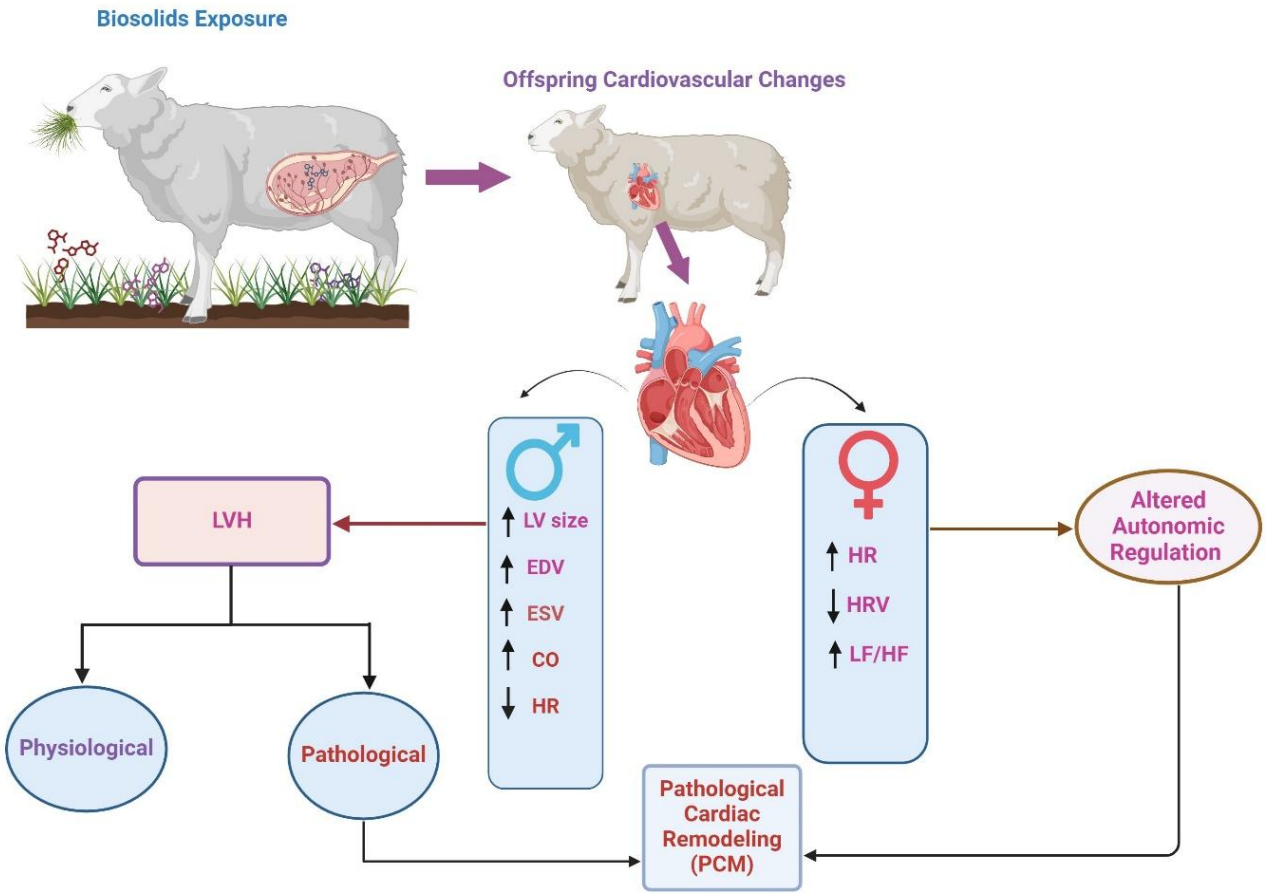

Figure 2-9. In utero exposure to real-life mixture of ECs in the form of biosolids and their sex-specific cardiovascular effects in resulting offsprings which indicate pathological cardiac remodelling and plasticity, particularly in males which can lead to cardiac dysfunction in adult offsprings. Figure generated in BioRender.com.

2.5.1 Influence of paternal genotype on offspring CV function

Considering that experimental outcome and adult onset of CVDs could be confounded by parental genotypes in the current study, we controlled paternal genotype and balanced it across the control and biosolids exposed groups. Interestingly we observed a significant sire-sex interaction with regard to a number of CV function markers including MABP, HR and CO as well as the impact of EC exposure on this interaction. The MABP was influenced by sire and was higher in males compared to females. In comparison, HRs and CO in males appeared to be affected by both maternal biosolids exposure and sire family which indicates that there is a genetic component to EC susceptibility. It is emphasized that the sires of the sheep in the current study were not exposed to biosolids. Previously using the same BTP model, an influence of paternal genotype has been reported on key energy markers of metabolism in male offspring (Ghasemzadeh-Hasankolaei et al., 2024). In the context of CVDs, epidemiological and human longitudinal case-controlled studies have reported conflicting results regarding effects of parental link of CVDs. While some found associations between offsprings and early paternal and late maternal age CVDs (Sesso et al., 2001), others have asserted that compared to paternal CV health, maternal health was a more robust predictor of offspring CV health (Muchira et al., 2020) and some have reported no substantial parental effect on CVD incidence (Weijmans et al., 2015). Several transgenerational studies have also investigated the relationship between CVD risk in parents and risk factors for CVDs in offspring. While some studies have reported no association between offspring and either parent in relation to CVD risk (Lawlor et al., 2003), (Adams et al., 2005) others have reported that decreasing offspring birth weight was linked to an increased risk of CVD in both parents (Smith et al., 1997), (Smith et al., 2005), (Davey Smith et al., 2007) or with CVD in the mothers (Smith et al., 2000a), (Smith et al., 2000b), (Smith et al., 2001)) . To the best of our knowledge, this is the first study of its kind to investigate the influence of paternal genotype in the context of mixed EC exposure susceptibility on structural and functional CV parameters. Further studies assessing the underlying mechanism of such relationship are required.

2.6 Conclusion

Our current study using real-life EC exposure model demonstrates that maternal exposure to a complex mixture of environmental chemicals (ECs) can influence the developing cardiovascular system and its regulation, and that the changes observed in young adult sheep (2.5yrs old) may predispose affected individuals to cardiovascular diseases (CVD) later in life. Importantly, some of the observed changes were sexually dimorphic manifested as significant differences in key structural and functional indices of cardiovascular function between EC mixture-exposed males and females with males exhibiting lower heart rates, increased left ventricular diameter, greater systolic and diastolic volumes, and increased cardiac output compared to females. These differences are not observed in the controls. These findings suggest that several cardiovascular markers may be affected by EC mixture exposure in males, and that these could potentially predispose males to a higher risk CVD in adulthood. In addition, the finding that paternal genotype contributes to the adverse impact on male offspring is supportive of genetic susceptibility to exposures to ECs on CV functioning. Future research should focus on the potential mechanisms involved in the programming of CV changes and cardiac remodeling due to gestational EC mixture exposure, particularly in males. Understanding the sexually dimorphic effects of in-utero chemical exposure on cardiovascular functioning could then allow for the development of targeted interventions and future preventive strategies.

2.7 Limitations

In this study, we have employed biosolids which are derived from human and industrial waste as a source of ECs. While this model reflects the human exposome, its complexity makes full EC characterization impossible and of limited value as the interactions between component ECs are not known or understood. As such, the study reports the effects of exposure to a real-life EC mixture and does not measure or ascribe effects to specific ECs. Humans are exposed to hundreds of chemicals, with measures available for only a subset of them. Considering that these ECs may have additive, synergist antagonistic

effects and measures of only a subset will not provide true picture of exposure to real-life exposome, we focused on studying the cumulative burden of the exposome. Further the mixture will contain a wide mixture of ECs, and this may vary with time and geographical location. Moreover, we have only looked at EC exposure during one period of time and while we know that prenatal EC exposure can disrupt the postnatal cardio metabolic events, to what extent these postnatal CV changes contribute to the final CV pathology could not be determined from this study. In addition, the effects of continual EC exposure across a lifetime have not been determined.

Chapter 3 Sexually dimorphic effects of in-*utero* exposure to a real-life environmental chemical mixture on markers of cardiovascular function in adult sheep

3.1 Abstract

Cardiovascular diseases (CVD) are a major sexually dimorphic cause of mortality and morbidity. Prenatal exposure to environmental chemicals (ECs) can program the adult onset of CVD. Using a real-life EC exposure sheep model, this study investigated structural and molecular underpinnings of the sex-specific effects of prenatal EC mixture exposure via mothers grazing on biosolids treated pasture (BTP) in left ventricular (LV) tissues. EC mixture exposure had no impact on plasma TG and TC levels, LV cardiomyocyte number or collagen scoring in both sexes. However, a significant increase ($P<0.05$) in fibrosis was evident in interstitial, perivascular and replacement fibrosis in BTP males. A significant upregulation of inflammatory (MHC-DRB1, MHC-DYA), apoptosis (CASP3) markers, together with elevated IGF-1 and IGF1-R expression was restricted to EC exposed males only. These findings extend our earlier results on sex-specific differences in prenatal EC exposure programming of adult CV functioning, particularly in males.
Keywords; Cardiovascular diseases, Environmental Chemicals, Sheep, CASP3, IGF-1, IGF1-R.

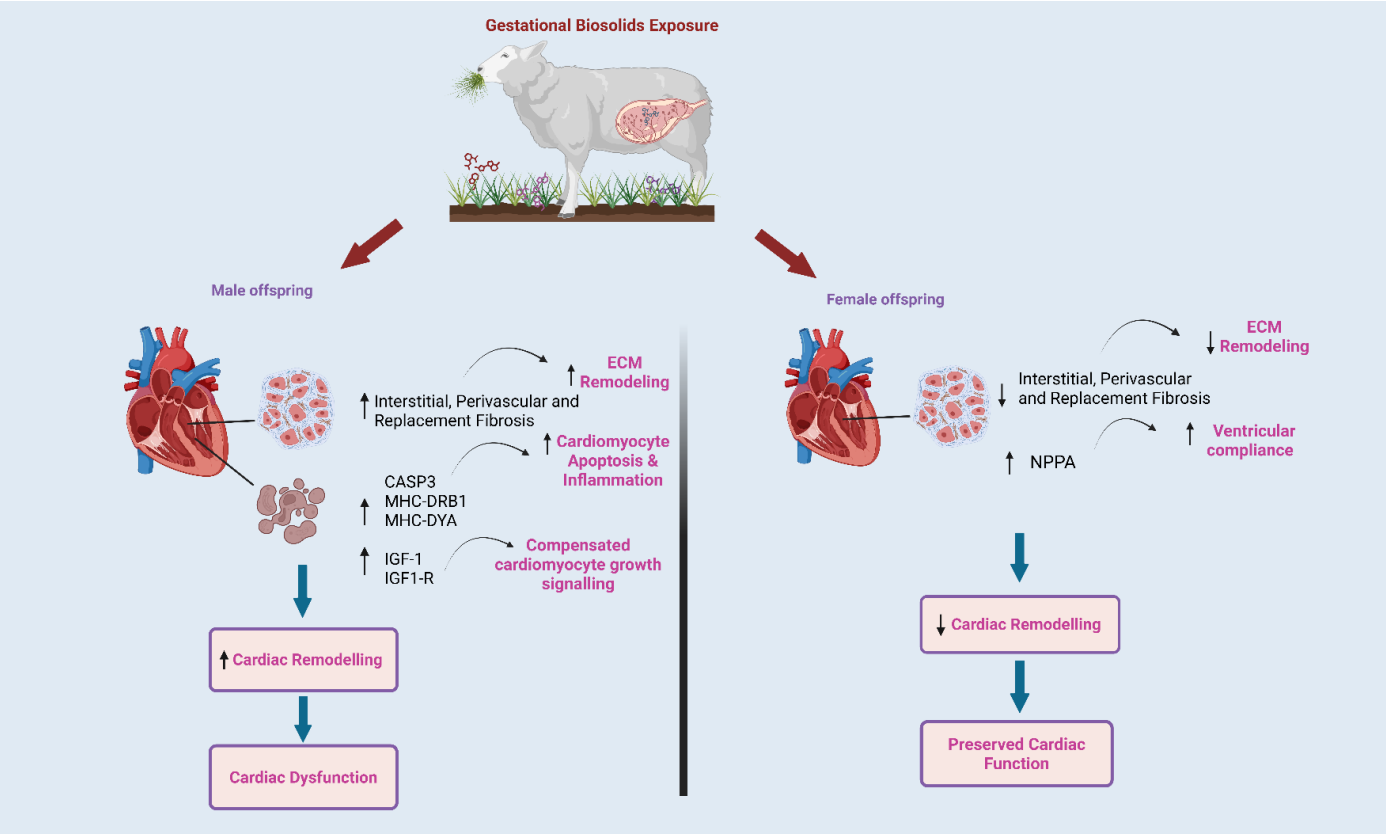

Figure 3-1. Graphical Abstract. Figure generated on BioRender.com.

3.2 Introduction

Cardiovascular diseases (CVD) area major public health concern globally. Sex differences exist in CVD risk, with males at greater risk than females (Ghali et al., 2003), however underlying mechanism and aetiology of such differences are not as well known (Arata et al., 2023). Recent studies suggest that while 2%–7% of the general population have no CVD risk factors, over 70% are exposed to multiple modifiable and non-modifiable risk factors (Dahlöf, 2010). One such modifiable, non-communicable, CVD risk factors, is exposure to environmental chemicals (ECs). The environment contains a ubiquitously mixture of ECs including persistent organic pollutants (PCBs, dioxins, pesticides, flame retardants), bisphenol A, phthalates, triclosan, heavy metals (arsenic, lead, mercury), air pollutants (including particulate matter, poly aromatic hydrocarbons (PAHs), perfluoroalkyl substances (PFAS), bacterial toxins and tobacco smoke ((Bodin et al., 2015b; Lavezzi and Ramos-Molina, 2023). Many of these ECs are suspected or known to have endocrine disrupting actions (Diamanti-Kandarakis et al., 2009; Klingelhöfer et al., 2025).

Relative to the origin of CVD, adverse in-utero exposure to various insults including exposure to exogenous chemicals during development can lead to changes in fetal organ structure and function, and subsequently increased risk of adverse health outcomes, such as CVD, in later life (Barker et al., 1993; Grandjean et al., 2008; Heindel et al., 2015). Several preclinical and clinical studies have demonstrated that in-utero exposure to a single EC can affect CV function mediated via a variety of mechanisms. For instance, prenatal exposure to the synthetic oestrogen diethylstilbesterol (DES) is associated with a higher risk of coronary artery disease and myocardial infarction in the offspring (Troisi et al., 2018). Similarly, in-utero exposure to bisphenol A (BPA) has been linked with changes not only in maternal blood pressure, cardiac microRNA expression but also induce fetal cardiac fibrosis in rats (Rasdi et al., 2020). In-utero exposure to BPA is reported to alter fetal cardiac transcriptome (Chen et al., 2025b; Koneva et al., 2017), disrupt key genes and pathways that predispose individuals to

diabetic cardiomyopathy (DCM) (Lingjuan et al., 2025), fetal and adult cardiac fibrosis (Belcher et al., 2015) and increased expression of genes implicated in cardiac hypertrophy (Chapalamadugu et al., 2014). Bisphenol S (BPS) exposure has also been reported to alter haematological parameters in adulthood and increase CVD risk (Pal et al., 2017). Exposure to ECs such as heavy metals (aluminium, lead, cadmium) during early life and adulthood have been associated with cardiac fibrosis, structural and mitochondrial abnormalities, dyslipidaemia, and myocardial inflammation (Jabeen et al., 2023; Jabeen et al., 2022; Lin et al., 2023b; Liu et al., 2023; Sajjad et al., 2019).

In vitro studies in human cardiomyocytes have also reported adverse effects of individual and EC mixture exposures. For instance, cardiomyocyte toxicity following exposure to BPS, phthalates and microplastics have been reported (Lee et al., 2025; Ma et al., 2025b; Zhou et al., 2025). Exposure to BPA and its analogues have been found to be associated with delayed repolarization, and pro-arrhythmic effects whereas exposure to Perfluoroalkyl sulfonate (PFOS) reduces the viability of cardiomyocytes and promote apoptosis (Ma et al., 2023; Wang et al., 2024). Exposure to Bisphenols A, S and F mixture results in additive adverse effects on cardiomyocyte differentiation and a significant increase in type 1 and 3 collagen expression (Zhou et al., 2021) stressing the need to study EC mixture effects.

Apart from adverse in-utero impact of ECs on CV system in offspring in animal studies, and in vitro cardiomyocyte studies mentioned above, diverse effects on CV functioning and risk factors have also been reported in human epidemiological studies. For instance, a positive association has been reported between serum BPA concentrations and increased risk of hyperlipidaemia, known risk factors for CVD (Yao et al., 2022); poor cardiovascular function during childhood and adolescence were found to be associated with methylmercury (Hg) exposure (Lopes-Araújo et al., 2023); insulin resistance and cardiovascular mortality were linked to phthalate exposure in affected population (Bulka et al., 2019). Studies addressing the sex specific effects of ECs on CV system are also conflicting in that either sex are affected differently in different studies (Aboul Ezz et al., 2015;

Belcher et al., 2015; Bruno et al., 2019; Gao et al., 2015; Lejonklou et al., 2017; Xu et al., 2022). While the effects of in-utero EC exposure on the fetal environment and development and adult consequences are not fully understood, EC's have been linked with changes in gene expression, altered protein expression, and the number and/or location of cells during fetal development (Barouki et al., 2012).

Of even more importance, most studies have investigated the impacts of exposure to single ECs, in a single sex on the CV system predominantly in rodent species. Studies addressing sex specific effects of EC mixture such as that occurring in real-life on CV system are of importance as ECs, even at low individual concentrations, can exhibit additive, synergistic or antagonistic effects (Demeneix and Slama, 2019). Such real-life exposure models are limiting. One model of real-life EC exposure is the biosolids treated pasture (BTP) sheep model. Biosolids, which is derived from human wastewater treatment, contain a complex mixture of low concentrations of ECs (Newmeyer et al., 2024) and is commonly used as a fertilizer in agriculture and land remediation. The composition and relevant concentrations of different ECs present in biosolids has been summarised by (Popoola et al., 2023) and includes BPA, PFAS, phthalates and many heavy metals. We recently reported sex specific adverse CV functional outcomes from prenatal exposure to biosolids, born to sheep grazed on BTP (Khan et al., 2025a). Other studies conducted as part of the same long-term experiment with the BTP model, have documented adverse effects on testicular and hypothalamic gene expression (Hasankolaei et al., 2025), metabolic systems (Filis et al., 2019; Ghasemzadeh-Hasankolaei et al., 2024), offspring growth dynamics (Evans et al., 2023) and lipid profile (Thangaraj et al., 2025). The aim of current study was to investigate possible underlying mechanisms through which developmental exposure to a real-life EC mixture could affect CV in adult male and female offsprings.

3.3 Material and methods

3.3.1 Ethical statement

All the experimental work was conducted in accordance with the United Kingdom's Animals (Scientific Procedures) Act 1986, under Project License PF10145DF. Experimental animals were kept at Cochno Farm and Research Centre, University of Glasgow, under normal husbandry practices. All study animals were treated humanely with due consideration to the alleviation of pain, suffering, distress and lasting harm throughout the study.

3.3.2 Experimental animals and study design

Easy-care ewes (n=320) were randomly allocated into two groups. The first group, designated as control (C) (n=160), was grazed on pastures treated with inorganic fertilizer. The second group, designated as biosolids (B) (n=160) was grazed on biosolids treated pastures (BTP) where biosolids were applied at conventional rates (4 tonnes/ha) twice a year in April and September. The ewes in each group were of equal parity, and there were no significant differences in body condition between the groups before conception or at the time of parturition. To control for potential paternal effects, ewes were mated by artificial insemination using semen from four unrelated rams raised exclusively on C pasture. This gave rise to four different sire groups/families within the first generation (F1) offspring. The pregnant ewes were maintained on their respective pastures until approximately two weeks before parturition, when they were housed and fed according to normal husbandry practice with B ewes fed silage harvested from BTP. Post-parturition, both C and B ewes (and respective C and B F1 lambs) were maintained together outdoors on C pastures up until weaning. After weaning, male and female offsprings were maintained separately on C pastures.

3.3.3 Animal euthanasia and tissue sampling

At approximately 2.5 years old, a subgroup of the F1 offspring (Male: C n=9, B n=12; Female: C n=10, B n=10) adult (avoiding siblings) were euthanized by intravenous administration of barbiturate (140 mg/kg Dolethal, Vetroquinol, UK).

Prior to euthanasia, males and females were weighed and jugular blood samples collected (BD Vacutainer Plus, BD, USA) Blood samples were centrifuged (3000×*g*, 15 min at 4 °C) and plasma harvested and stored at -20°C for later use in lipid profile analysis. Hearts from each animal were harvested, allowed to drain completely of blood, trimmed of pericardial fat, and weighed. After weighing, hearts were dissected and a tissue slice from apical region of left ventricular wall was fixed in 10% neutral buffered formalin (NBF, Thermo Scientific-16499713) for histological studies. Separately, left ventricle tissue samples from each animal were frozen in liquid nitrogen and stored at -80°C for cardiac gene expression studies.

3.3.4 Left ventricle histology

For each animal, two 5µm thick sections, 50µm apart were cut using a Microtome (Leica Biosystems, model RM2125RT), mounted and subjected to H&E staining for the determination of cardiomyocyte (CM) count per field (Fig.3-3A). For each animal, six images at 20x magnification were acquired (3 images per each section) using Leica DM4000B microscope. Cardiomyocyte number in each image was quantified using image analysis software QuPath (Version: 0.3.2) and averaged for each animal. An additional 5µm thick section was stained with Masson's trichrome for the assessment of fibrosis. After staining with Masson trichome, complete slide scans were obtained and were scored blindly by a veterinary anatomical pathologist on an ordinal scale (0-5) (Pawlinski et al., 2002).The different fibrosis scores were described as "0" (No obvious signs of fibrosis), "1" (Perceptive), "2" (Mild), "3" (Moderate), "4" (Severe) and "5" (Very Severe). In addition, different types of myocardial fibrosis were assessed and categorized as interstitial, perivascular, or replacement fibrosis, as well as a combination of these types. Interstitial fibrosis refers to collagen deposition between cardiomyocytes, perivascular fibrosis which involved accumulation of collagen around blood vessels, and replacement fibrosis where damaged or dead cardiomyocytes were substituted by fibrotic tissue.

3.3.5 mRNA extraction, cDNA synthesis, and quantitative real- time (qRT) PCR

For mRNA extraction, samples were homogenized in 500µl TRIzol® reagent lysis buffer using a FastPrep-24 5G homogenizer (MP Biomedicals, Germany) at 4 m/s for 45 seconds as described previously (Hasankolaei et al., 2025) and mRNA extracted using Qiagen RNeasy® RNA extraction mini kit spin columns (Qiagen, Hilden, Germany) as per the manufacturer's instructions. RNA purity was assessed with a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA). For cDNA synthesis, 500ng of mRNA was reverse transcribed using a QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). Among the different markers/genes studied included Atrial Natriuretic Peptide Precursor A (NPPA), Atrial Natriuretic Peptide Precursor B (NPPB) which encodes Atrial Natriuretic Peptide (ANP) and Brain Natriuretic Peptide (BNP) respectively and are key regulators of cardiac function. Other CV function genes investigated were Serine/Threonine Kinase 1 (AKT1), Collagen type 1 alpha 1 (COL1A1), Collagen type 3 alpha 1 (COL3A1), Mechanistic Target of Rapamycin Kinase (mTOR), MYC, protooncogene transcription factor (cMYC), BCL2 Associated X, apoptosis regulator (BAX), Caspase3 (CASP3), MHC Class II antigen DY alpha (DYA), MHC-DR beta chain 1 (DRB1), Bos Taurus solute carrier 2 member 4 (SLC2A4), Insulin like growth factor 1 (IGF-1), Insulin like growth factor 1 receptor (IGF1-R), Estrogen receptor 1 (ESR1) and Estrogen receptor 2 (ESR2). qPCR was performed using 12.5ng/µl of cDNA template, 9µl of master mix consisting of SYBR green, primers for target genes (Table 3-1) and RNAase free water on a Stratagene 3000 qPCR machine. All the primers were designed using NCBI Primer-BLAST and had been previously validated in their respective positive controls. Mean cycle threshold (CT) values for each sample were calculated, and relative expression for each marker was quantified after normalization to the expression of a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). GAPDH was selected as a reference gene owing to its greater stability which has been previously validated in normal and diseased human heart tissue (Li et al., 2017) as well as in sheep cardiac tissue samples (MohanKumar et al., 2017; Vyas et al., 2016). Its stability was confirmed by lack

of significant variation in the CT values between the control and treatment groups in the data set.

3.3.6 Lipid profile assessment

Plasma triglycerides (TG) and total cholesterol (TC) concentrations were analysed by the Veterinary Diagnostic Services Laboratory of the University of Glasgow, School of Biodiversity, One Health and Veterinary Medicine (Lab Ref:573311) (https://www.gla.ac.uk/schools/bohvm/vetdiagnostics/). Detection limits (TG=≤0.1mmol/L, TC=≤0.5mmol/L) %CV (TG=≤5%, TC=≤3%).

Table 3-1. Cardiovascular function markers/genes and associated qPCR primer sequences

Marker/Gene	Forward sequence	Reverse sequence	Accession No.
Natriuretic Peptide A (NPPA)	CCTCCTCTTTGTGGCGTTTC	TTGTCCTCCAAACGGTCCAG	NM_001160027.1
Natriuretic Peptide B (NPPB)	CCTGCTTCTCCTCTTCTTGC	GAGTCCCAGGTTTCTTCCAG	NM_001160026.1
Serine/threonine kinase 1 (AKT1)	CTTCATCATCCGCTGCCTG	AAGTCCATCGTCTCCTCCTC	NM_001161857.1
Collagen type I alpha 1 (COL1A1)	CTGCCCTTTCTGCTCCTTTC	ACTTGGGTGTTTCGGCATTG	XM_027974705.2
Collagen type III alpha 1 (COL3A1)	ATCTCCTGGTTCAAGCGGTG	ACCCATTTCACCTTTGCCAC	XM_004004514.5
Mechanistic target of rapamycin kinase (mTor)	AATGCTATGGAGGTCACGGG	TCCATCAGCCTCCAGTTCAG	NM_001145455.1
BCL2 associated X, apoptosis regulator (BAX)	GCCTCCTCTCCTACTTTGGG	CTCAGCCCATCTTCTTCCAG	XM_027978592.2
Caspase 3 (CASP3)	GCTATGGGTGTGTGTGGAAG	TGATGACTCTTACCCTCTTTGG	XM_015104559.3
MHC class II antigen DY alpha (DYA)	CCCATCTTGACCTTGACTGAG	ATTCCTCCATAAAGTCTGCCC	NM_001123398.2
MHC DR beta chain 1 (MHCII-DRB1)	AAGATGATGAGTGGAGTTGGG	CTGTTGGCTGAAGGGTAGGG	NM_001123402.1

Bos taurus solute carrier family 2 member 4 (SLC2A4)	GCTTGGCTTCTTCATCTTCACCTT	TGCTCAGACCACCCTTCCCTCCAG	NM_174604.1
Insulin like growth factor 1 (IGF1)	TCACATCCTCCTCGCATCTC	CACACGAACTGGAGAGCATC	NM_001009774.3
Insulin like growth factor 1 receptor (IGF1R)	TCAAGGACATCGGGCTCTAC	ATGTAGTTGTTGGATGCGGC	XM_027957015.2
MYC proto-oncogene, bHLH transcription factor (MYC)	GCTAAGTTGGACAGTGGCAG	GTGTCCGCCTCTTGTCATTC	NM_001009426.1
Estrogen receptor 1 (ESR1)	ACTGTGCAGTGTGAATGAC	TATAAAACCAAGCCTCACCT	XM_042253635.1
Estrogen receptor 2 (ESR2)	CCAGGGAAGACAGGAACACT	CTGAACCTGACACGCTGATG	XM_042251939.1
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	ATGCCTCCTGCACCA	AGTCCCTCCACGATG	XM_060411594.1

3.3.7 Statistical analysis

Heart weight (HW), heart to body weight ratio (HBR), cardiomyocyte number, gene expression data and lipid profile were analysed by Two-way ANOVA considering sex and treatment as explanatory variables (Prism Windows 5.04). Bonferroni post-hoc tests were applied for pairwise comparisons of group means. Ordinal (fibrosis scores) and categorical data (fibrosis types) were analysed separately for males and females using the Mann–Whitney U test and Fishers exact test in R (RStudio version 2022.02.2+485) respectively. In view of the variability associated with outbred species and the small sample size, sex-specific analyses were also undertaken and data analyzed by using a student t-test for which normality was assessed using Shapiro wilk test. Statistical significance was defined as $P < 0.05$. All graphs were generated in GraphPad (Prism Windows 5.04) and expressed as mean ± SEM. Heatmaps, principal component analysis (PCA) and

partial least squares – discriminant analysis (PLS-DA) were performed in Metaboanalyst (https://www.metaboanalyst.ca/).

3.4 Results

3.4.1 Impact of in-utero B exposure on heart weight and heart to body weight ratio

Two-way ANOVA revealed a significant effect of sex (P<0.05) on mean body weight and heart weight which was significantly greater in males than that of females (Fig.3-2A). There was no significant effect of exposure or exposure-sex interaction (p>0.05) in mean body weight in males (C=85.94±3.83, B=90.41±2.31) and females (C=76.9±3.27, B=73.11±3.62) and heart weight. Heart to bodyweight ratio (HBR) was not significantly (P>0.05) affected by EC exposure, sex and there was no exposure by sex interaction (Fig.3-2B). Sex stratified analyses also found no significant effect of exposure in either sex.

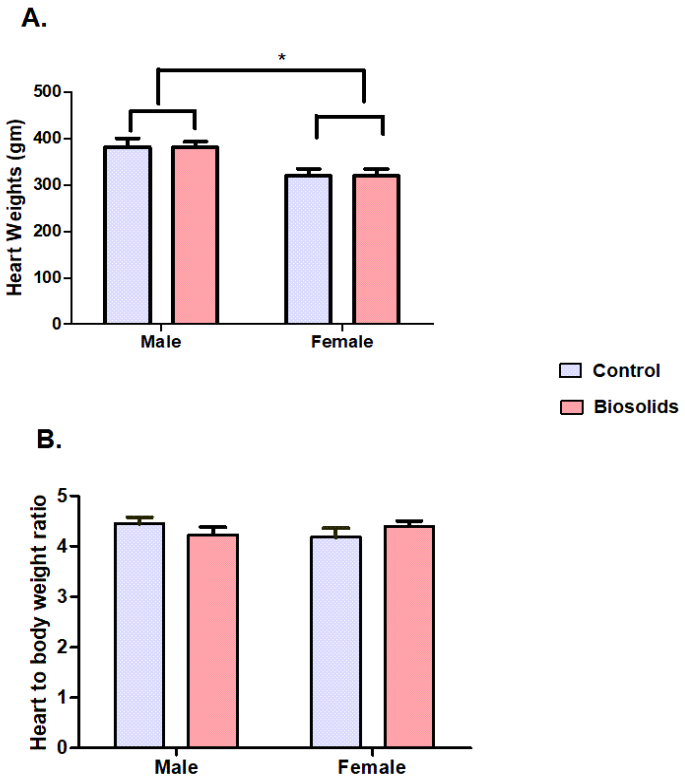

Figure 3-2. Heart weights (A) show no significant effect of exposure (P= 0.97), while significant (P= 0.0002) sex effect was observed. (B) Heart to bodyweight ratio shows no significant effect of exposure (P=0.9), sex (P=0.7) or exposure-sex interaction (P=0.1). * P<0.05. Data analysed by Two-way ANOVA and presented as Mean ± SEM.

3.4.2 Cardiomyocyte count

Results from Two-way ANOVA demonstrated an apparent pattern ($p = 0.09$) towards a sex difference, with males exhibiting a lower number of cardiomyocytes/field compared to females (Fig.3-3B) and no significant exposure effect or exposure-sex interaction ($p>0.05$). Sex stratified analyses revealed no significant effect of exposure ($p>0.05$) on cardiomyocyte number in either sex.

3.4.3 Impact of in-utero B exposure on LV collagen deposition

Representative Masson trichome stained images from Control and Biosolids exposed males and females are shown in Fig. 3-4. No statistically significant ($P>0.05$) exposure or sex difference was observed in collagen scores (Fig. 3-5A). Sex specific analysis found the mean fibrosis score to be higher in the B compared to C group, in both males (C = 2.65 ± 0.28, B = 2.83 ± 0.40, d = 0.3) and females (C = 2.20 ± 0.35, B = 2.40 ± 0.33, d=0.1). In the males, there was also a significant effect of EC exposure in the qualitative distribution of fibrosis (p=0.04), with more advanced form of fibrosis (interstitial+perivascular+replacement) seen in B compared to C group (Fig.3-5-B).

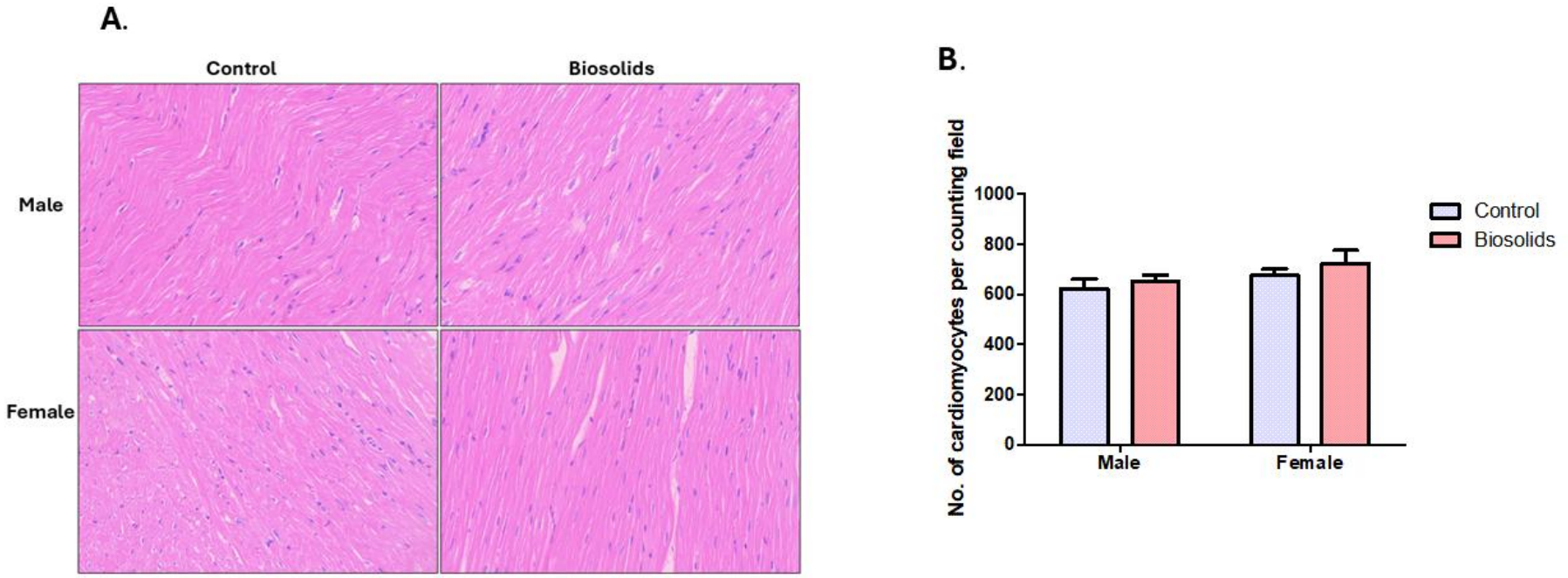

Figure 3-3. Haematoxylin and Eosin-stained images from C and B males and females (A) and Mean Cardiomyocyte number (B). No significant difference was seen in cardiomyocyte count per field in C and B in both sexes, P-value exposure (P=0.26), sex (P= 0.09), interaction P= 0.81). Data analysed by Two-way ANOVA and presented as Mean ± SEM.

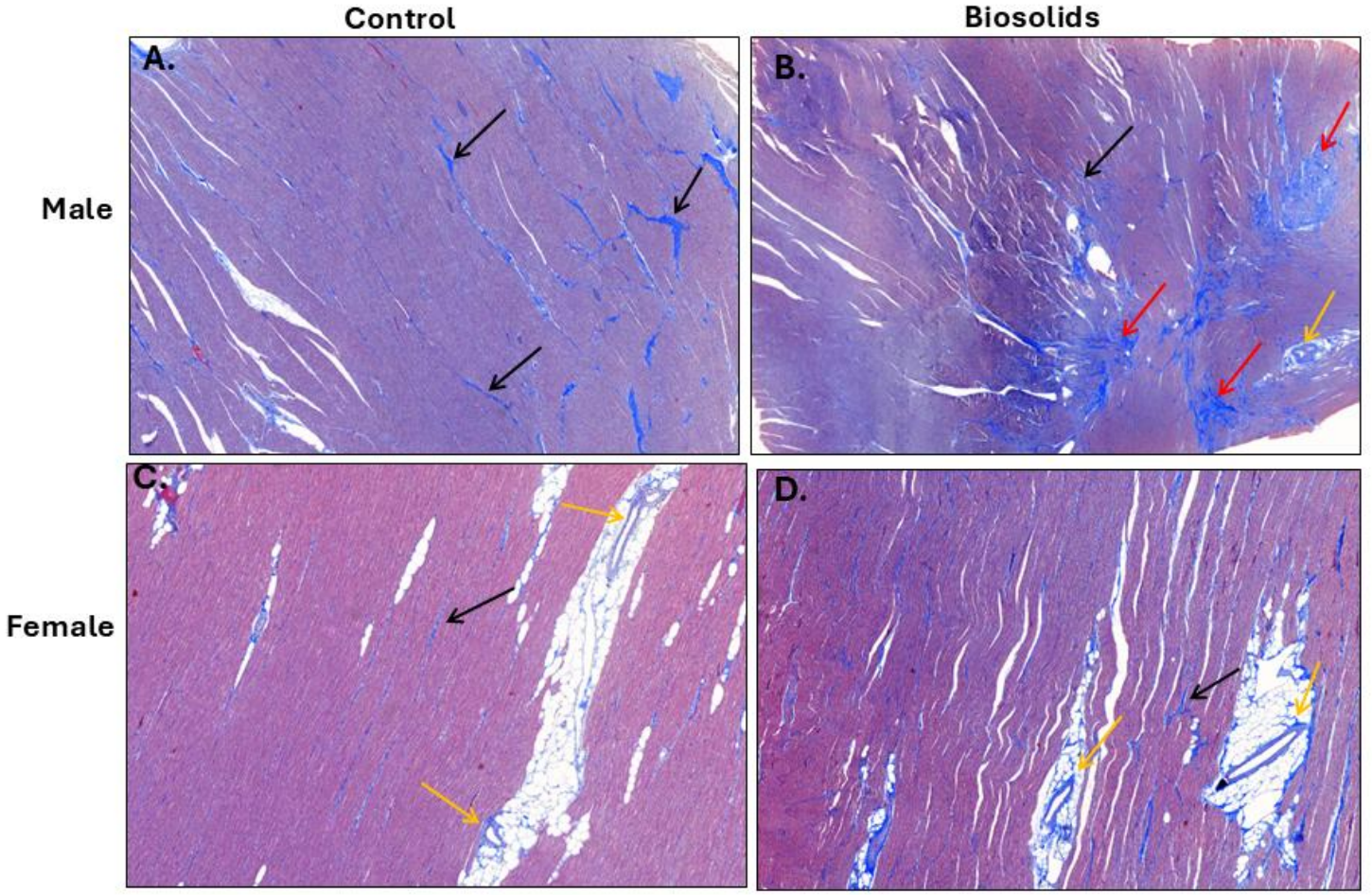

Figure 3-4. Representative Masson trichome stained images from Control and Biosolids exposed males and females showing interstitial (black arrows), Perivascular (orange arrows) and Replacement (red arrows) fibrosis. A) Diffuse interstitial only fibrosis (Score=3, Moderate). B) Interstitial, perivascular and replacement fibrosis (Score=5, Very Severe). C) Interstitial and perivascular fibrosis (Score=2, Mild). D) Perivascular and interstitial fibrosis (Score=4, Severe). Scale bar=50μm.

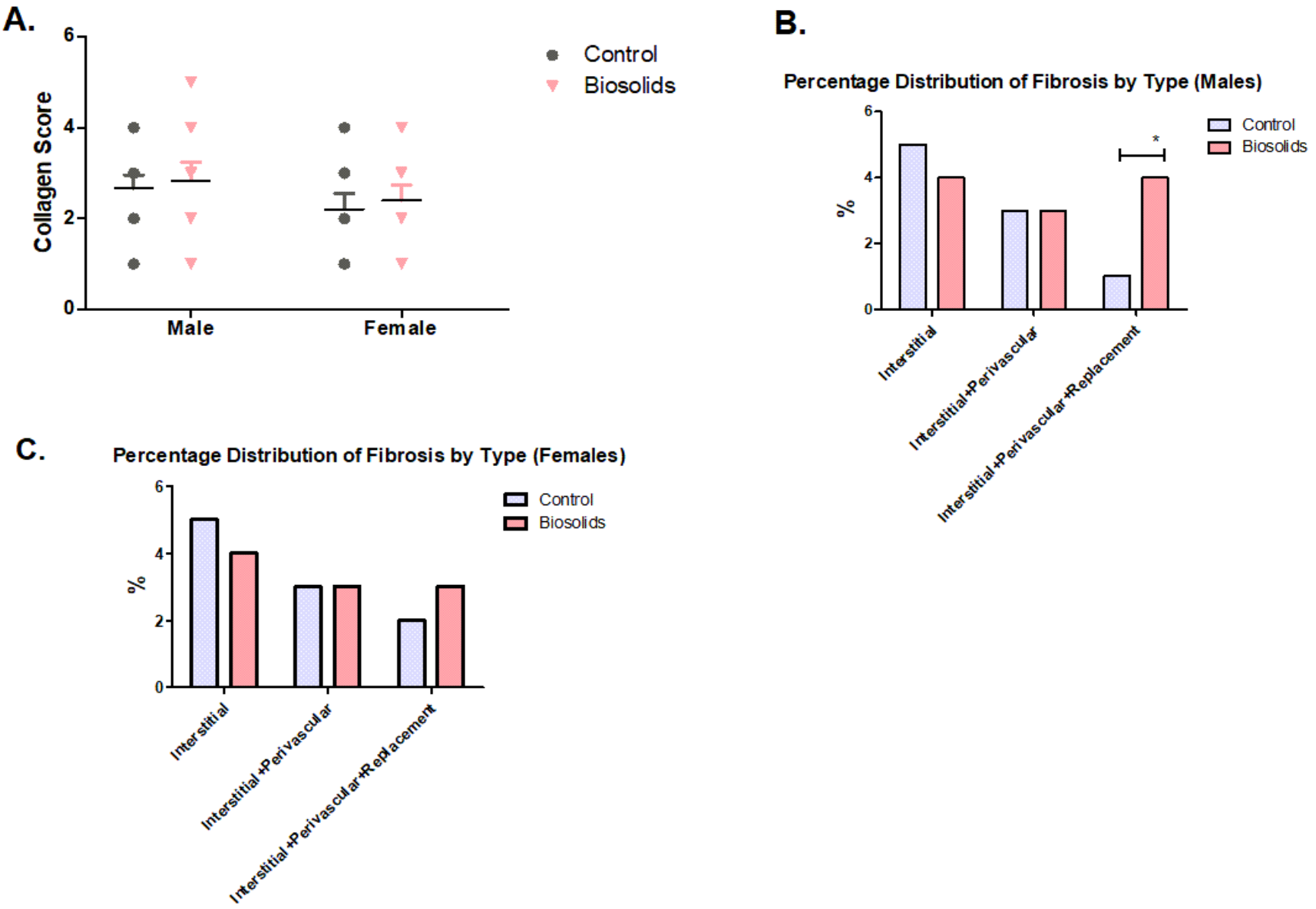

Figure 3-5. Collagen Score (A) and relevant percentage of fibrosis types in males (B) and females (C) showing high levels of mixture of interstitial, perivascular and replacement fibrosis in B groups. * Indicates $p<0.05$.

3.4.4 Impact of in-utero B exposure on molecular pathways involved in LV structure and function

3.4.4.1 Molecular markers of cardiac stress and fibrosis

Two-way ANOVA unveiled an overall, significant sex difference in the expression of mRNA for NPPA and NPPB ($p < 0.05$) (Fig. 3-6A-B). There was no significant effect of EC exposure or an EC exposure-sex interaction on the expression levels of either NPPA or NPPB ($P>0.05$). Sex stratified analysis further revealed an apparent pattern ($p=0.08$) towards an increased expression of NPPA in B compared to C females (Fig.3-6A). ANOVA revealed significant sex differences in mRNA expression of cardiac fibrosis markers COL1A1 and COL3A1. COL1A1 expression was significantly higher in males, whereas COL3A1 was higher ($P < 0.05$) in females (Fig. 3-6C-D). Again, no significant effects of EC exposure or EC exposure-sex interaction were observed in the expression of COL1A1 and COL3A1. Sex specific analysis revealed an apparent pattern towards increased COL1A1 expression ($p=0.1$, $d=0.6$) in B compared to C males while there were no significant exposure effects on COL3A and COL1A1 in females.

3.4.4.2 Apoptotic markers

No significant exposure, sex effect or EC exposure-sex interaction was observed in the expression of BAX or CASP3 as indicated by Two-way ANOVA (Fig.3-7A). Sex specific analysis showed that mean CASP3 expression was significantly ($P<0.05$) upregulated in the B compared to C in males, but no significant EC exposure effect was observed in females (Fig.3-7B).

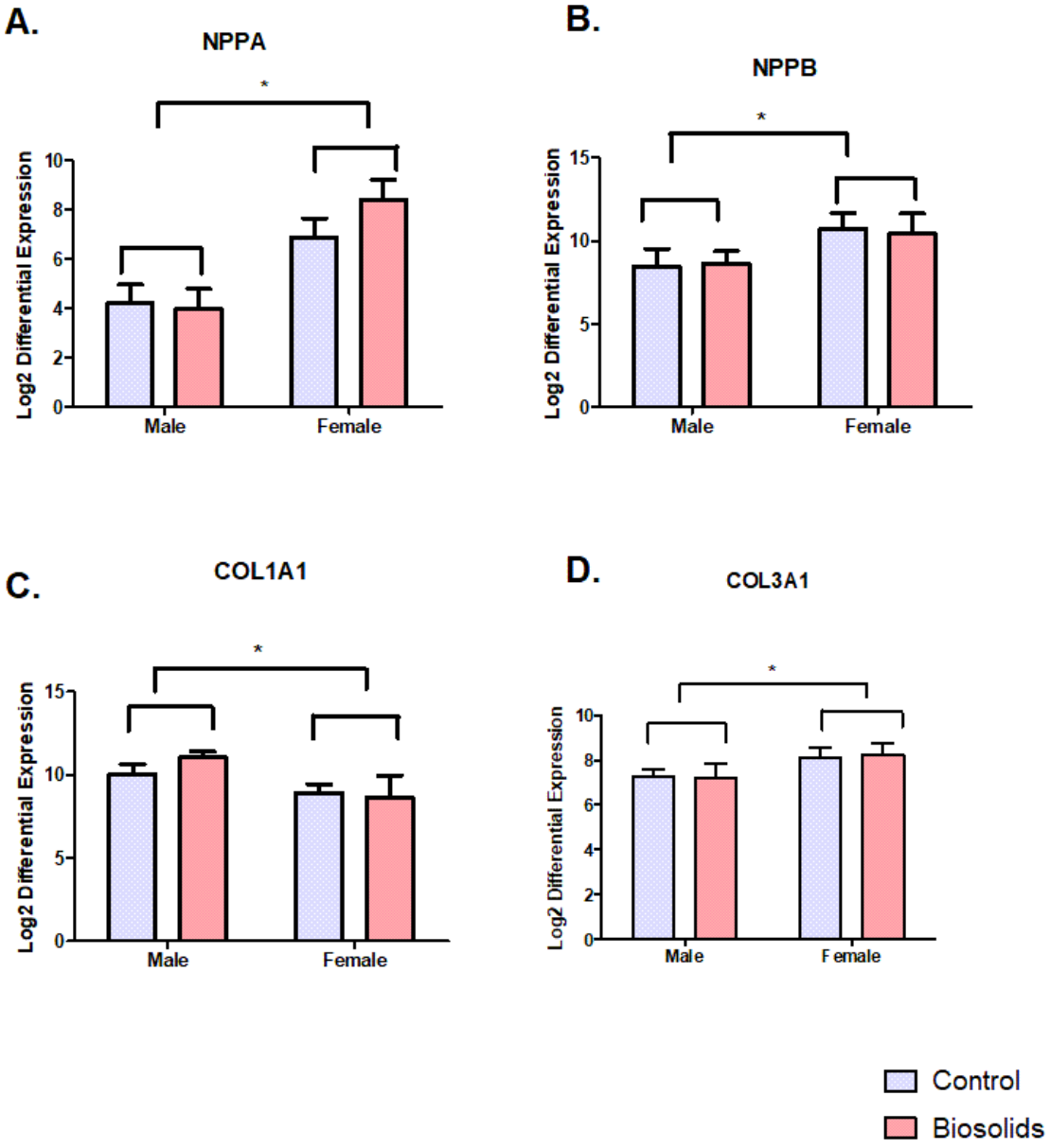

Figure 3-6. Mean relative expression levels of cardiac stress and fibrosis markers (relative to GAPDH). (A) Atrial Natriuretic Peptide Precursor A (NPPA) expression was significantly higher in females than males; P-value Sex= 0.0001, Exposure=0.41, Interaction= 0.26. There was a trend for NPPA being highly expressed in B females compared to C upon sex-specific analysis (P=0.08). (B) Atrial Natriuretic Peptide Precursor B (NPPB) was also highly expressed in females relative to males; P-Value Sex= 0.05, Exposure=0.95, Interaction=0.81. (C) Significant sex effect can be seen in the expression of Collagen type 1 alpha 1 (COL1A1); P-Value Sex= 0.02, Exposure=0.64, Interaction=0.40 and (D) Collagen type 3 alpha 1 (COL3A1); P-Value Sex= 0.05, Exposure=0.90, Interaction=0.86. Males (n C=9, B=12), Females (n C=10, B=10). * Indicates P<0.05. Data analysed by Two-way ANOVA and shown as Mean ± SEM. Sex-specific analysis were performed using a student t-test.

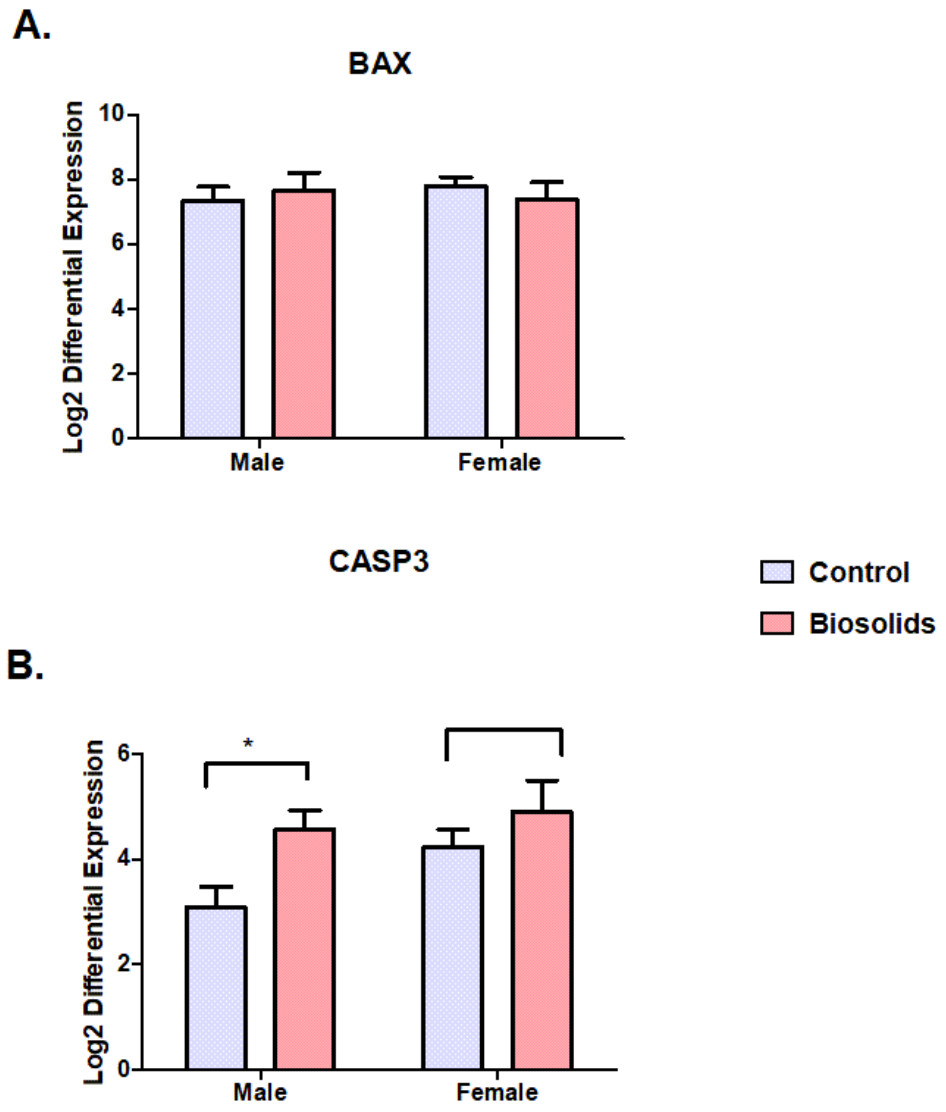

Figure 3-7. Relative expression of key cardiac apoptotic markers. (A) No significant effect of exposure, sex or exposure-sex interaction was noted in the expression of BCL2 Associated X, apoptosis regulator (BAX); P-Value, Exposure=0.89, Sex= 0.84, Interaction= 0.45. (B) Significant exposure effect in Caspase3 (CASP3) was observed; P-Value Exposure=0.01, Sex= 0.10, Interaction=0.36 along with significant sex-specific effect of exposure in males (P=0.01). Males (n C=9, B=12), Females (n C=10, B=10). * Indicates P<0.05. Data analysed by Two-way ANOVA and shown as Mean ± SEM. Sex-specific analysis were performed by using student t-test.

3.4.4.3 Markers of cardiac hypertrophy and cardiomyocyte growth

Two-way ANOVA revealed no significant effects of EC exposure, sex, or EC exposure sex interaction ($P > 0.05$) with regard to the expression of the AKT1 and cMYC (Fig.3-8B-C). A significant effect ($P<0.05$) of sex was observed in the expression of mTor, IGF-1 and IGF1-R, levels being higher in females compared to males (Fig.3-8A, D, E). A significant ($P<0.05$) effect of EC exposure and an EC exposure-sex interaction ($P<0.05$) was observed in the expression of IGF-1, which was significantly higher in the B compared to C males but unaffected in females (Fig.3-8D). Finally, a significant EC exposure-sex interaction ($P<0.05$) was seen in IGF1-R expression, with B males having higher levels of IGF1-R expression compared to C males, and B females exhibiting lower levels of IGF1-R expression compared to C females (Fig.3-8E).

3.4.4.4 Markers of cardiac inflammation

There was an overall significant effect of EC exposure in the expression of MHCII-DRB1and DYA which were observed to be significantly higher in the B compared to group as shown by Two-way ANOVA, with no exposure-sex interaction ($p>0.05$). A significant sex effect was also observed in the expression of DRB1 and DYA, with females having significantly ($p<0.05$) higher levels of mRNA expression, of both markers, compared to males (Fig. 3-9 A-B). Sex stratified analysis revealed significantly higher ($p<0.05$) expression of DRB1 and DYA in B compared to C males (Fig. 3-9 A-B).

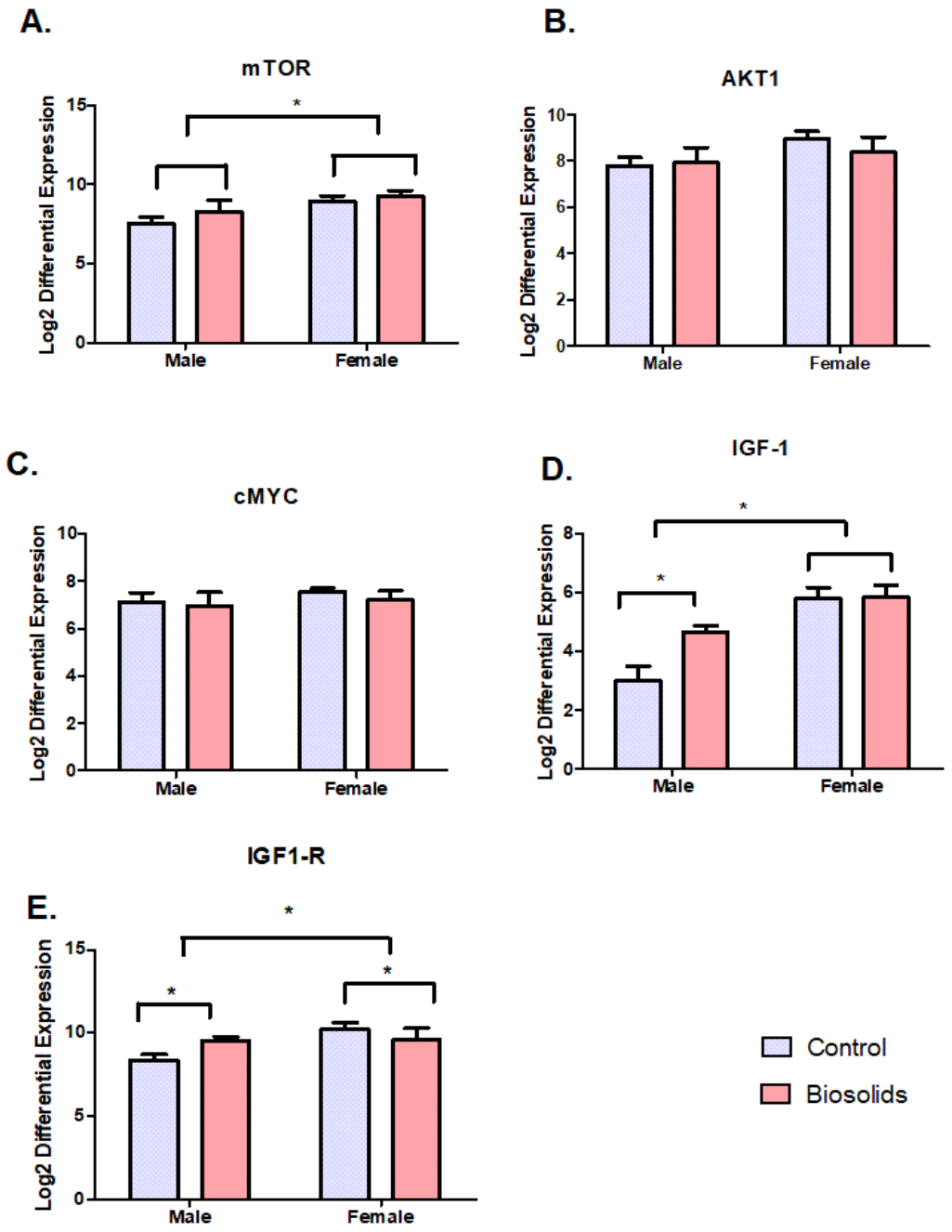

Figure 3-8. Relative expression of markers for cardiac hypertrophy and cardiomyocyte proliferation. **(A) Mechanistic Target of Rapamycin Kinase** (mTor) expression was significantly higher in females relative to males; P-Value Sex=0.02, Exposure=0.33, Interaction=0.69. **(B)** No significant effects of exposure, sex or exposure-sex interaction were noted in the expression of **Serine/Threonine Kinase 1** (AKT1); P-Value Exposure=0.68, Sex= 0.15, Interaction= 0.51 and cMYC **(C),** P-Value Exposure=0.54, Sex= 0.40, Interaction= 0.83. **(D) Insulin like growth factor 1** (IGF-1) expression was significantly higher in B compared to C males and females relative to males P-Value Exposure= 0.03, Sex= 0.0001, Interaction= 0.03. **(E) Insulin like growth factor 1 receptor** (IGF1-R) expression was significantly higher in females compared to males with significant exposure-sex interaction; P-value Exposure= 0.54, Sex= 0.03, Interaction=0.05. Males (n C=9, B=12), Females (n C=10, B=10). * Indicates P<0.05. Data analysed by Two-way ANOVA and shown as Mean ± SEM.

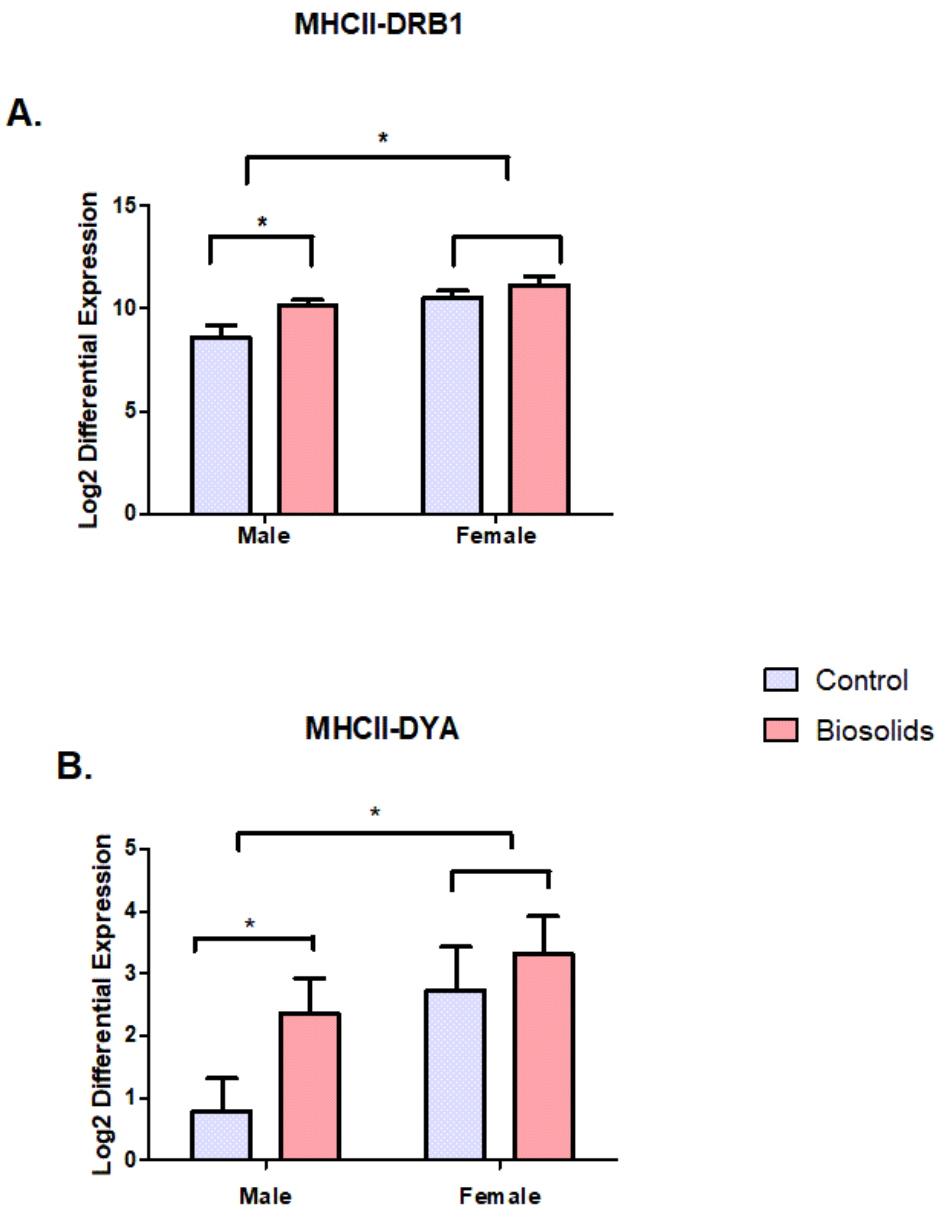

Figure 3-9. Relative expression of markers involved in cardiac Immune/inflammation cascades. **(A) MHC-DR beta chain 1 (**DRB1) was expressed significantly higher in B compared to C group and females relative to males. P-Value, Exposure=0.008, Sex=0.0005, Interaction=0.24. Sex-specific analysis shows significantly upregulated DRB1 levels in B compared to C males (P=0.01). **(B)** MHCII-DYA expression was also seen to be significantly higher in B compared to C group, and overall females relative to males; P-Value Exposure=0.05, Sex=0.02, Interaction=0.42. Upon sex-specific analysis, similar to DRB1, **MHC Class II antigen DY alpha** (DYA) was also significantly upregulated in B compared to C males only (p=0.05). Males (n C=9, B=12), Females (n C=10, B=10). * Indicates P<0.05. Data analysed by Two-way ANOVA and shown as Mean ± SEM. Sex-specific analysis were performed by using student t-test.

3.4.4.5 Glucose metabolism and ER signalling

Two-way ANOVA identified no significant effect of EC exposure ($p>0.05$ but a significant sex effect in SLC2A4. The expression of SLC2A4 was significantly ($P<0.05$) higher in females compared to males. An apparent pattern towards an EC exposure-sex interaction ($P=0.08$) was observed for SLC2A4 expression which was higher in B males but lower in B females, compared to their respective controls (Fig.3-10A). Significant ($P<0.05$) sex differences were observed in the expression of ERα/ESR1 and ERβ/ESR2 both being significantly higher in females compared to males but there was no significant effect of EC exposure or EC exposure-sex interaction relative to the expression of either mRNA (Fig.3-10 B-C). In addition, sex stratified analysis showed no significant EC exposure effect in either sex. A summary of key findings on various markers/genes which were differentially expressed are presented in table 3-2.

Table 3-2. Summary of differential expression of genes/markers related to cardiac function.

Group	Marker/Gene	Change direction	P-value (Exposure)	P-value (Sex)	P-value (Interaction)
C-B (Female) (*t*-test)	NPPA	Up	0.08	-	-
C-B (Male) (*t*-test)	CASP3	Up	0.01	-	-
C-B (Male) (*t*-test)	MHCII-DRB1	Up	0.01	-	-
C-B (Male) (*t*-test)	MHCII-DYA	Up	0.05	-	-
C-B, M-F (Two-way ANOVA)	IGF1	Up in B males	0.03	0.0001	0.03
C-B, M-F (Two-way ANOVA)	IGF1-R	Up in B males, Down in B females	0.54	0.03	0.05

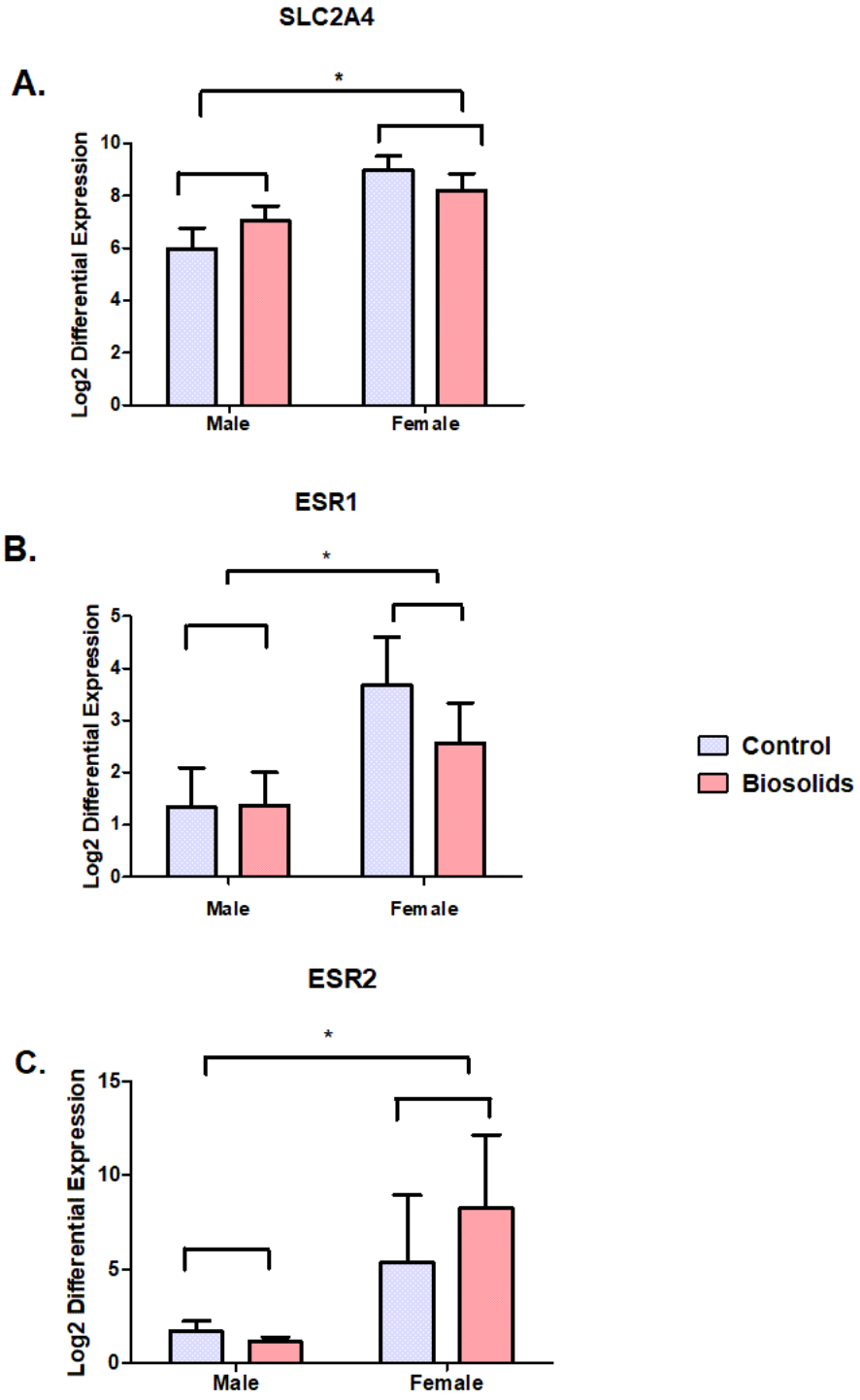

Figure 3-10. Relative expression of genes related to glucose metabolism and estrogen signalling shown. (A) Bos Taurus solute carrier 2-member 4 (SCL2A4) expression was significantly high in females relative to males and a pattern for exposure-sex interaction was noted, P-Value, Exposure=0.81, Sex=0.002, Interaction=0.08. (B) Estrogen receptor-1 (ESR1) and (C) Estrogen receptor-2 (ESR2) expression was significantly high in females compared to males with no effects of B exposure in either sex P-Value ESR1, Exposure=0.48, Sex=0.02, Interaction=0.46, P-Value ESR2 Exposure=0.65, Sex=0.04, Interaction=0.51. Males (n C=9, B=12), Females (n C=10, B=10). * Indicates $P<0.05$. Data analysed by Two-way ANOVA and shown as Mean ± SEM. Sex-specific analysis were performed by using student t-test.

3.4.5 PCA and PLS-DA analysis of gene expression data

Heatmaps showing the differential expression of markers related to CV function, in the C and B males, and females, are presented in Fig. 3-11 (A) and 3-11 (B) respectively. Principal Component Analysis (PCA) revealed no distinct clustering with either the C or B groups. Some animals, however, did sit outside the majority clusters of C and B animals (Appendix Fig 2 & 3). Partial least square discriminate analysis (PLS-DA) revealed partial separation of the C and B groups in the males. Differences in IGF1, IGF1R, COL1A1, CASP3, DYA, DRB1, SCL2A4, NPPA and NPPB expression contributed most strongly towards this group separation, as shown by the length and direction of loading vectors in the biplot (Fig.3-11 C). In the females, differences in the expression level of ESR2 was the major contributor towards group separation, as shown in (Fig. 3-11 D). In PLS-DA, the top genes/ markers differentially expressed in C and B males and females were identified by variable importance in projection (VIP). In the males, the top five differentially expressed genes, expression being higher in the B animals, were IGF1, DYA, MHC1, CASP3 and IGF1-R. In the females the top five differentially expressed genes were ESR-2, NPPA, ESR-1, SCL2A4 and CASP3 (Appendix Fig. 4 & 5).

3.4.6 Plasma lipid profile

Data analysis of the lipid profile revealed significant sex differences in plasma triglycerides (TG) and total cholesterol (TC). TG and TC concentrations were significantly higher in males compared to females. There was, however, no significant effect of EC exposure or exposure-sex interaction in either sex. Sex specific analysis also did not show any EC exposure effect in either sex (Fig.3-12).

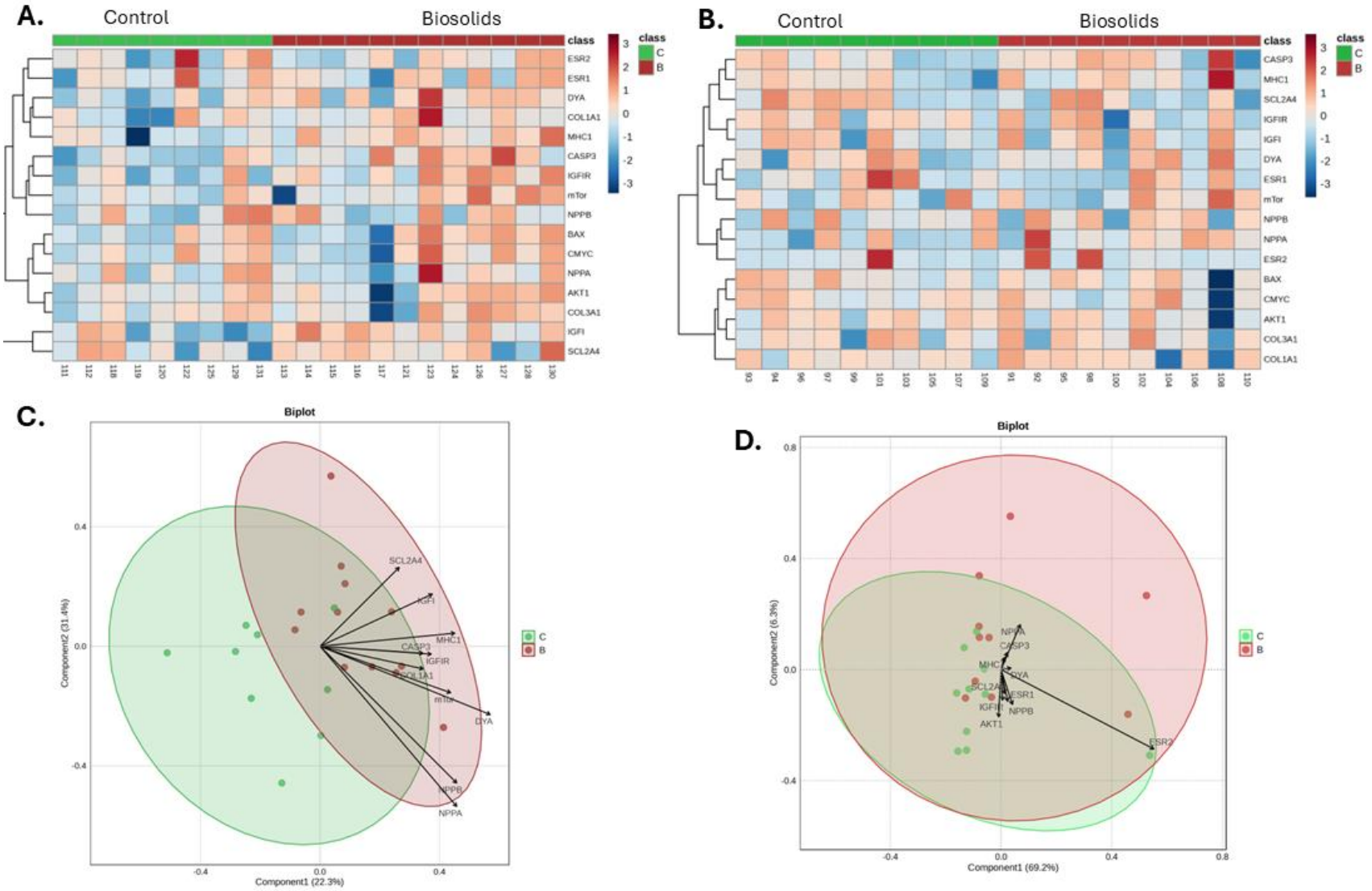

Figure 3-11. Heatmaps from males (A) and females (B) showing differential expression of markers/genes in C and B groups. Biplots from PLS-DA analysis in males (C) and females (D) shows the markers/genes driving group separations in C and B.

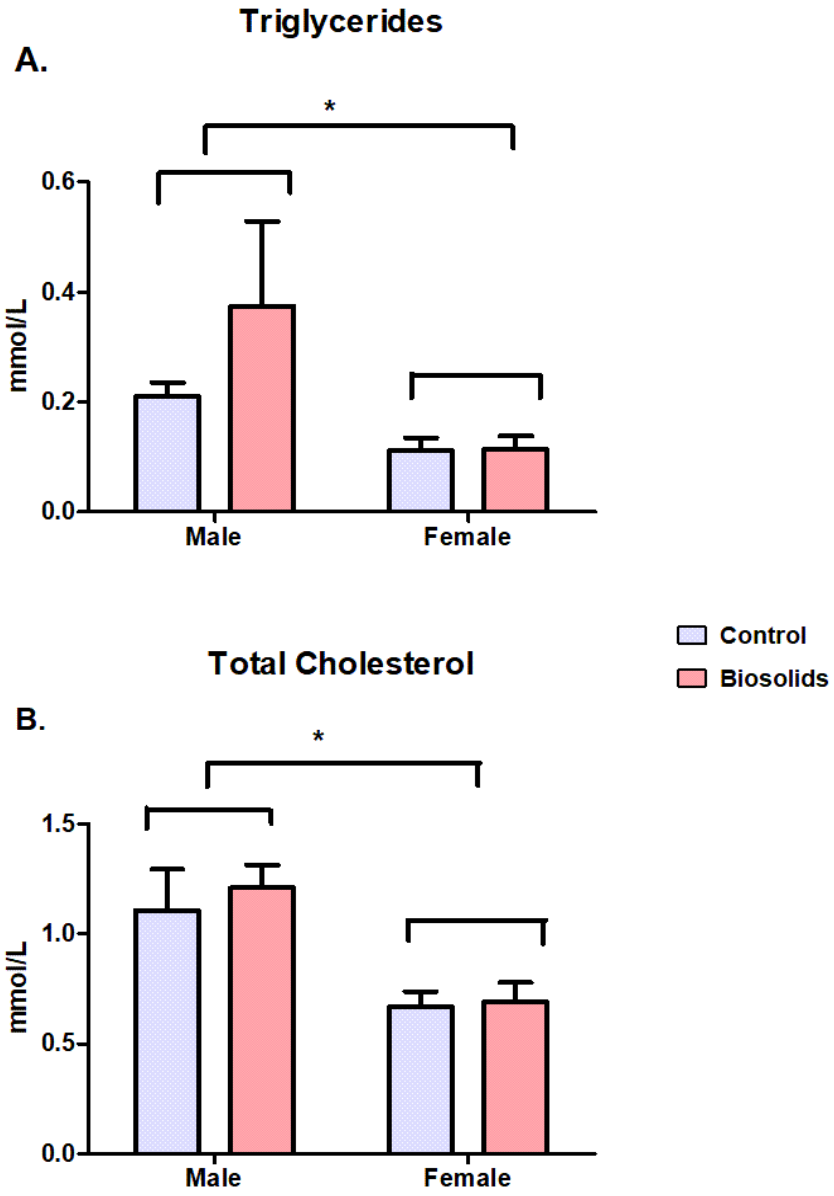

Figure 3-12. Plasma Triglycerides **(A)** and Total Cholesterol **(B)** levels in C and B males and females. P-value, Exposure=0.34, Sex=0.04, Interaction=0.39. * Indicates P<0.05. Data analysed by Two-way ANOVA and shown as Mean ± SEM.

3.5 Discussion

The present study investigated whether maternal exposure to a real-life EC mixture, in the form of grazing biosolids treated pasture, had sex-specific effects on cardiac histology, lipid profile and the expression of genes associated with cardiac structure and function, in adult sheep offspring. Findings revealed significant effects of maternal EC exposure in males in the form of histological changes including more advanced fibrosis, and significantly higher expression of markers implicated in cardiac inflammation and apoptosis (CASP3, DRB1, DYA) along with IGF-1 and IGF1-R, markers for cardiomyocyte growth and survival, indicating a possible compensatory mechanism towards EC induced cardiac remodeling. EC exposed females exhibited less severe form of fibrosis and an apparent pattern towards an increased ANP expression.

As reported in humans (Molina and DiMaio, 2012, 2015; Westaby et al., 2023), mean heart weight in the sheep in the current study was significantly greater in males compared to females. Heart weight, however, was not affected by maternal biosolids exposure. This result is consistent with studies of the effects of prenatal bisphenol-A (BPA) and Di (2-ethylhexyl) phthalate (DEHP) exposure (Wang et al., 2025a), and prenatal exposure to a mixture of poly aromatic hydrocarbons (PAHs) (Zhang et al., 2021) in male and female adult rats. It might be expected that heart weight could be influenced by body size, and that the effects of sex could be due to this relationship, at least in part. Consistent with this premise, when heart weight was assessed as a ratio against body weight, the sex difference seen in the current study was indeed lost, but again there was no effect of EC exposure. In contrast, (Zhang et al., 2021) found a decrease in the HW/BW ratio in male (but not female) mice following in utero exposure to PAH mixture suggestive of potential effects of PAHs on cardiac structure. The lack of such an effect in the males in our study may be reflective of species differences or alternatively the effects of PAHs may have been neutralized by other chemicals present in biosolids. It is important to note that like heart weights, body weights across both sexes were not significantly affected by maternal B exposure in the present study. The lack of an effect of EC exposure on the

weight of the heart is of interest when seen alongside the results of functional studies conducted in the same animals, where we reported significantly increased left ventricular dimensions, end-diastolic and systolic volumes, and cardiac output in EC exposed males (Khan et al., 2025a). The observation of these functional changes despite heart weight being unaffected suggests that EC exposure may differentially impact aspects of cardiac structure and or cardiac function. This possibility is supported by findings from a study on gestational rosiglitazone treatment where no significant change in heart weight was noted despite a decrease in the size of fetal cardiomyocytes in sheep (Lie et al., 2014). Such discrepant finding of disruption in cardiac function without presence of cardiac hypertrophy as noted by no change in cardiac weight could also signify that there may be underlying compensatory mechanism preceding the onset of hypertrophy.

In response to physiological and pathological stimuli, it is known that cardiac remodeling can occur including changes in cardiomyocyte number or function (Wu et al., 2017). Since cardiomyocytes have limited potential of proliferation after birth, structural and functional adaptation and density of existing myocytes remain central to maintain cardiac compliance (Woodcock and Matkovich, 2005). In this study, cardiomyocyte number was assessed by quantification of the number of cardiomyocytes in a given area. Consistent with the findings of a study involving combined exposure to BPA and PFOS during gestation in rats (Zhou et al., 2020), we observed no effect of exposure to an EC mixture, on cardiomyocyte number per field, in either sex. Likewise, in another study which primarily lacked sex-specific investigation, fetuses treated with cortisol during gestation resulted in no significant changes in myocyte number(Lumbers et al., 2005). Furthermore, a study on altered neonatal nutritional insult in rats, it was observed that adult males and females have a comparable number of cardiac myocytes (Bai et al., 1990). Conversely, sex-specific effects of gestational excess of hormonal treatment such as testosterone in sheep have documented cardiomyocyte hyperplasia but in female fetuses only (Ghnenis et al., 2022). This contrast in the undetectable changes in cardiomyocyte numbers could be explained by several factors such as

differences in the composition of ECs, the level of exposure, and importantly, the developmental points at which the animals were investigated in respective studies. It can also be speculated that because cardiomyocyte proliferation is rare after birth, any subtle prenatal EC exposure changes in cardiomyocyte density may have been masked by postnatal remodeling or compensatory mechanisms in both sexes. In addition to in-vivo studies, several *in vitro* studies involving direct exposure of stem cells derived cardiomyocytes have documented the effects of ECs on several aspects of cardiomyocytes functionality. BPA exposure has been shown to impair cardiomyocytes proliferation and contractility (Escarda-Castro et al., 2021; Lamberto et al., 2023; Ma et al., 2023), while atrazine has been associated with increased cardiomyocyte apoptosis and inflammation (Wang et al., 2025b; Zhao et al., 2024). Additionally, exposure to complex mixtures of ECs has been linked to disruptions in cardiomyocyte function, including alterations in beating patterns and calcium signalling (Sirenko et al., 2017). While these isolated cardiomyocyte studies and the current study serve as an important tool in interrogating the direct toxicological and in vivo consequences of EC exposure on cardiac structure/function respectively, further in vivo cardiomyocytes toxicity studies involving greater sample size are essential to gain an understanding of the impacts of EC exposure on cardiac physiology in the individual as a whole.

In addition to assessing changes in cardiomyocyte number per field, we also evaluated whether prenatal EC exposure resulted in any effects on myocardial fibrosis and myocardial collagen deposition. Cardiac fibrosis is a consequence of increased accumulation of extracellular matrix (ECM) proteins and expansion of the myocardial interstitium (Berk et al., 2007), predominantly (90%) by collagen type1 and collagen type 3 (10%) (Fan et al., 2012). Cardiac fibrosis is mainly classified into three forms i.e., interstitial where collagen deposition results in expansion of the space between cells, perivascular where there is a thickening of the area surrounding blood vessels and replacement fibrosis where damaged or dead cells are replaced by scar tissue (Anderson et al., 1979). These forms of fibrosis can be seen on their own or in combination and while they can be an essential aspect of cardiac repair process, excess fibrosis can lead to organ

damage and impaired cardiac function (Burt et al., 2014). In our study, we observed no statistically significant EC exposure effect or sex difference in the amount of fibrosis present in the adult offspring heart. These results contrast with those observed in a rat model where prenatal exposure to BPA (Hu et al., 2016) and nicotine (Yu et al., 2016) resulted in significant myocardial fibrosis in adult male offspring and mouse model of heart failure where (Withaar, 2024) reported increased levels of replacement fibrosis in males compared to females. However, an apparent pattern was observed in the current study for increased collagen deposition in animals prenatally exposed to a real-life EC mixture and this was accompanied by a shift in the pattern and complexity of fibrosis. Specifically, EC exposed animals exhibited more complex pattern of qualitative change, which included a mixture of interstitial, perivascular, and replacement fibrosis, and would suggest that there are EC effects on the underlying mechanisms driving early or subclinical remodelling. Interestingly as in the model of heart failure, mentioned above (Withaar, 2024), this qualitative change in fibrosis was more pronounced in males than females, indicating a possible sex-specific susceptibility to EC induced changes in collagen architecture. In the current study alongside EC induced changes in the pattern of fibrosis, an increase was also seen in the expression of mRNA for the fibrosis marker collagen 1 type 1 (COL1A1). As with the histological findings this EC induced difference in gene expression was again only seen in the males. The other cardiac fibrosis marker studied COL3A1 was not affected by treatment but was found to be expressed at a higher level in the females relative to males. These observed gender differences in COL1A1 and COL3A1 expression can be explained by the predominant type of these collagens during different phases of cardiac injury process. COL3A1 is considered as juvenile collagen and is present in the early phase of cardiac injury, whereas COL1A1 is a stiffer fibrillar protein and is more commonly associated with cardiac remodelling (Singh et al., 2023). The observed pattern towards an increased expression of COL1A1 in B males in the current study may indicate cardiac fibrotic remodelling associated with developmental EC exposure in males. Cardiac fibrosis in males, has been reported following exposure to several ECs including PCBs (Wang et al., 2021), BPA (Belcher et al., 2015; Hu et al., 2016; Reventun et al., 2020), nonylphenol

(Guo et al., 2024; Liu et al., 2021), and nicotine (Yu et al., 2016) in rodent species. When combined with the results of the current study, these indicate that male hearts may have a greater sensitivity towards the development of fibrosis in response to EC exposure compared to females. One of the hallmark of pathological cardiac remodelling is presence of fibrosis and the fact that an increased level of fibrosis seen as a mixture of interstitial, perivascular and replacement alongside greater ventricular dimensions (without any changes in wall thickness) in B males (Khan et al., 2025a) reinforce the possibility of pathological left ventricular eccentric hypertrophy in these animals.

In addition to their contractile function, cardiomyocytes secrete a family of peptide hormones including atrial natriuretic peptides (ANP) and brain natriuretic peptide (BNP) which are important in the regulation of diuresis and exert natriuretic and vascular smooth muscle relaxation effects (Clerico et al., 1999; Sagnella, 1998). In humans, it has been shown that ANP and BNP can be cardioprotective, that their secretion is driven by female sex steroid hormones, and it has been proposed that this may explain or contribute to the lower CVD risk in females (Clerico et al., 2002) (Murphy et al., 2011). The results of the current study would support this proposal as the expression of the genes encoding ANP and BNP in the sample collected from the left ventricle and the expression of ER1 and ER2, were greater in females in comparison to males. The cardioprotective role of estrogen is suggested to be mediated via traditional (ERα/ERß) as well as G-protein-coupled (GPR30) receptors located on the mitochondrial membrane. Activation of these receptors can initiate transcriptional changes within the nuclear and mitochondrial genes, which influences mitochondrial function, cell survival and enhances cardio-protection (Iorga et al., 2017; Klinge, 2008; Lagranha et al., 2010). These ERs mediated protective effects on CV function have been reported by animal studies in male (Bopassa et al., 2010; Zhai et al., 2000) and female (Pavón et al., 2012; Xue et al., 2007; Zhu et al., 2013) separately. The higher ER2 expression observed in B compared to C females in the present study could have important pharmacological implications, since the synthesis, secretion and metabolism of hormones is coordinated by the hypothalamic–pituitary–adrenal (HPA), and

hypothalamic–pituitary–gonadal (HPG) axes, and it has been reported that endocrine disrupting chemicals such as prochloraz and propylthiouracil can lead to altered hormonal signalling (Liu et al., 2011; Ma et al., 2016). In addition, ECs may also act as selective estrogen receptor modulators (SERM) in cardiac tissue. SERM are substances known to exhibit tissue-selective pharmacological actions which means that they can act as an agonist in some tissues (bone, liver, heart) but antagonists in other tissue (brain and breast) or their action may be both agonistic and antagonistic in nature, such as in uterus (Das et al., 2022; Lewis and Jordan, 2005; Sant et al., 2025). ECs, particularly those with endocrine disrupting action can bind selectively to different ERs and mimic estrogenic activity. The resultant effects of ECs on ERs could be variable and ER type dependent for example; BPA has been demonstrated to act as pure agonist in the presence of Erα but a pure antagonist in the presence of Erβ (Bolli et al., 2008; Pellegrini et al., 2014). As the current study used an EC mixture, in which individual ECs could have had both agonistic and antagonistic actions, the role of individual ECs as selective ER agonist/antagonist could not be established. The observation that mTor, IGF1, and IGF1R which are markers associated with cardiomyocyte growth and SCL2A4 a marker of glucose metabolism was also higher in females compared to males in the current study would also go along with higher cardiac resilience, metabolic efficiency and reparative capacity in females compared to males. Maternal EC exposure did not have any significant effect on the expression of NPPA, NPPB and mTor in either sex. We did, however, observe an apparent pattern towards increased NPPA expression in B compared to C females. ANP expression has previously been reported to be increased in the ventricles of female sheep perinatally exposed to BPA (Koneva et al., 2017; MohanKumar et al., 2017) and testosterone (Ghnenis et al., 2022). In the study of (Ghnenis et al., 2022), the testosterone effect on ANP expression in the females was also mirrored in the males fetuses and in both sexes testosterone also increased the expression of BNP. The apparent pattern towards increased expression of ANP in females developmentally exposed to a real-life EC mixture may indicate a protective response possibly mediated via ER signalling (Jankowski et al., 2001) and aligns with their less severe fibrosis compared to the males.

Another important finding we observed in the present study was the significantly greater expression of proapoptotic and inflammatory markers CASP3, MHC-DRB1 and MHC-DYA in developmentally EC exposed males but not females. The higher expression of these markers' signals sex specific cardiac proapoptotic and inflammatory cascades driven by maternal EC exposure in males. Among the caspases, CASP3 is central executioner in the apoptotic pathway (Thornberry and Lazebnik, 1998). EC exposure has been shown to induce inflammation/fibrosis, and apoptosis via CASP3 activation in male rats, whether in the form of particulate matter in hyperlipidemic models (Wang et al., 2019), or secondhand smoke (Kuo et al., 2005). Interestingly, in the study of (Kuo et al., 2005) and in the current study, this was also associated with an increased levels of insulin like growth factor 1 (IGF1), a survival candidate, which can be a compensatory mechanism to the evolving cardiac apoptosis and inflammation processes. The higher expression of CASP3 in developmentally EC mixture exposed males may indicate cardiomyocytes apoptosis signaling adverse cardiac remodeling. The concurrent expression of higher inflammatory and immune markers MHC-DRB1 and MHC-DYA along with CASP3 in males exposed to a real-life EC mixture during in-utero development further mirrors inflammatory changes parallel to apoptosis. While we did not evaluate the antioxidant status in the present study, the observed increase in apoptosis and inflammatory markers in the LV tissue in the males could be due to EC induced oxidative stress. The redox mechanisms remain central to the normal functioning of the heart however, an excess of reactive oxygen species (ROS) generated during this process can lead to a redox imbalance which may result in cardiac remodeling and toxicity (M Costa et al., 2011). This possibility is more likely in the heart as it has low antioxidant reserves compared to other organs (Costa et al., 2013). In male mice, exposure to ECs such as BPA and DEHP have been shown to result in ROS generation and to be associated with a reduction in the activity of antioxidant enzymes including glutathione, catalase and malondialdehyde (Aboul Ezz et al., 2015; Shen et al., 2023) which are involved in neutralizing ROS, and mitigating cardiac toxicity. Separately, BPA exposure has been shown to result in downregulation of glutathione, superoxide dismutase, catalase, and

glutathione peroxidase enzymes in the hearts of male wistar rats (Khodayar et al., 2020). The situation may differ in females, whose cardiovascular health may benefit from higher estrogen levels and a better antioxidant profile (Zhu et al., 2013). Several other EC exposure animal studies have also reported inflammation, apoptosis and oxidative stress in males (Jing et al., 2019; Nemmar et al., 2007; Xu et al., 2022). In contrast, a study on developmental BPA exposure has demonstrated that BPA induced inflammation and myocardial cell degeneration in offsprings of both sexes in rats (Gear et al., 2017). This could reflect differences in the effects of single vs mixtures of EC exposure, or species (rodent vs sheep). Again, it was interesting to witness the lack of upregulation in apoptosis and inflammatory markers such as CASP3, MHC1, and DYA in the EC exposed females which were markedly increased in EC exposed males, and are also supported by our previous in vivo observations which demonstrated that developmental exposure to a real-life mixtures of ECs had a lesser effect on CV function markers in females compared to in males (Khan et al., 2025a).

As well as gene expression and histology, we also investigated whether prenatal exposure to a real-life EC mixture had affected the plasma lipid profile, a known contributing factor for the development of adult CVD. Sex differences were detected in TG and TC levels, which were significantly higher in males compared to females. This result mirrors well documented sex differences in humans (Abbott et al., 1983; Magkos and Mittendorfer, 2009; von Hafe, 2019) which have been largely attributed to differences in sex steroid hormones (von Hafe, 2019). We did not see any effects of developmental EC exposure on the plasma lipid profile of adult offspring in the current study. This contrasts with an earlier study in which an EC exposure effect was seen in in the TG levels in prepubertal male offspring (Ghasemzadeh-Hasankolaei et al., 2024) and sex-specific alterations in the lipid profile of resulting offspring (9.5 weeks of age) in sheep (Thangaraj et al., 2025) indicating potential sexually differentiated effects on lipid metabolism which may contribute to the development of adult onset of CVD. It is, however, a recognized limitation of this study that our conclusions with regards to lipid profile are based on a single plasma sample per animal, which may not capture dynamic or any subtle changes in lipid homeostasis.

3.6 Conclusion

Findings from the present study on developmental EC mixture exposure indicate histological alterations in the form of ECM remodeling and changes in key markers of CV function in adult male offspring. These changes were characterized by the presence of advanced fibrosis types and increased expression of proapoptotic and inflammatory markers in the left ventricles of EC exposed males which mirrors adverse cardiac remodeling resulting from gestational EC mixture exposure. The cardiac changes observed in this study are consistent with the cardiac phenotype we previously reported in EC exposed males (Khan et al., 2025a) where changes such as greater ventricular dimensions, increased systolic and diastolic volumes and high cardiac output were observed. Together seen with molecular results from gene expression in the present study, the concurrent upregulation of IGF-1 and IGF1-R, markers of enhanced cardiomyocyte growth and survival, can be seen as compensatory mechanism in response to cardiomyocyte apoptosis and inflammation as evidenced by upregulation in CASP3, DYA and DRB1 in EC exposed males and indicates pathological cardiac eccentric remodeling due to gestational EC exposure. In comparison to males, the CV function markers in female offspring were less affected by gestational EC exposure in the present study as well as that of (Khan et al., 2025a) and signals sex-specific protective role of estrogens in cardiovascular remodeling. Future EC mixture studies should focus on the sex-specific molecular underpinnings and the interplay between the pathways involved in the differential expression of CV function markers particularly in male offsprings which may help to understand the sexually dimorphic CVD risk in humans.

3.7 Limitations

While the BTP model utilized in the current study reflects real-life EC mixture exposure where ECs in biosolids can exhibit an additive, synergistic and antagonistic effects, the sex-specific effects observed in the present study could not be ascribed to a particular class of ECs. Additionally, with regards to lipid profile, differences in ruminant lipid metabolism compared to humans could also

influence how such changes manifest in the CVD risk of EC exposure. Given that some animals may be more susceptible than others, as observed in our previous work, a larger population size may be required.

Chapter 4 Developmental exposure to a complex environmental chemical mixture is associated with transgenerational, sex-specific cardiovascular remodelling

4.1 Abstract

Cardiovascular diseases (CVD) constitute a major and sexually differentiated cause of death across the world. It is proposed that adverse conditions such as exposure to exogenous environmental chemical (EC) during early growth may perturb the developing cardiovascular system and lead to transgenerational adult onset of CVD. The work presented here in evaluated sex-specific intergenerational impacts of EC exposure on adult cardiovascular functioning by grand maternal grazing of sheep on pasture fertilized with sewage sludge (Biosolids). EC exposure did not have any transgenerational effects on blood pressure in either sex. Heart rate variability metrics RMSSD and SDNN were significantly lower ($P<0.05$) in B compared to C group, and a significant exposure-sex interaction ($P<0.05$) was evident in heart rate (HR) observed to be lower in B males and higher in B females compared to their respective controls. Echocardiography results showed increased left ventricular wall thicknesses and pulmonary artery diameter in B males while these were lower in B females. In conclusion, EC exposure during development may lead to intergenerational sex-specific changes in cardiovascular structure and function and these are more pronounced in males than females.

4.2 Introduction

Cardiovascular disease (CVD) is one of the leading causes of mortality and morbidity worldwide with its prevalence nearly doubled (523 million in 2019) compared to 271 million in 1990, while CVD associated mortality has increased from 12.1 million (1990) to 18.6 million (2019) (Kim et al., 2020; Roth et al., 2020). It is well known that conditions which alter the *in utero* environment can shape long-term health outcomes in an individual including an increased risk of developing CVD (Barker, 2004a; Barker, 2004b), Among these early-life insults, exposure to environmental chemicals (ECs) is gaining attention as an important yet underexplored risk factor that contributes to adult CVD risk. ECs such as bisphenol A (BPA), phthalates, parabens, triclosan, heavy metals and persistent organic chemicals which are widely present in the environment, are known to exert adverse health effects on endocrine (Coiffier et al., 2023), metabolic (De Long and Holloway, 2017; Saedi et al., 2023; Wang et al., 2014),reproductive cardiovascular (CV) system (Singh et al., 2021; Svoboda et al., 2022).

Epidemiological studies provide evidence on the CV impacts of adult human EC exposure. This include exposure to polychlorinated biphenyls (PCBs) which has been associated with coronary heart disease (Blackowicz et al., 2024) and hypertension (Akinyemi and Obeng-Gyasi, 2025). BPA exposure has been linked with reduced heart rate variability (HRV), a risk factor for CVD (Bae et al., 2012). Likewise exposure to heavy metals such as lead (Pb) and cadmium (Cd) is suggested to result in atherosclerosis, dyslipidaemia, stroke and heart failure (Peters et al., 2010; Revis et al., 1981; Solenkova et al., 2014).While little is known about prenatal EC exposure and CVD in humans, evidence is mounting on the prenatal exposure effects in the resulting offspring which can lead to adult onset of CVD. For example exposure to BPA (Khalil et al., 2014), Phthalates (Lu et al., 2018; Trasande et al., 2016), Diethylstilbesterol (DES) (Troisi et al., 2018),dichloro diphenyl dichloroethylene (DDE) (Vafeiadi et al., 2015) and air pollution, particularly PM2.5 and black carbon (Lin et al., 2023a) are associated with elevated blood pressure , a major risk factor for adult CVD. It has also been

reported that fetuses exposed to perfluoroalkyl substances (PFAS) and DES are at higher risk of developing congenital heart defects and coronary artery disease respectively (Ou et al., 2021; Troisi et al., 2018).

Diverse cardiovascular effects of both prenatal and adult EC exposure have also been reported in preclinical animal studies. DEHP exposure in adult mice has been linked to altered autonomic regulation with a decrease in basal HRV (Jaimes III et al., 2017). Chronic low-level exposure to mercury has been reported to promote endothelial dysfunction in adult rat (Wiggers et al., 2008), Prenatal BPA exposure has been associated with adverse cardiac effects in rodent, zebra fish and primate models (Brown et al., 2018; Chapalamadugu et al., 2014; Fonseca et al., 2022; Gear et al., 2017; Yujiao et al., 2023). Prenatal exposure to PCBs causes toxic effects that impair normal heart development in zebrafish (Li et al., 2014). Importantly, animal studies have indicated that these cardiotoxic effects of EC exposure can be trans-multigenerational, (Lombó et al., 2015; Manikkam et al., 2012; Manikkam et al., 2013).

The cardiac effects driven by EC exposure occur in sex-specific manner in both animals and humans (Svoboda et al., 2022), however; there is little understanding of the mechanisms that underlie these differences as most studies only investigated the cardiac effects of EC exposure in a single sex. Traditional animal cardiotoxicity models also do not accurately replicate real-life human EC exposure, where individuals are continuously exposed to complex mixtures of ECs, often at low individual concentrations. An experimental paradigm which is more translationally relevant to real-life human EC exposure is the biosolids treated pasture (BTP) sheep model. Biosolids are a commonly used agricultural fertilizer derived from wastewater treatment. However, because of their origin, they contain a mixture of anthropogenic ECs (Rhind et al., 2010; Rhind et al., 2002; Venkatesan and Halden, 2014) such as heavy metals, personal care products, veterinary medicines, pharmaceuticals, bisphenol A (BPA), phthalates, per- and polyfluoroalkyl substances (PFAS), flame retardants (polybrominated diphenyl ethers, PBDEs) and organophosphorus compounds (Clarke and Smith, 2011). Sheep grazed on BTP can be utilised to understand the impacts of

gestational exposure to EC mixtures on the offspring. We have previously reported sex-specific CV effects of real-life EC exposure in lambs born to ewes grazed on BTP during gestation (Khan et al., 2025a) where gestational exposure to a complex EC mixture resulted in increased left ventricular dimensions, high systolic and diastolic volumes, and high cardiac output in adult male but not female offspring. This work was supplemented with histological and molecular studies which demonstrated adverse changes including upregulated inflammatory and apoptotic markers along with a high distribution of mixture of interstitial, perivascular and replacement fibrosis in the left ventricular tissues from F1 males but again no significant effects in F1 females (Khan et al., 2025b). The aim of the present study was to determine whether the in vivo effects of exposure to a complex mixture of ECs seen in F1 animals on CV function, persist into the second generation (F2).

4.3 Material and methods

4.3.1 Ethical statement

All experimental animal work was conducted at Cochno Farm and Research Centre, University of Glasgow. Experiments were conducted under the United Kingdom's Animals (Scientific Procedures) Act 1986, under the specific authority of Project Licence PF10145DF. All animals were humanely treated throughout the study, with due consideration to alleviation of pain, suffering, distress and lasting harm.

4.3.2 Experimental design

Easy care ewes (n=320) were acquired and randomly allocated into two groups. The first group, designated as control (C) (n=160), was grazed on pasture treated with inorganic fertilizer. The second group, designated as biosolids (B) (n=160) was grazed on biosolids treated pastures (BTP) where biosolids were applied to the land at conventional rates (4 tonnes/ha) twice a year in April and September. The ewes in each group were of equal parity, and there were no significant differences in their body condition scores between the groups before

conception or at the time of parturition. To control for potential paternal effects, ewes were mated by artificial insemination using semen from four unrelated rams raised exclusively on C pasture. This gave rise to four different sire groups/families within the first generation (F1) offspring. The pregnant ewes were maintained on their respective pastures until approximately two weeks before parturition, when they were housed and fed according to normal husbandry practices with B ewes fed silage harvested from BTP. Post-parturition, both C and B ewes (and respective C and B F1 lambs) were maintained together outdoors on C pastures until weaning, after which, male and female offspring were maintained separately on C pastures. At 18 months old, F1 C and B ewes were mated by artificially insemination with semen from F1 rams from their respective experimental groups, avoiding close family pairings. The resultant F2 offspring (both male and female) were maintained only on C pasture until adult stage (2-year-old) and in vivo CV measurements were collected from males (n C=10, B=10) and females (n C=12, B=12).

4.3.3 In vivo cardiovascular experiments

4.3.3.1 Blood pressure measurements

Non-invasive blood pressure measurements were carried out as described previously (Khan et al., 2025a). Briefly, three consecutive measurements of systolic (SBP), diastolic (DBP) and mean arterial blood pressure (MABP) and heart rate (HR) were taken at the same time of day (7:00 am-09:00 am) for 15 consecutive days in the month of September from each animal, while in a standing position, using a standard veterinary blood pressure cuff (Midmark Inc. USA) and digital BP monitor (Cardel 9401 Midmark Inc. USA).

4.3.3.2 Heart rate variability (HRV) measurements

The electrical activity associated with the heart was recorded using an ACTi heart 5 (CamNtech Inc. USA) heart rate monitor as described previously (Khan et al., 2025a). Analysis of HRV data was performed in KUBIOS software (Kubios HRV Standard 3.4.1, Kubios Oy, Finland). Time domain and frequency domain analysis

parameters including root mean square of successive differences (RMSSD), standard deviation of normal N-N intervals (SDNN), high frequency power (HF), low frequency power (LF), LF/HF ratio and HR measurements were acquired for each animal.

4.3.3.3 Echocardiography

Right parasternal echocardiography was conducted as described previously (Khan et al., 2025a). Representative still images are shown in Fig.4-1 (A-D). Right parasternal long axis (PLAX) 2-D and short axis (PSAX)), along with 1-D M-Mode scans were acquired from each animal, which included four chambered (4-C), left ventricular outflow tract (LVOT), short axis left ventricle at mitral valve and chordae tendineae (SA-LV) and short axis aorta (SA-Ao) views. Each parameter was measured for three consecutive cycles and averaged accordingly. Among the structural and functional parameters studied were, left atrial diameter in end systole (LAD), mitral valve annulus at end systole (MVA), pulmonary artery (PA) and aortic diameter at end diastole (Ao), and left ventricular diameter in diastole (LVDd) and systole (LVDs), interventricular septum thickness in diastole (IVSd) and systole (IVSs), left ventricular free wall thickness in diastole (LFWd) and systole (LFWs), left ventricular ejection fraction (LVEF), fractional shortening (FS), end-diastolic (EDV) and end-systolic (ESV) volumes, stroke volume (SV), cardiac output (CO) and HR (resting).

4.3.4 Statistical analysis

Data were analysed in R (RStudio 2025.09.1+401), by multi factorial analysis of variance (ANOVA) with treatment and sex as the main explanatory variables. Birth weight and litter size had no effects on any of the parameters in the model and so were not included. Parental genotype was included in the model as explanatory variable. All data graphs were generated in GraphPad prism (Prism Windows 5.04) and presented as Mean ± SEM. $P < 0.05$ was considered statistically significant.

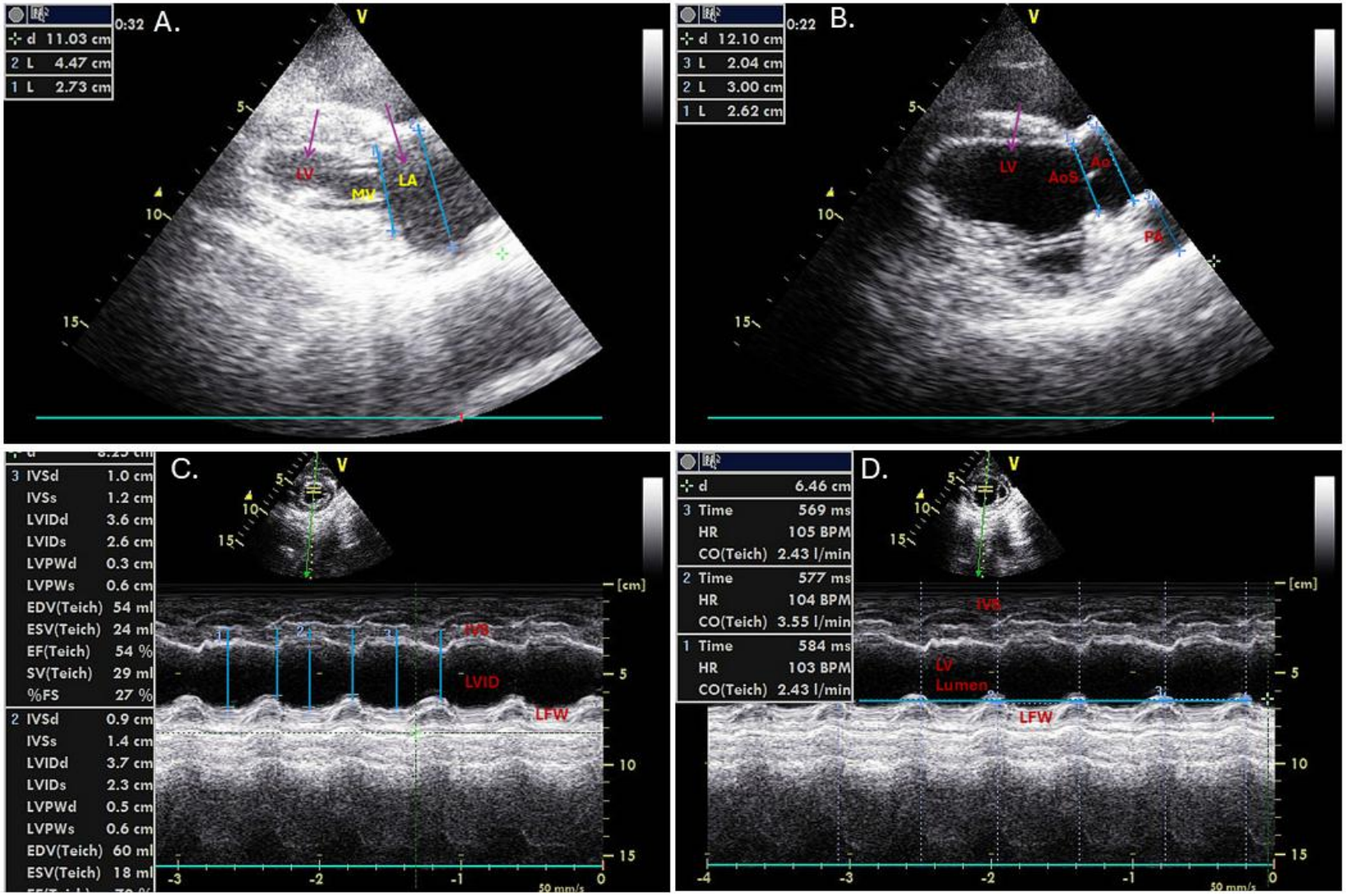

Figure 4-1. Right parasternal echocardiographic views from F2. A) A 4-Chambered view with left atrium (LA), mitral valve annulus (MVA) and left ventricle (LV) in cross section. B) Left ventricular outflow tract (LVOT) view showing Aorta (Ao), Aortic sinus (AoS) and pulmonary artery (PA) in cross section. C) M-mode view showing inter-ventricular septum (IVS), left ventricular internal lumen diameter (LVID), and left ventricular free wall (LFW) measurements in cross section. D) An M-mode view showing consecutive measurements for heart rate (HR) and cardiac output (CO).

4.4 Results

4.4.1 Transgenerational effects of in-utero EC exposure on blood pressure

SBP, DBP, MABP and HR were not significantly different between B and C in either males or females ($P>0.05$) Fig. 4-2. (A-D). An apparent pattern towards significance ($P=0.08$) was observed for exposure-sex interaction in SBP where SBP was lower in B males and higher in B females relative to their C. Significant sex differences ($P<0.05$) were also evident in SBP, DBP, MABP and HR which were greater in males compared to females, and HR was greater in females compared to males (Fig. 4-2. A-D).

4.4.2 Transgenerational effects of in-utero EC exposure on heart rate variability

Analysis of time domain HRV parameters indicated that RMSSD and SDNN were significantly lower in animals in the B compared to the C group. There was no significant ($P>0.05$) effect of sex or exposure-sex interaction on either RMSSD or SDNN (Fig.4-3. A-B) however, there was an apparent pattern for sex difference ($P=0.07$) in SDNN being lower in females compared to males. Frequency domain parameters HF and LF were not significantly different between B and C (Fig. 4-3. C-D). However, the LF/HF ratio, was observed to be higher in females compared to males (Fig. 4-3. E). Finally, mean HR was observed to be significantly higher in B compared to C group and a significant exposure-sex interaction ($P<0.05$) was also noted in mean HR, which was lower in B males, but higher in B females compared to their respective controls (Fig. 4-3. F).

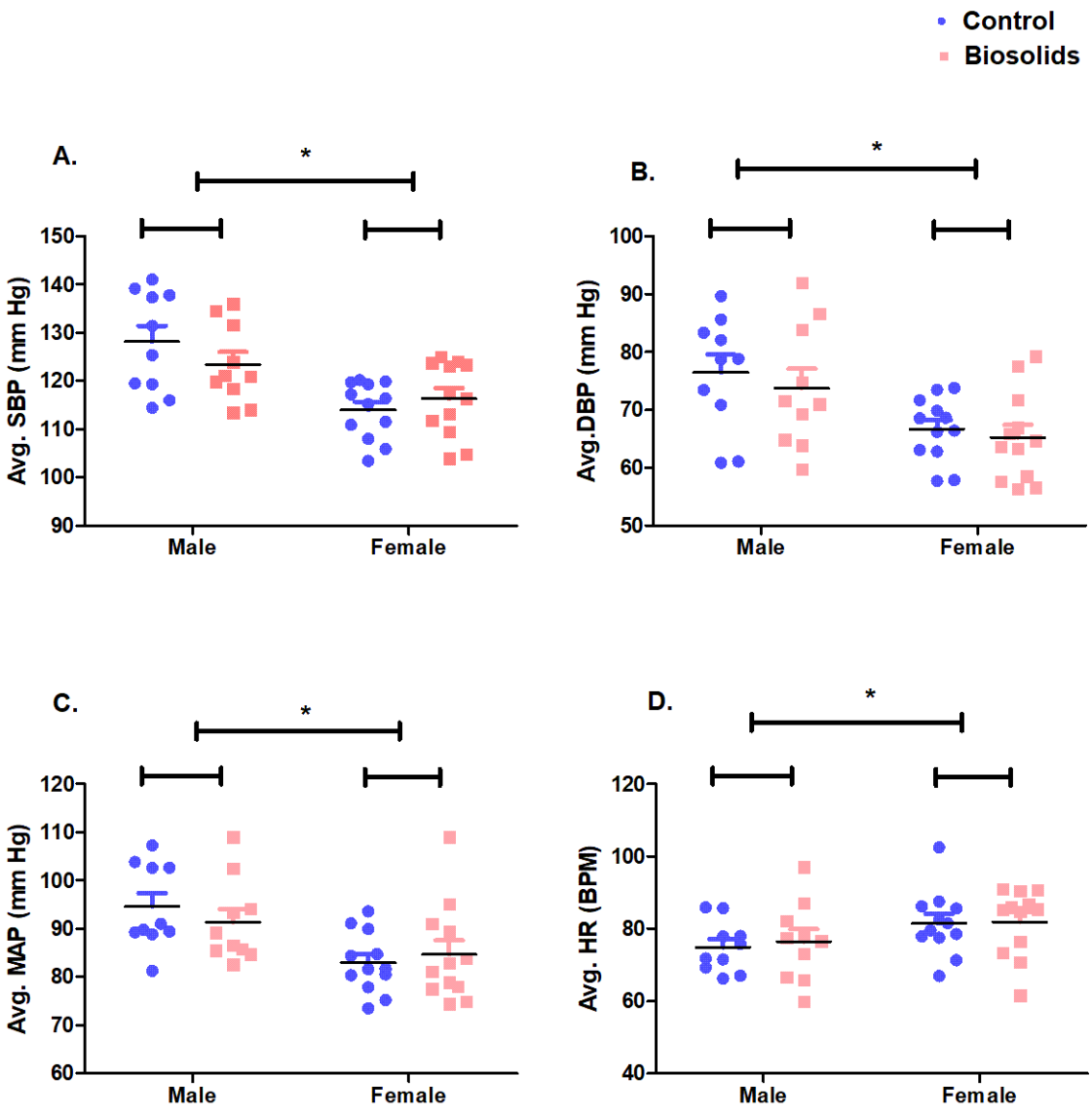

Figure 4-2. Scattered plots showing data from blood pressure measurements. (A) Systolic blood pressure (SBP) showed overall high SBP in males compared to females with no significant exposure or exposure-sex interaction, P-value; Exposure = 0.16, Sex = 0.003, Interaction = 0.08. Diastolic blood pressure (DBP) (B) and mean arterial blood pressure (MABP) (C) also showed significant sex differences with significantly high DBP and MABP in males compared to females, P-values DBP; Exposure= 0.59, Sex = 0.01, Interaction = 0.67, P-values MABP; Exposure = 0.95, Sex = 0.005, Interaction = 0.31. (D) Average heart rate (Avg.HR) were significantly higher in females relative to males, P-value; Exposure = 0.62, Sex = 0.002, Interaction = 0.67. Data analysed by multifactorial ANOVA and presented as Mean ± SEM. * indicate significant ($P < 0.05$) differences between groups (control vs biosolids) and sex (male vs female).

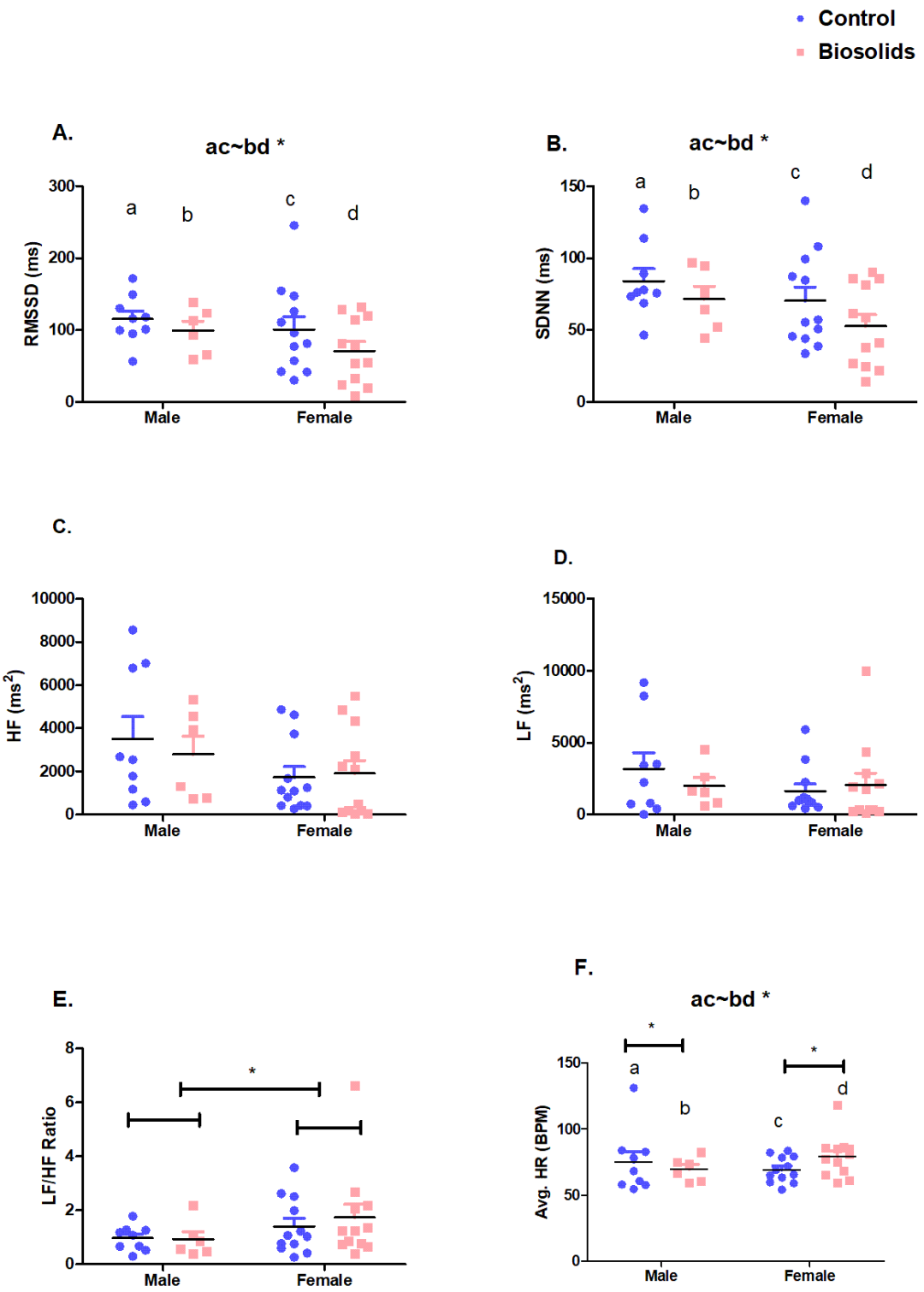

Figure 4-3. Scattered plots showing (A) Root mean square of successive differences (RMSSD) showed lower RMSSD in B compared to C group (ac~bd), P-value Exposure = 0.04, Sex = 0.10, Interaction = 0.18. (B) Standard deviation of the IBI of normal sinus beats (SDNN) was also significantly lower in B compared to C (ac~bd), P-value Exposure = 0.04, Sex = 0.07, Interaction P = 0.50. (C) Absolute high frequency power (HF) showed no significant exposure, sex differences or interaction, P-value; Exposure = 0.66, Sex = 0.36, Interaction = 0.77. (D) Absolute low frequency power (LF) also showed no significant exposure, sex differences or interaction, P-value; Exposure = 0.85, Sex = 0.69, Interaction = 0.64. (E) LF/HF ratio showed significant sex differences with females having high LF/HF compared to males, P-value; Exposure = 0.20, Sex = 0.02, Interaction = 0.66. (F) Heart rate (HR) showed overall significant exposure effect (ac~bd) and exposure-sex interaction, P-value, Exposure = 0.03, Sex = 0.86, Interaction = 0.004. Data presented as Mean ± SEM. * indicate significant ($P < 0.05$) differences between groups (control vs biosolids) and sex (male vs female).

4.4.3 Transgenerational effects of in-utero EC exposure on echocardiography parameters

The structural cardiac parameters LAD, MVA, Ao, IVSd and IVSs were not significantly different between B and C groups ($P > 0.05$) (Fig. 4-4. A-E). However, a significant exposure-sex interaction ($P < 0.05$) was evident in PA, LFWd and LFWs, which were higher in B males, but lower in B females, compared to their respective controls (Fig. 4-4. F, I, J). Additionally, significant sex differences were detected in LVDd and LVDs, LFWd and LFWs, with males exhibiting higher LVDd and LVDs and lower LFWd and LFWs compared to females (Fig.4-4. G-J). Functional cardiac parameters were not different in B or C groups in both sexes (Fig. 4-5 A-F). However, EDV, ESV, SV, and CO were significantly ($P<0.05$)) greater in males compared to females (Fig.4-5. C-F).

4.4.4 Influence of parental genotype

Genotype analysis revealed a significant influence of parental (F1) genotype on mean HR ($P = 0.05$), a significant genotype-exposure ($P =0.005$) and genotype-sex interaction ($P =0.02$). The mean HR was higher in females compared to males and lower in B compared to C males but higher in B compared to C females. A significant influence of genotype was also observed on LAD ($P=0.05$), FS ($P=0.04$) and LVEF ($P=0.03$) and a significant genotype-exposure interaction was also noted for PA ($P=0.02$). The relevant genotype data are presented in Fig. 4-6.

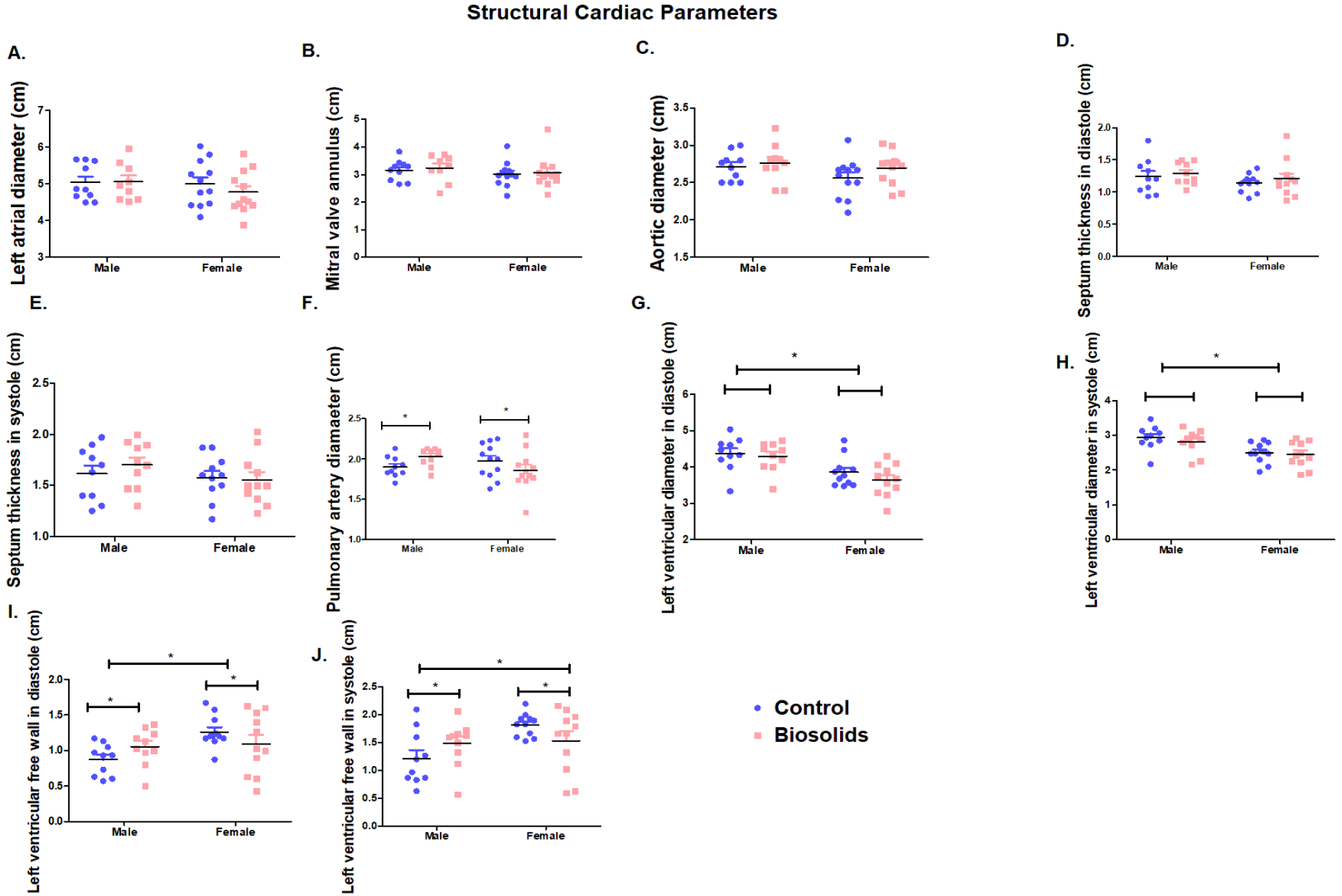

Figure 4-4. Structural cardiac parameters from echocardiography of F2 males and female sheep, where the F0 ewes had been exposed to ECs by grazing on BTP during gestation. No significant differences were observed in left atrial diameter (A), mitral valve annulus (B), aortic diameter (C), inter-ventricular septum thickness in diastole (D) and systole (E) between B and C animals. Pulmonary artery diameter (PA) (F) showed a significant exposure-sex interaction (P = 0.01). Left ventricular internal diameter in diastole (G) and systole (H) showed a significant effect of sex (P = 0.001 and P = 0.003) respectively. Left ventricular free wall in diastole (I) and systole (J) showed a significant effect of sex (P=0.01, 0.02) and exposure-sex interaction (P=0.04, 0.04). * Indicate significant ($p < 0.05$) differences between groups (control vs biosolids) and sex (male vs female).

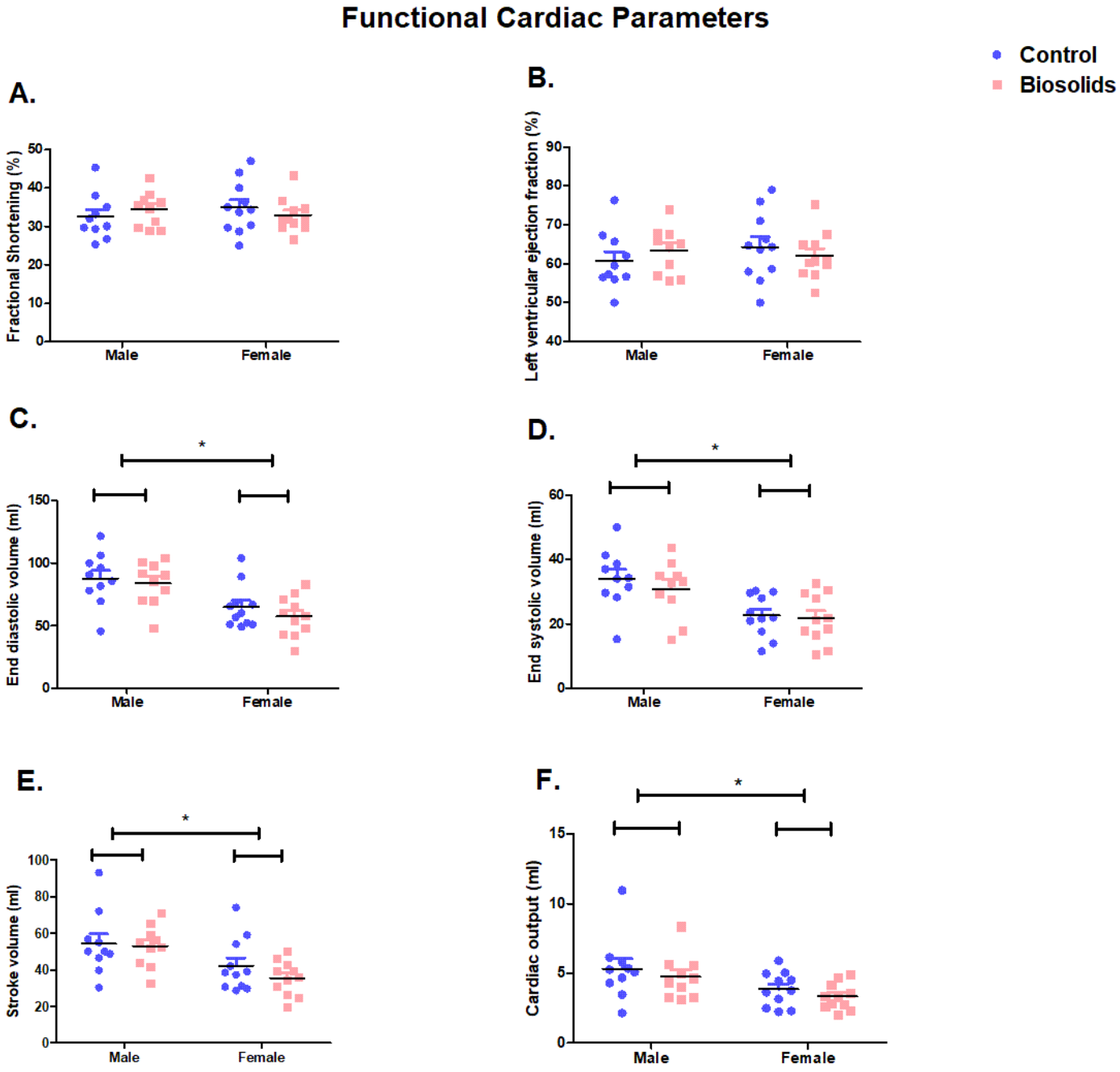

Figure 4-5. Functional echocardiography measures from F2 males and female sheep, where the F0 ewes had been exposed to ECs by grazing on BTP during pregnancy. Fractional shortening (A) was not significantly different between B and C groups (P-value; Exposure = 0.93, Sex = 0.76, Interaction=0.28). Left ventricular ejection fraction (B) showed no significant difference between B and C, and no sex interaction (P-value; Exposure = 0.97, Sex = 0.60, Interaction=0.27). A significant effect of sex can be observed in end-diastolic (C), (P = 0.001) and end-systolic volume (D), (P = 0.0007). Stroke volume (E) and cardiac output (F) also showed significant sex differences (P = 0.005, 0.01). * Indicate significant differences (P < 0.05) between groups (control vs biosolids) and sex (male vs female).

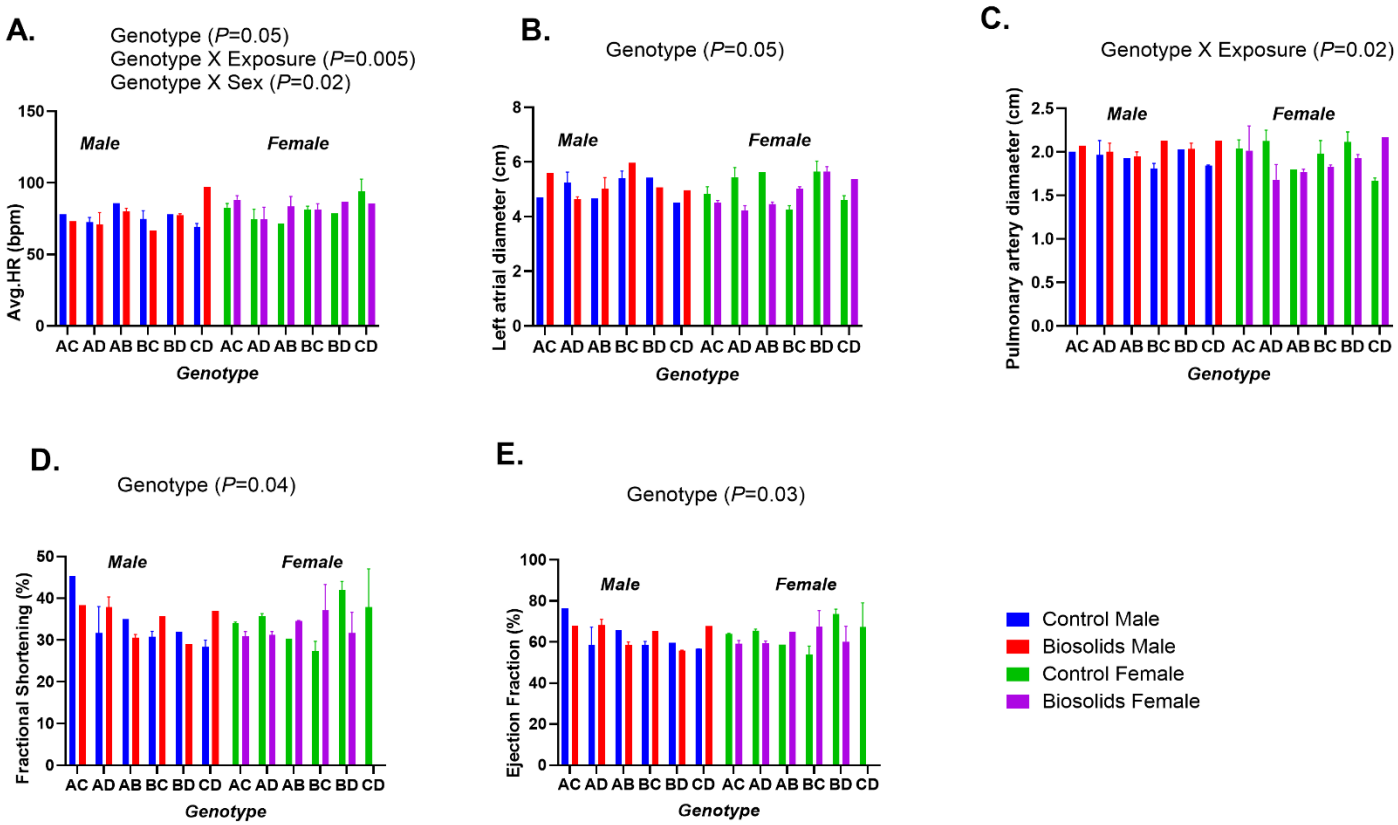

Figure 4-6. Parental Genotype influence on cardiac parameters in the F2 offspring. Significant overall influence of parental genotype as well as A genotype-exposure and genotype-sex interaction was observed on average heart rate (HR). Significant effect of genotype was also seen in left atrial diameter, fractional shortening and ejection fraction (B, D, E). (C) Significant genotype-exposure interaction also observed in pulmonary artery diameter (PA).

4.5 Discussion

The present study was aimed at investigation of sex-specific intergenerational effects of mixed EC exposure during *in utero* development on the CV system, specifically, the F2 generation of sheep from flocks where the F0 mothers had either been grazed on biosolids treated pasture which contains a diverse mixture of anthropogenic ECs or control pasture that had only received inorganic fertilisers. Major findings from this study included significant difference in mean HR which was lower in B males but higher in B females compared to controls as well as changes in PA diameter and LV wall thickness which were observed to be higher in B males and lower in B females compared to their respective controls. Sex-specific multi-generational effects of EC exposure on CV function or its associated risk factors have not been extensively investigated in mammals. In zebrafish it has been reported that paternal BPA exposure can lead to cardiac malformations in the F1 and F2 generations (Lombó et al., 2015), the study however included only males. Following gestational exposure to mixture of EDCs BPA, DEHP and DBP in rat, adverse health outcomes including obesity, a risk factor for CVD was reported in the F3 generation (Manikkam et al., 2013) however sex-specific changes were not evaluated. The mechanisms associated with such multigenerational inheritance are not fully understood but may involve epigenetic programming (Benincasa et al., 2024; Nilsson et al., 2018; Svoboda et al., 2022).

As with our previous study in the F1 generation (Khan et al., 2025a), sexual dimorphism in the effects of EC exposure was observed in resting HR in the F2 generation. In the F2 female B group, their higher HR was accompanied by an apparent pattern of lower RMSSD, SDNN and high LF/HF ratio which could indicate high sympathetic dominance. In the F2 males B group, however, despite similar patterns in RMSSD and SDNN, HR was lower than in the controls which could suggest enhanced autonomic compensation. This was also shown by an overall lower LF/HF ratio in the males compared to females which would support the possibility of a higher sympathetic influence in females. While HR is tightly regulated by the dynamic balance between the sympathetic and

parasympathetic branches of the autonomic nervous system (Rajendra Acharya et al., 2006a), an autonomic imbalance may lead to increased morbidity and mortality from a host of conditions and diseases, including CVD (Thayer et al., 2010). Previous studies of the effects of mixed EC exposure on the autonomic regulation of CV function are lacking, however, it has been shown that single EC exposure, such as early life lead (Halabicky et al., 2022) and mercury (Gribble et al., 2015) exposures in humans as well as DEHP exposure in male mice (Jaimes III et al., 2017) can result in dysregulated HRV that is indicative of alterations in autonomic function and can lead to CV health implications such as CVD .

The results of the current study indicated that in the grand offspring of ewes exposed to an EC mixture during gestation, while there were no effects of EC exposure on body weight there were significant sexually differentiated differences in a number of structural parameters of the heart including PA diameter as well as LFWd and LFWs. PA diameter was smaller in the B females but greater in B males. This pattern of the effects of EC exposure on PA diameter in the F2 animals is consistent with what we reported in the F1 generation of animals (Khan et al., 2025a) and provides evidence that maternal EC exposure may program multigenerational effects on pulmonary vasculature. However, not all the changes in cardiac structure we saw in the F1 generation born to EC exposed mothers were seen in the F2 generation. For example, we had reported that the ventricular dimensions were significantly greater in F1 males whose mothers had been EC exposed during gestation (Khan et al., 2025a), but LVDd and LVDs were similar between the F2 EC exposed and control males. The increased left ventricular dimensions in F1 EC males were associated with significant changes in functional parameters including higher EDV, ESV and CO. In the current study it is of interest to note that despite no significant differences in ventricular dimensions, structural difference in measures such as LFWd and LFWs were increased in F2 B males and decreased in F2 B females compared to their controls. The thickened LV free wall in the absence of chamber enlargement or changes in functional parameters such as EF, FS, and CO could indicate early concentric hypertrophy (Grossman et al., 1975) where the myocardium may be undergoing compensatory structural adaptation.

Concentric left ventricular hypertrophy in response to EC exposure has previously been reported to occur in female mice exposed to arsenic in drinking water (Sanchez-Soria et al., 2012) and in both males and females prenatally exposed to DDT (La Merrill et al., 2016).

Consistent with findings in the F1 generation (Khan et al., 2025a), the results of the current study demonstrated significantly higher SBP, DBP, and MABP in males compared with females. As reported in human and animal studies, these sex differences are likely to contribute to the increased incidence of age adjusted CVD risk in males compared to females (Chen and Meng, 1991; Maris et al., 2005; Reckelhoff, 2001; Sandberg and Ji, 2012). Sex differences also existed in LVDd and LVDs parameters where males exhibited large ventricular dimensions compared to females which could be due to greater heart weights in males. We did not see any generational effect on systemic BP and structural parameters LVDd and LVDs. Adverse maternal exposures beyond ECs, such as a high-fat diet during gestation, have been shown to induce elevated blood pressure across F1, F2, and F3 male offspring (Ponzio et al., 2012), as well as increased left ventricular mass in F1, F2, and F3 males and in F1 and F2 females (Ferey et al., 2019). Since obesity is associated with high fat diet during gestation (Desai et al., 2014; Sun et al., 2012), and because ECs have been shown to induce transgenerational obesity (Manikkam et al., 2014; Manikkam et al., 2013), there is a possibility that EC exposure and high fat diet may converge on shared epigenetic and metabolic pathways implicate.

The results of the current study highlighted a significant genotype and exposure interaction regarding parameters such as resting HR and PA diameter. This finding supports our previous work with the F1 generation (Khan et al., 2025a). This genotype-exposure relationship is of paramount importance and not only predicts the CV effects of maternal EC exposure but also helps to establish how gestational effects on offsprings CV programming are genotypically determined. It is of note that significant genotype effects were observed in other cardiac structural and functional parameters in the F2 generation, including LAD, FS and LVEF but these parameters were unaffected by ancestral EC exposure. This

relationship of parental genotype on offsprings cardiac function parameters is supported by a study in mice where higher ejection fraction (EF) and E/A ratio were associated with difference in parental mice strains (Knight et al., 2009). Similarly in rats, cardiac hypertrophy and hypertension have been linked to heterozygous parental strains (Marsh et al., 2007; Reffelmann and Kloner, 2003). This implies that certain genotypes may be susceptible to onset of cardiovascular diseases in the offspring.

4.6 Conclusion

In conclusion, the results of this study add to the body of work generated by this transgenerational trial and extends our previous findings that prenatal exposure to a real-life EC mixture can elicit sex-specific CV alterations, some of which persist into the F2 generation despite those sheep having no direct exposure to ECs. Overall, it would appear that fewer parameters in the F2 offspring, were affected compared to F1 as a result of grandmaternal gestation EC exposure, however, the observed changes in wall thicknesses in males and PA diameter in females suggest EC exposure can result in heritable cardiac remodelling processes that may in turn influence adult CVD susceptibility. This highlights the need for additional studies that investigate the transgenerational effects of EC mixture exposure, the need that future work incorporates sex specific effects, a large sample size and that additional studies are required that focus on the molecular and epigenetic mechanisms to define the underlying mechanisms and identify early indicators of CVD risk transmission.

Chapter 5 Transgenerational effects of real-life EC mixture exposure on markers of cardiovascular function in adult sheep

5.1 Abstract

Despite considerable advancements in diagnostics and therapeutics, cardiovascular diseases (CVD) continue to play a leading role in global mortality. Sex differences in global burden of CVD are prevalent, and the mechanisms are poorly understood. Exposure to environmental chemicals (EC) during gestation is a growing public health concern, particularly the development of adult CVD. We have previously reported sex-specific adverse effects of developmental EC exposure on cardiovascular function in F1 adult offsprings. By allowing grandmaternal grazing of sheep on biosolids treated pasture (BTP), this study investigated transgenerational effects of EC exposure on left ventricular histology and gene expression in F2 male and female adult offspring. Biosolids exposure did not affect left ventricular cardiomyocyte number or collagen levels in either sex. There was a significant ($P<0.05$) increase in left ventricular mRNA expression of myocardial stress (NPPB), inflammation (DYA), fibrosis (COL3A1) and hypertrophic signalling (AKT1) along with increased expression of insulin signalling markers (IGF1 and IGF1-R) in B males while females exhibited an increase in COL1A1 expression. These findings particularly in males are concerning which indicates sexually dimorphic and intergenerational cardiovascular risk associated with developmental EC exposure.

5.2 Introduction

Despite considerable advancements in preventive and treatment strategies for cardiovascular diseases (CVD) over the last 30 years, CVDs continue to be the leading cause of death worldwide (Roth et al., 2020). Multiple risk factors are involved in CVD including non-modifiable risk factors such as age and genetics, as well as modifiable risk factors such as hypertension, diabetes and obesity, and lifestyle factors such as smoking and lack of physical activity (Arafa et al., 2021; Cercato and Fonseca, 2019; De Rosa et al., 2018; Fuchs and Whelton, 2020). In addition, it is proposed that exposure to anthropogenic environmental chemicals (ECs) may contribute to the development and severity of CVDs (Cosselman et al., 2015; Landrigan et al., 2016).

Data from the U.S. Centre for Disease Control and Prevention (CDC) have shown that almost all Americans carry detectable levels of ECs in their bodies, including substances with known endocrine disrupting, neurotoxic and carcinogenic actions (Control and Prevention, 2014). Due to the ubiquitous nature of ECs, human beings are constantly exposed to persistent organic chemicals such as polychlorinated biphenyls (PCBs), and nonpersistent plasticizers including bisphenols and phthalates (Sly et al., 2016). Specific concerns have been expressed relative to developmental exposure to ECs, as exposure during sensitive periods of development has been associated with adverse health outcomes in the resulting offspring including low birth weight, diabetes, cancer and importantly, adult onset of CVD (Betts, 2007; Govarts et al., 2016; Thornburg, 2015; Wang et al., 2016b).

Several human epidemiological studies have documented an association between ECs exposure and CVD. Among these, exposure to BPA, both during the prenatal and adolescence period, has been associated with hypertension as well as coronary and peripheral artery diseases in adulthood (Bae et al., 2012; Bae et al., 2017; Chrysant, 2015; Gore et al., 2015; Melzer et al., 2012a; Melzer et al., 2012b; Shankar et al., 2012b). Maternal exposure to polyaromatic hydrocarbons

(PAH) has also been linked with an increased risk of hypertension and congenital heart diseases in the offspring (Chen et al., 2025a; Elhassan et al., 2024) and the incidence of metabolic syndrome, which is an important predictor of adult CVD has been reported to be increased in children exposed to polyfluoroalkyl substances (PFAS) (Fossa et al., 2025; Jacobs Jr et al., 2022; Pool et al., 2021). A number of experimental animal studies have confirmed that ECs can have adverse effects on cardiac structure/function and attempted to address the specific mechanisms of action that underlie the effects of EC exposure. For example, BPA exposure in rats has been reported to induce cardiac fibrosis and cell proliferation by activation of estrogen receptor and ERK1/2 dependent pathways (Hu et al., 2016) and increased expression of connective tissue growth factor protein (CTGF) which is involved in extracellular matrix (ECM) remodelling (García-Arévalo et al., 2021). BPA exposure in adult rats has also been linked with structural and functional CV changes as a result of reactive oxygen species (ROS) generation, apoptosis, inflammation and fibrosis (Sewelam et al., 2024). Fibrosis and cardiac hypertrophy were also reported in mice exposed to polychlorinated biphenyl (PCB) (Wang et al., 2021) and increased systolic and diastolic blood pressure was seen in rats chronically exposed to estradiol, independently of effect on heart rate (Subramanian et al., 2017). Although not extensively investigated as many animal-based experiments are single sex, evidence is accumulating on sex-specific effects of developmental ECs exposure on cardiac structure and function. For example, prenatal BPA exposure induced inflammatory changes and higher expression of genes involved in the regulation of cardiac function such as ANP, BNP, Col1A1, Col3A1, TGF-ß, and CTGF, in males compared to female mice (Marrone et al., 2025). Cardiac hypertrophy was also observed in female but not male mice prenatally exposed to BPA and DES (Patel et al., 2015) while prenatal PAHs exposure resulted in cardiomyocyte apoptosis and reduced expression of transforming growth factor (TGF-ß) in males but not females. All these studies reinforce the importance of studying the sexually dimorphic nature of ECs induced CV changes.

Current evidence on EC-contribution to CV effects is therefore limited by the lack of sex-specific real-life EC exposure studies, in which ECs are present at low levels and can exhibit additive, synergistic and antagonistic effects. Many of the studies have only used single ECs and have predominantly been conducted using rodent models which have a short lifespan and do not fully consolidate the chronic low-level human EC exposure. Furthermore, few studies have addressed the possibility that the effects of ECs on CV function may be due to alterations to the fetal environment which results in permanent alterations possibly via epigenetic mechanisms which can be transmitted across generations (Chamorro-Garcia et al., 2017; Dunn and Bale, 2011; Svoboda et al., 2022). The biosolids treated pasture (BTP) sheep model provides a realistic approach to study the effects of mixed EC exposure in a translationally relevant large animal model. Biosolids, derived from wastewater treatment is commonly used as an agricultural fertilizer and in land remediation which contains a complex mixture of ECs albeit at low individual concentrations, akin to the human exposome (Rhind et al., 2010; Rigby et al., 2021). After its application to the land, a mixture of different endocrine disrupting chemicals including BPA, DEHP, DPP, PAHs, DEHP etc. have been detected in the soil, summarized by (Bellingham and Evans, 2024). As well as soil, ECs were also detected in tissue samples following BTP exposure (Rhind et al., 2011; Rhind et al., 2005a). Using a series of in vivo and in vitro studies, we have previously used the BTP sheep model to address the sex-specific effects of real-life EC mixture exposure on CV structure and function. The results have demonstrated greater ventricular dimensions, high systolic and diastolic volumes and high cardiac output (Khan et al., 2025a) as well as significant changes in extracellular matrix remodelling in the form of high levels of interstitial, perivascular and replacement fibrosis accompanied by higher expression of inflammatory and apoptotic markers in males but not females (Khan et al., 2025b). The present study was focused on investigating the sexually dimorphic transgenerational effects of EC mixture exposure on structural and molecular markers of cardiac function in the second generation (F2) adult offspring of sheep.

5.3 Material and methods

5.3.1 Ethical statement

All the experimental animal work was performed according to the United Kingdom's Animals (Scientific Procedures) Act 1986, under Home Office Project License PF10145DF. Experimental animals were maintained at Cochno Farm and Research Centre, University of Glasgow under standard husbandry conditions. All the animals were humanly treated during experimental procedures with due consideration to the alleviation of pain, suffering, distress and lasting harm.

5.3.2 Experimental animals and study design

Easy-care ewes (n=320) were randomly assigned to either control (C) or biosolids (B) groups (n=160/group). Ewes from the C group was grazed on pasture treated with inorganic fertilizer while B group ewes were grazed on pasture treated with biosolids at conventional rates (4 tonnes/ha) twice a year in April and September. For breeding and to control for potential paternal effects, F0 ewes were artificially inseminated with semen from four unrelated rams raised on C pasture, to generate four sire groups/families in the first generation (F1). Until approximately two weeks before parturition, ewes were maintained on their respective pasture after which they were brought inside and fed according to normal husbandry practices with B group feed derived from BTP. After parturition, F0 ewes and F1 lambs (C & B) were maintained on C pasture until the age of weaning. Post-weaning, male and female offsprings were maintained separately on C pasture, to avoid inbreeding. At the age of 18 months, C and B (males & females) were artificially mated with semen from rams from their respective group avoiding immediate family pairings. The resulting F2 offspring were maintained on C pasture throughout their life.

5.3.3 Euthanasia and tissue sampling

A subgroup of F2 adult male (C, n = 10: B, n = 9, 2y old) and female (C, n = 12: B, n = 12, 2.5y old) offspring were euthanised by intravenous administration of barbiturate (140 mg/kg Dolethal, Vetroquinol, UK). Prior to euthanasia blood samples were collected (BD Vacutainer Plus, BD, USA), centrifuged (3000 ×*g*, 15 min at 4 °C) and plasma harvested and stored at −20°C for later lipid profile assessment. Following euthanasia, hearts from each animal were harvested, trimmed of excess fat, weighed and dissected for left ventricular wall sampling as described previously (Khan et al., 2025b). For each animal, left ventricular tissues were either fixed in 10% neutral buffered formalin (NBF, Thermo Scientific, 16499713) before processing to paraffin blocks or frozen in liquid nitrogen for histological and gene expression studies, respectively.

5.3.4 Left ventricular histology

Left ventricular tissue sections (each 5µm thickness) from each animal were cut using a microtome (Leica Biosystems) and stained with haematoxylin and eosin (H&E) (Fig.5-2A) for the purpose of determination of cardiomyocyte count/field as previously described (Khan et al., 2025b). Additionally, 5µm thick sections were cut and stained with Masson trichome for the assessment of fibrosis. The level of fibrosis was scored on an ordinal scale (0-5) and defined as “0” (No obvious signs of fibrosis), “1” (Perceptive), “2” (Mild), “3” (Moderate), “4” (Severe) and “5” (Very Severe). In addition to scoring, the distribution of different types of fibrosis i.e. interstitial, perivascular, replacement and a combination of these types were also examined as described previously (Khan et al., 2025b).

5.3.5 mRNA extraction, Reverse Transcription and quantitative real-time (qRT) PCR

Samples were initially homogenized using 500 µl TRIzol® reagent lysis buffer on a FastPrep-24 5 G homogenizer (MP Biomedicals, Germany) at 4 m/s followed by

mRNA extraction using Qiagen RNeasy® RNA extraction mini kit spin columns (Qiagen, Hilden, Germany) according to the manufacturer instructions. RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). The expression of selected cardiac function markers was evaluated by qPCR as described previously (Khan et al., 2025b). The markers/genes studied were Atrial Natriuretic Peptide Precursor A (NPPA), Atrial Natriuretic Peptide Precursor B (NPPB), Serine/Threonine Kinase 1 (AKT1), Collagen type 1 alpha 1 (COL1A1), Collagen type 3 alpha 1 (COL3A1), Mechanistic Target of Rapamycin Kinase (mTOR), MYC protooncogene transcription factor (cMYC), BCL2 Associated X, apoptosis regulator (BAX), Caspase3 (CASP3), MHC Class II antigen DY alpha (DYA), MHCII-DR beta chain 1 (DRB1), Bos taurus solute carrier 2 member 4 (SLC2A4), Insulin like growth factor 1 (IGF-1), Insulin like growth factor 1 receptor (IGF1-R) and Estrogen receptor 2 (ESR2). The primer sequences for the markers were the same as that of (Khan et al., 2025b).

5.3.6 Plasma triglycerides and total cholesterol

Triglycerides (TG) and total cholesterol (TC) concentrations in plasma were analysed by Veterinary Diagnostics Laboratory, University of Glasgow, School of Biodiversity, One Health and Veterinary Medicine, with detection limits and % CV values as previously reported (Khan et al., 2025b).

5.3.7 Statistical analysis

Heart weight, cardiomyocyte number, lipid profile and gene expression data were analysed by Two-way ANOVA with treatment and sex as explanatory variables (GraphPad Prism, Prism Windows 5.04). Bonferroni post-hoc tests were used for pairwise comparisons of group means. Fibrosis scores and categorical distribution of fibrosis were analysed by Mann–Whitney *U* test and Fishers exact test in R (RStudio version 2022.02.2 +485) respectively. PCA and PLS-DA analysis of gene expression was carried out in Metaboanalyst (https://www.metaboanalyst.ca/) as previously described (Khan et al., 2025b).

5.4 Results

5.4.1 Transgenerational effects of in-utero EC exposure on heart weight and heart to body ratio

Heart weights (HW) were not significantly different in the C and B groups in either sex (P>0.05) and there was no significant exposure-sex interaction (Fig 5-1 A). Significant sex differences (P< 0.05) were prevalent in HW where males had significantly greater HW compared to females (Fig.5-1 B). HW when normalized to bodyweights i.e. heart to body weight ratio (HBR) revealed no significant (P>0.05) effects of exposure, sex or exposure-sex interaction (Fig. 5-1B).

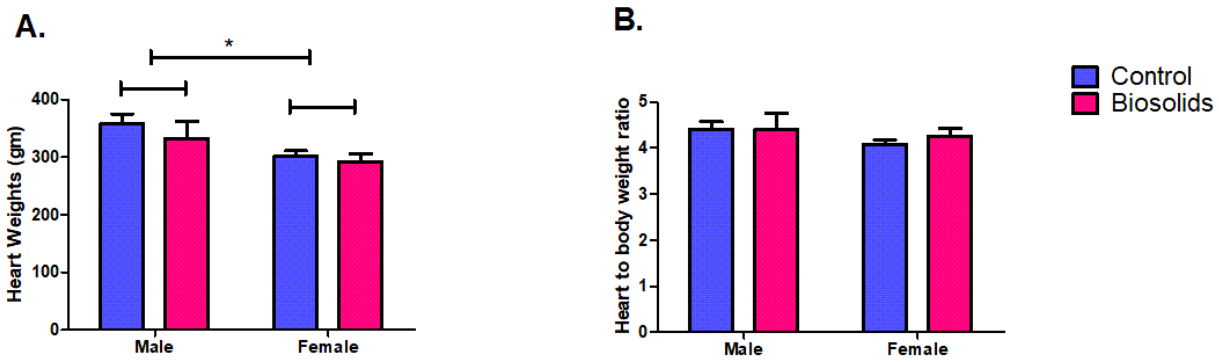

Figure 5-1. Heart weights and heart to body weight ratios A. Significant sex differences in heart weights are shown (P=0.008), while no significant transgenerational effect of B exposure or interaction was observed; P-value Exposure = 0.33, Interaction = 0.65. B. Heart to body weight ratio showing no significant treatment, sex or exposure-sex interaction effects; P-value Exposure= 0.65, Sex = 0.24, Interaction = 0.67. Males (Cn = 10, Bn = 9), Females (Cn = 12, Bn = 12). Data analysed by Two-way ANOVA and expressed as Mean ± SEM. * indicate significant (P < 0.05) differences between groups (control vs biosolids) and sex (male vs female).

5.4.2 Transgenerational effects of in-utero EC exposure on left ventricular cardiomyocyte number and collagen scoring

Left ventricular cardiomyocyte number/field was not significantly different in B exposure group and there was no exposure-sex interaction ($P>0.05$). A significant sex effect ($P< 0.05$) was noted in CM number with more cardiomyocytes per field in males compared to females (Fig. 5-2 B). Masson trichome stained images from C and B males and females are shown in Fig.5-3. No significant effects of exposure, sex or exposure-sex interaction were observed in the fibrosis scores (Fig. 5-4 A) and there was no statistically significant difference ($P>0.05$) in the distribution of different types of fibrosis between C and B groups in both sexes as shown (Fig. 5-4 B-C).

5.4.3 Transgenerational impact of in-utero EC exposure on molecular markers of cardiac function

5.4.3.1 Cardiac stress and fibrosis markers

Results revealed no significant ($P>0.05$) transgenerational effect of developmental EC exposure, sex or exposure-sex interaction in the relative expression of NPPA (Fig. 5-5 A). An apparent pattern($P=0.06$) for an exposure-sex interaction was noted in the expression of NPPB and post-hoc analysis revealed significantly ($P<0.05$) higher expression of NPPB in B compared to C males while there was no difference between C and B females (Fig.5-5 B). A significant ($P<0.05$) effect of sex was observed in the mRNA expression of the fibrosis marker COL1A1, with higher expression of COL1A1 in the males compared to the females. There was also a significant exposure-sex interaction in the expression of COL1A1 which was higher in B relative to C females while no significant difference was seen between B and C males (Fig.5-5 C). A significant ($P<0.05$) effect of exposure and an exposure-sex interaction was also seen in the expression of COL3A1 which was significantly higher in B compared to C males while there was no significant difference between B and C females (Fig.5-5 D).

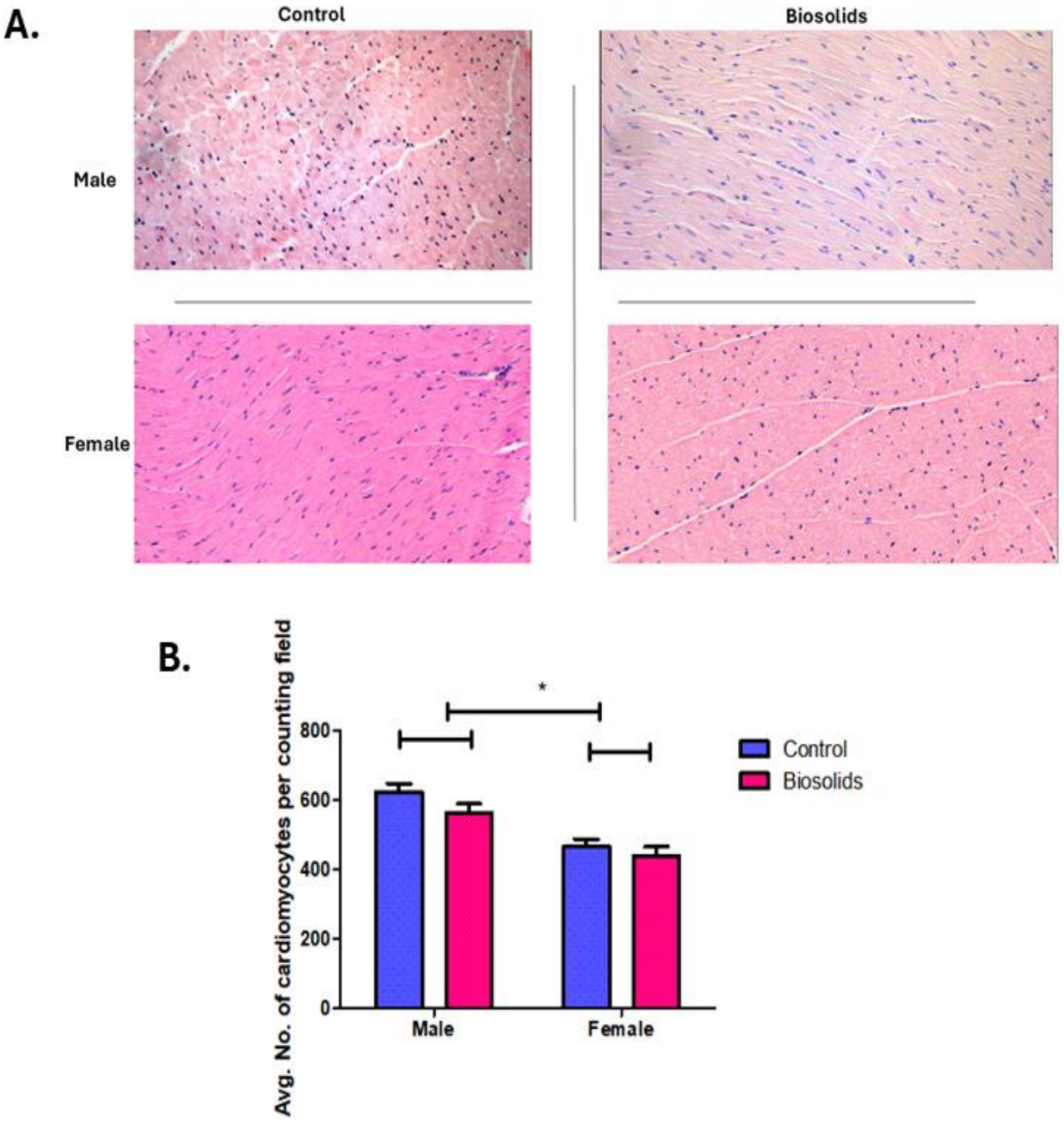

Figure 5-2.A. Haematoxylin and Eosin-stained images from males and females (Control and Biosolids). B. Average cardiomyocyte count per field.

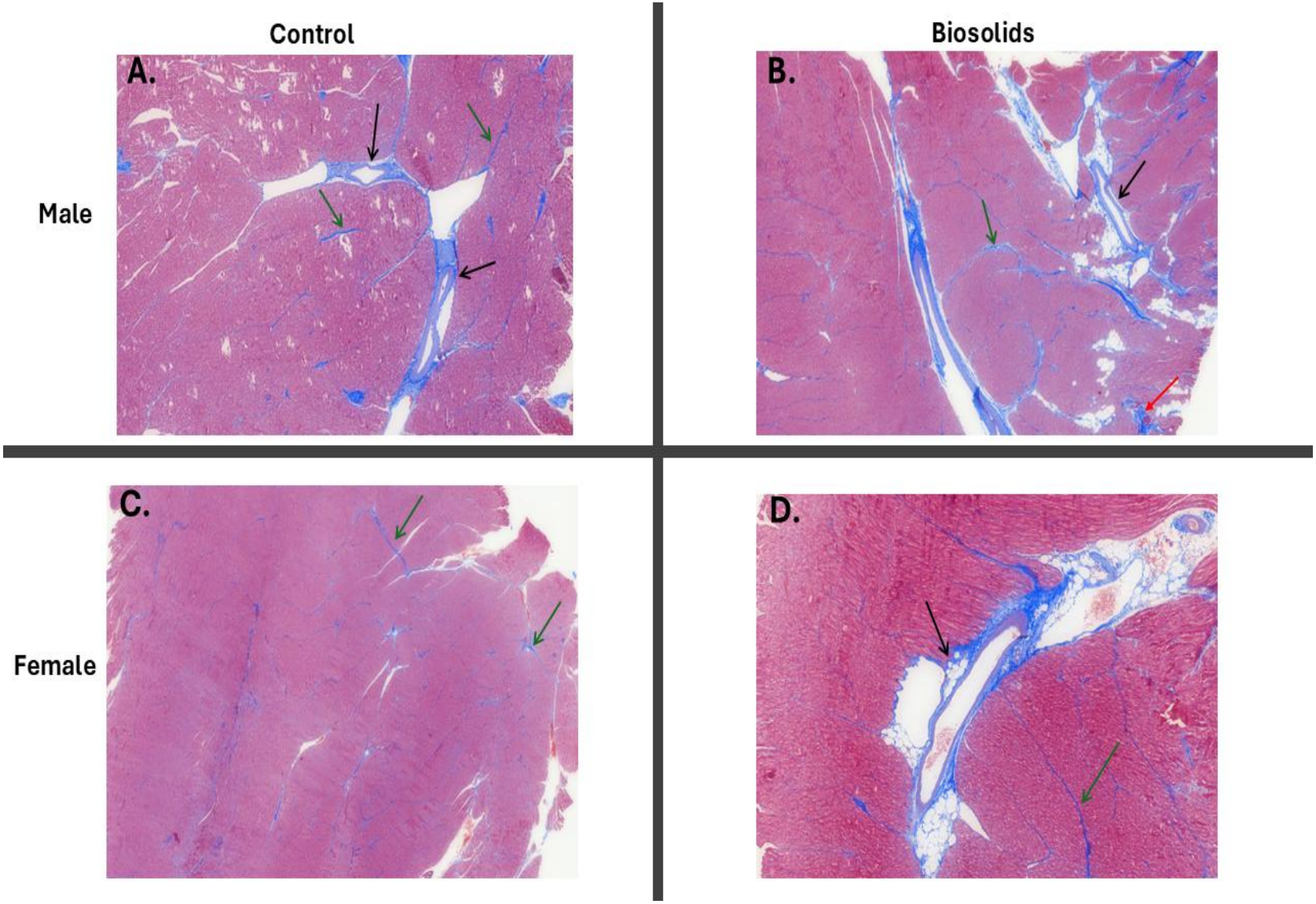

Figure 5-3. Representative Masson's trichome stained images from C and B males and females showing interstitial (green arrow), perivascular (black) and replacement fibrosis (red arrow). A. Mild interstitial and perivascular fibrosis (Score=2). B. Interstitial, perivascular and some replacement fibrosis (Score=4). C. Perceptive interstitial fibrosis (Score=1). D. Moderate interstitial and perivascular fibrosis (Score=3).

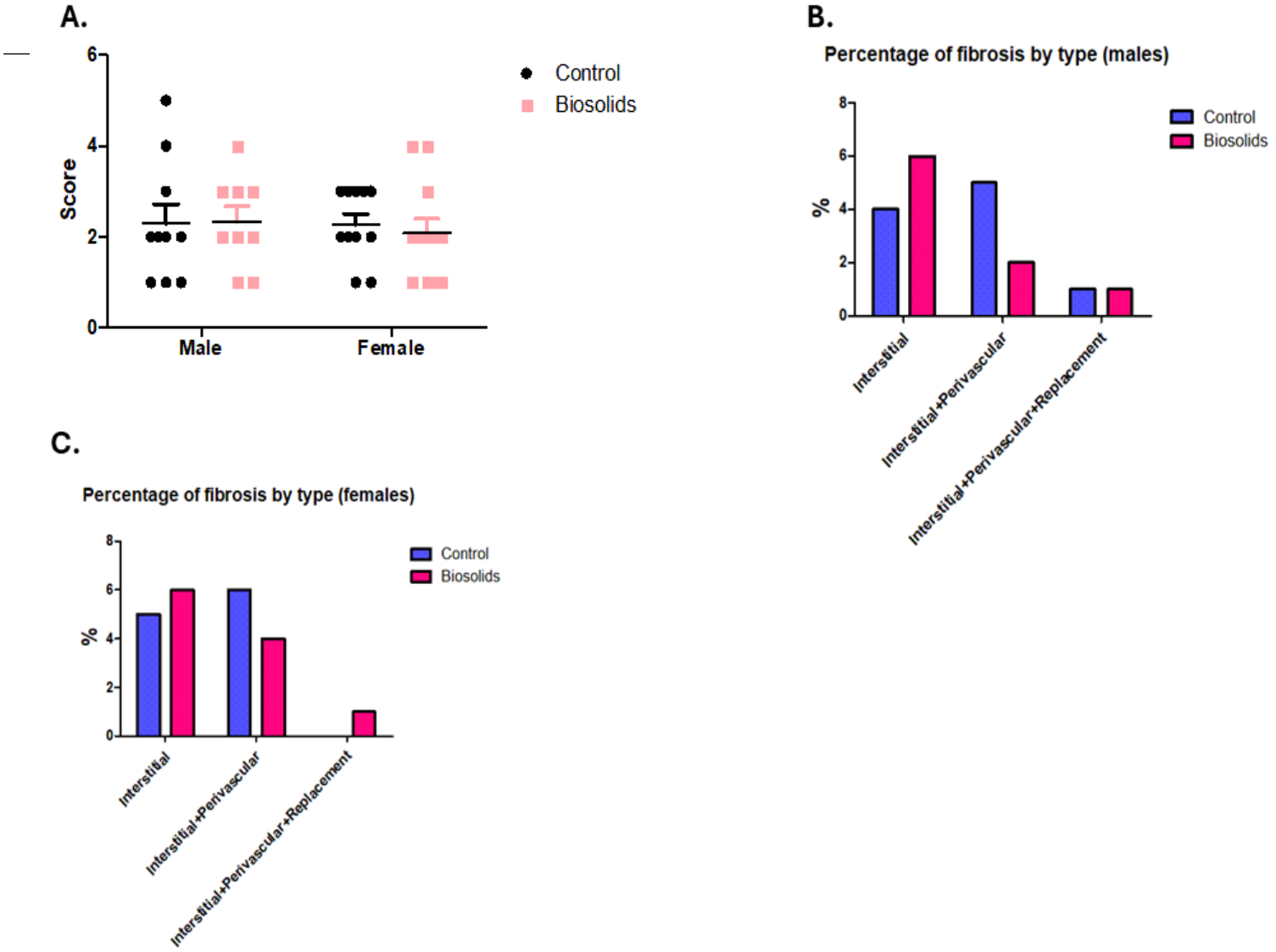

Figure 5-4. Fibrosis scoring **(A)** and percentage distribution of fibrosis types in males **(B)** and females **(C).**

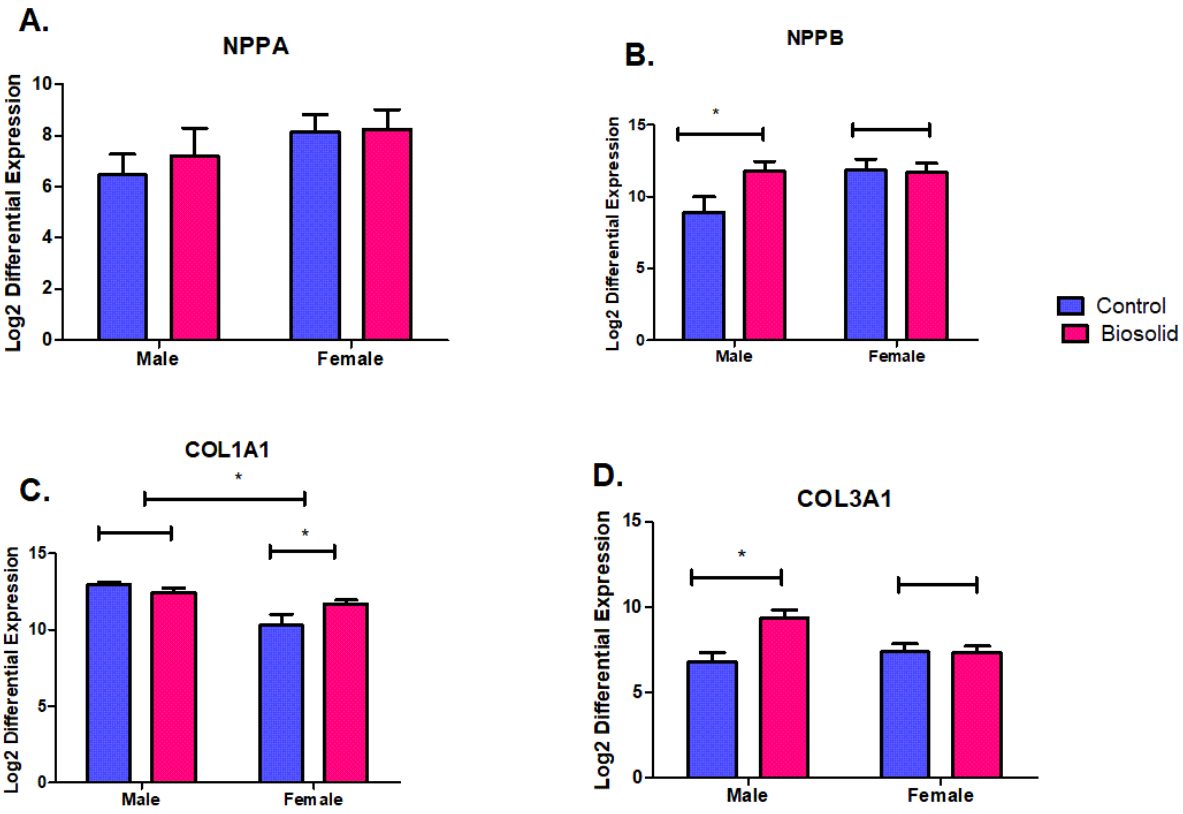

Figure 5-5. Differential expressions of cardiac stress and fibrosis markers (relative to GAPDH). A. mRNA expression of NPPA showing no significant effect of exposure, sex or interaction; P-value Exposure = 0.60, Sex = 0.11, Interaction = 0.71. B. shows significantly higher NPPB expression in B compared to C males but no significant effect of exposure in females; P-value Exposure = 0.10, Sex = 0.08, Interaction = 0.06. C. Shows overall significantly higher expression of COL1A1 in males relative to females and an exposure-sex interaction; P-value; Exposure = 0.32, Sex = 0.0006, Interaction = 0.03. D. Shows significant exposure-sex interaction with higher COL3A1 expression in B males compared to controls while no significant effect in females; P-value; exposure = 0.01, Sex = 0.13, Interaction = 0.008. Males (Cn = 10, Bn = 9), Females (Cn = 12, Bn = 12). Data analysed by Two-way ANOVA. Post-hoc Bonferroni test performed for pairwise group comparison. Data expressed as Mean ± SEM. * indicate significant ($P < 0.05$) differences between groups (control vs biosolids) and sex (male vs female).

5.4.3.2 Markers of cardiomyocyte apoptosis and inflammation

Data analysis indicated no significant ($P>0.05$) effects of exposure, sex or exposure-sex interaction in the expression of the cardiomyocyte apoptotic markers BAX and CASP3 (Fig. 5-6 A-B). An apparent towards significant exposure effect ($P=0.06$) was observed in CASP3 which was higher in B compared to C group. An overall significant effect of exposure ($P<0.05$) was seen in the expression of inflammatory marker MHCII-DRB1, which was higher in B compared to C animals, however, there was no significant ($P>0.05$) effect of sex or

exposure-sex interaction (Fig. 5-6 C). A significant effect of exposure and sex ($P<0.05$) and an apparent pattern for an exposure-sex interaction ($P=0.06$) was apparent in the mRNA expression of MHCII-DYA, which was significantly increased in B compared to C males, as well being significantly higher in males compared to females (Fig.5-6 D).

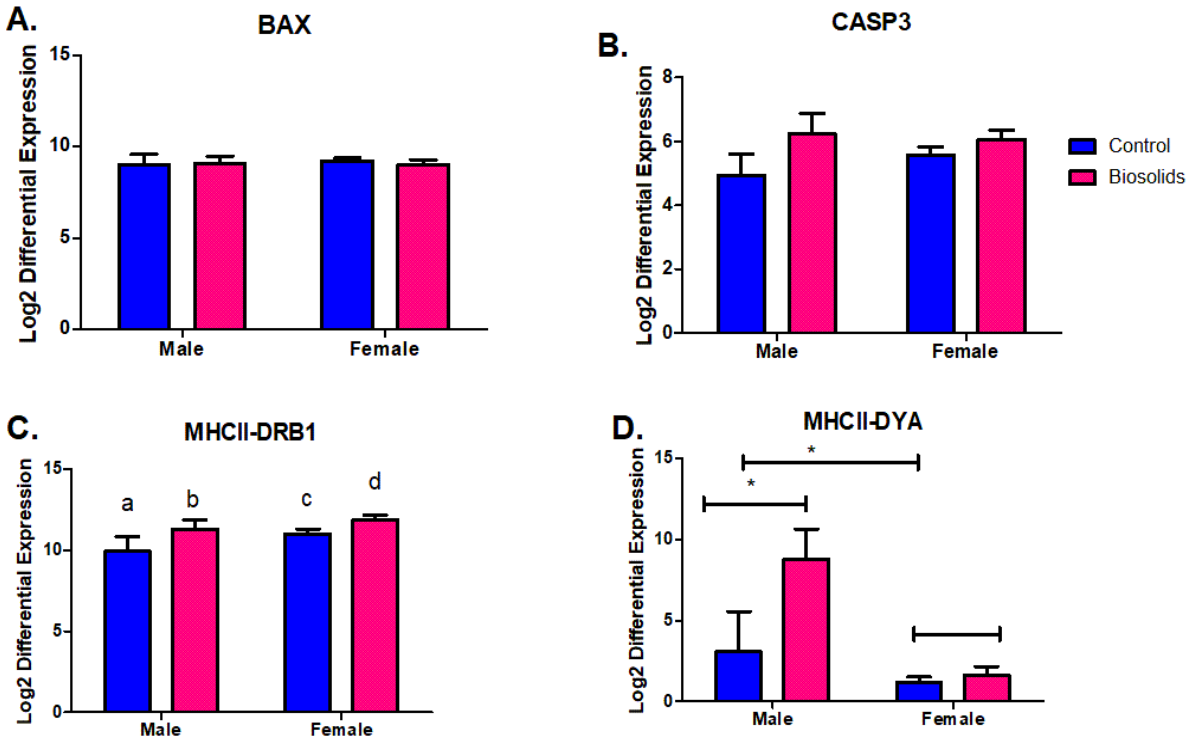

Figure 5-6. mRNA expression of markers of cardiomyocyte apoptosis and inflammation (relative to GAPDH). mRNA expression of BAX (A) and CASP3 (B) showing no significant effect of exposure, sex or exposure-sex interaction; P-value BAX; Exposure = 0.81, Sex = 0.82, Interaction = 0.71, P-value CASP3; Exposure = 0.06, Sex = 0.64, Interaction = 0.41. C. MHCII-DRB1 expression shows no significant difference in C and B groups in each sex but an overall (ac~bd) higher expression in B compared to C group was noted; P-value Exposure = 0.03, Sex = 0.11, Interaction = 0.62. D. Shows significant upregulation of MHCII-DYA in B compared to C males as well overall in males compared to females; P-value Exposure = 0.02, Sex = 0.002, interaction = 0.06. Males (Cn = 10, Bn = 9), Females (Cn = 12, Bn = 12). Data analysed by Two-way ANOVA and expressed as Mean ± SEM. * indicate significant ($P < 0.05$) differences between groups (control vs biosolids) and sex (male vs female).

5.4.3.3 Markers of cardiomyocyte growth, glucose metabolism and estrogen receptor signalling

Data analysis revealed a significant ($P<0.05$) impact of exposure and an exposure-sex interaction in the expression of the cardiomyocyte growth and survival marker AKT1, which was significantly upregulated in B compared to C males, but similar in B and C females (Fig.5-7 A). No significant ($P>0.05$) effect of exposure, sex or exposure-sex interaction was observed in the expression of mTOR (Fig.5-7 B). The expression of cMYC was significantly higher in females in comparison to males without any significant effect of exposure and there was no significant exposure-sex interaction (Fig. 5-7C). There was a significant ($P<0.05$) effect of exposure and an exposure-sex interaction in the expression of IGF1 (Fig.5-7 D) and IGF1-R (Fig.5-7 E) mRNA, IGF1 and IGF1-R were significantly upregulated in B compared to C males, but there was no significant effect of B exposure in females, but IGF1-R was overall higher in females relative to males (Fig.5-7 E). Finally, significant sex differences were observed in the expression of SCL2A4 (also known as GLUT4) which was higher in females compared to males ($P<0.05$) and an apparent pattern($P=0.09$) was noted for an exposure-sex interaction in the expression of SCL2A4 where B males had higher SCL2A4 expression compared to C males while there was no difference in the expression of SCL2A4 in females (Fig. 5-7 F). An apparent pattern ($P=0.06$) was observed in the expression of ESR2 mRNA, it being higher in males compared to females but there were no significant ($P>0.05$) effects of exposure or exposure-sex interaction (Fig. 5-7 G).

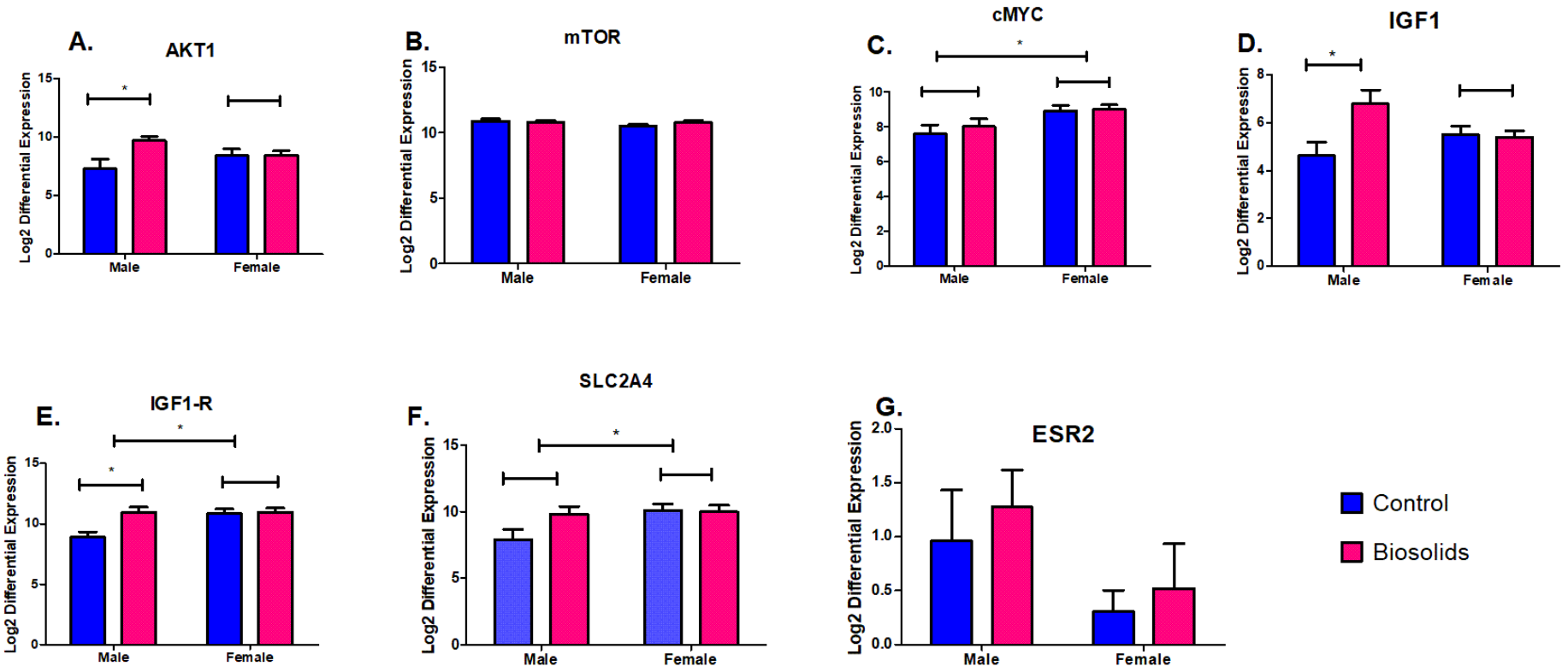

Figure 5-7. mRNA expression of cardiomyocyte growth, Insulin and steroid receptor signalling (relative to GAPDH). **A.** Shows significantly higher AKT1 expression in B compared to C males; P-value Exposure = 0.02, Sex = 0.93, Interaction = 0.03. **B.** Shows no significant effect of exposure, sex, or interaction in the mTOR expression; P-value Exposure = 0.55, Sex = 0.21, Interaction = 0.23. **C.** Shows significant sex differences in the expression of cMYC; P-value Exposure = 0.51, Sex = 0.004, Interaction = 0.67. **D.** Shows significantly higher IGF1 expression in B males compared to C males; P-value Exposure = 0.02, Sex = 0.55, Interaction = 0.01. **E.** Shows significantly higher expression of IGF1-R in B compared to C males and overall higher expression in females compared to males; P-value Exposure = 0.01, Sex = 0.02, Interaction = 0.02. **F.** SCL2A4 expression was significantly higher in females relative to males; P-value Exposure = 0.14, Sex = 0.05, Interaction = 0.09. **G.** Shows trend for higher ESR2 expression in males compared to females; P-value Exposure = 0.48, Sex = 0.06, Interaction = 0.89. Males (Cn = 10, Bn = 9), Females (Cn = 12, Bn = 12). Data analysed by Two-way ANOVA and expressed as Mean ± SEM. * indicate significant ($P < 0.05$) differences between groups (control vs biosolids) and sex (male vs female).

5.4.4 PCA and PLS-DA analysis of gene expression data

Heatmaps showing the markers that are differentially expressed between C and B groups in the males and females are shown in Fig. 5-8A & B respectively. Principle component analysis (PCA) revealed no distinct separation of the B and C groups in females (Fig. 5-8 D). In males however, there was a tendency towards group separation, with some animals from B group sitting outside the cluster formed by rest of the animals (Fig.5-8 C). Partial least square discriminate analysis (PLS-DA) demonstrated that NPPB, AKT1, COL3A1, and MHC-DYA contributed most strongly towards group differentiation in the males (Fig.5-9 A) whereas a strong overlap between C and B groups in females with COL1A1 being the major driver in group separation was evidenced in females as shown by the length and direction of the loading vectors (Fig.5-9 B). Variable importance in projection (VIP) scores (in PLS-DA) further identified the top markers/genes differentially expressed between C and B groups as MHC-DYA, NPPB, COL3A1, AKT1 and IGF1 in males and COL1A1, CASP3, ESR2 and DYA in females (Fig. 5-9 C-D).

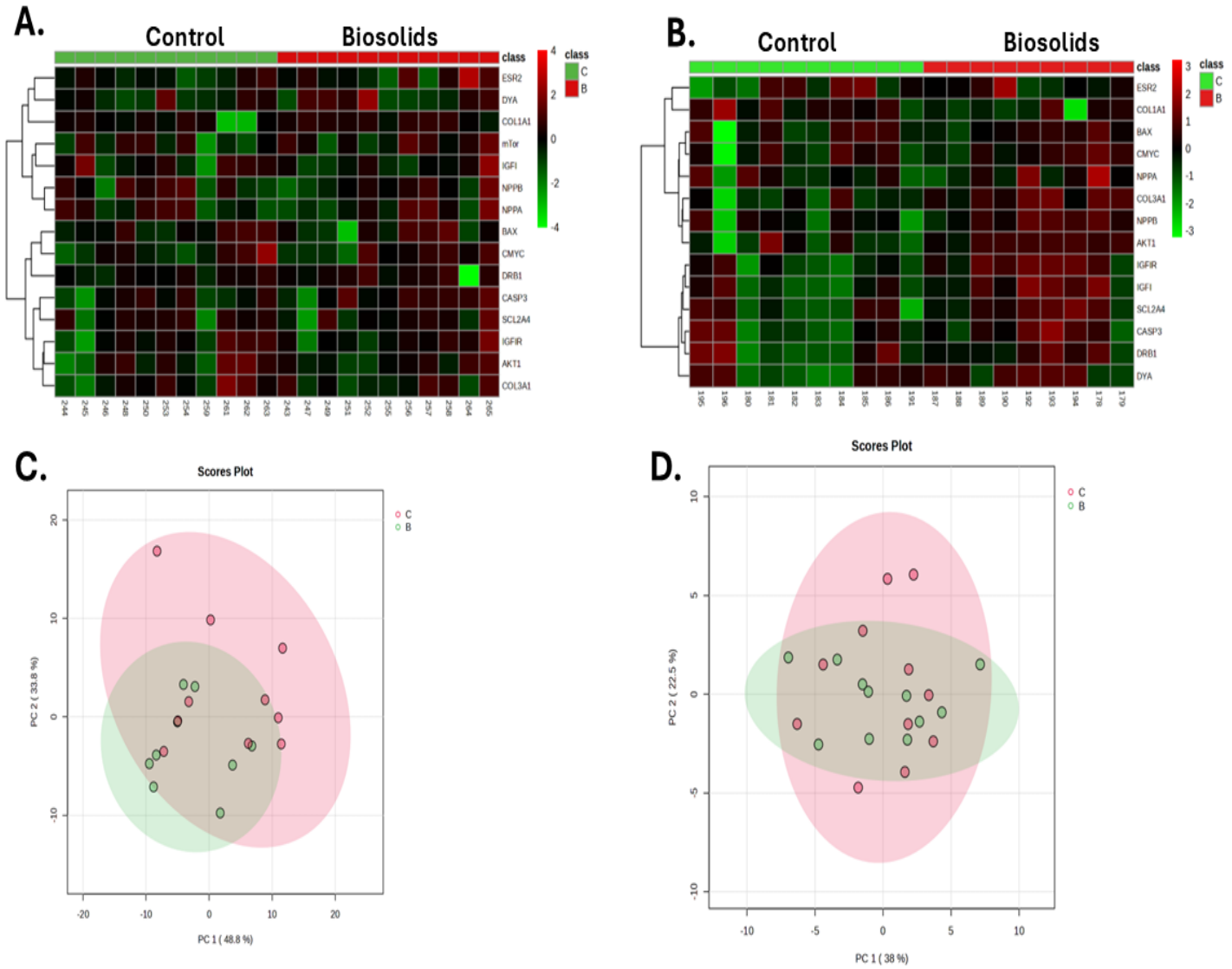

Figure 5-8. Heatmaps showing differential expression of cardiac function genes/markers in males (A) and females (B). C. PCA biplot showing partial group separation in males and no distinct separation in females (D).

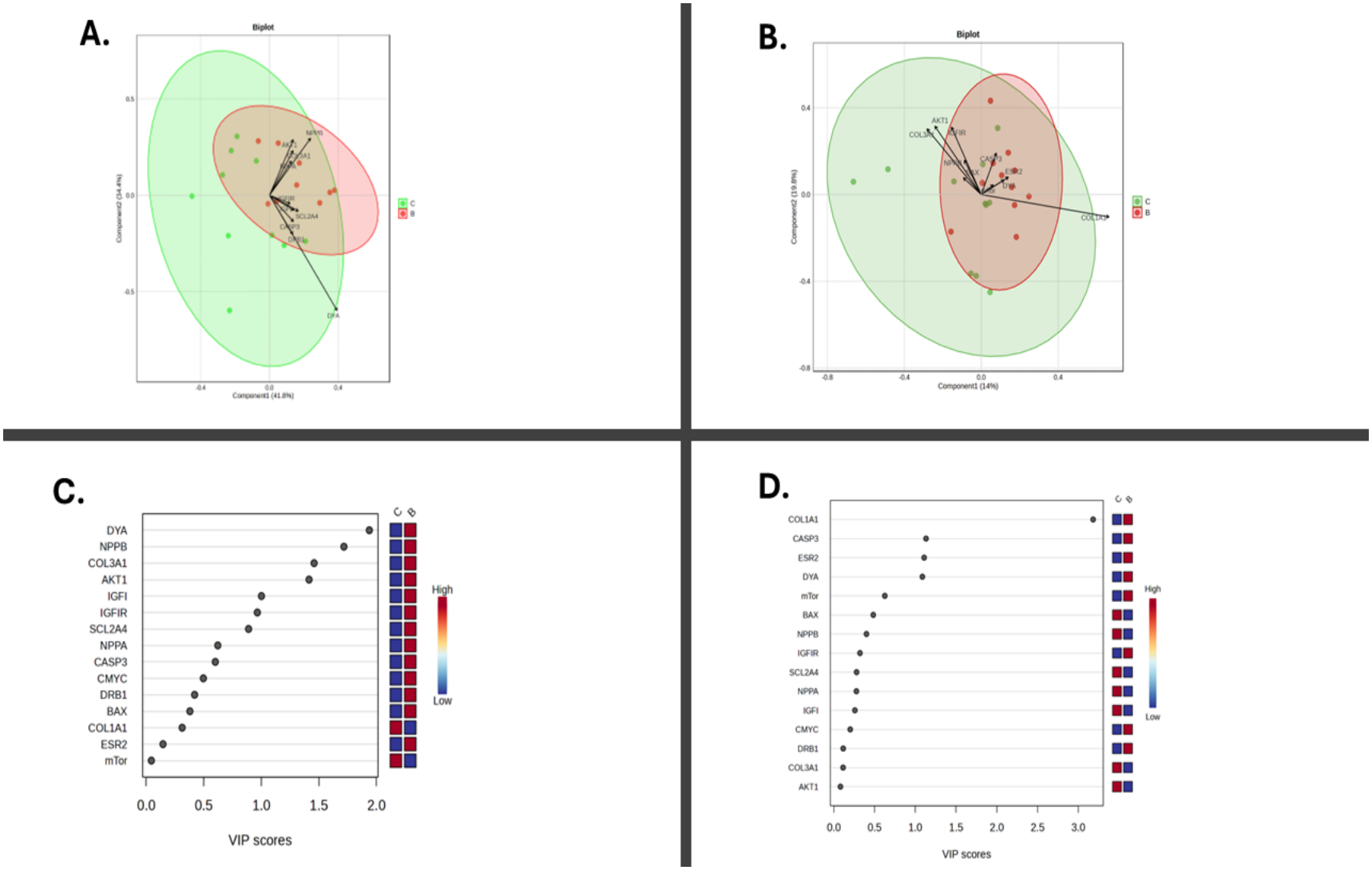

Figure 5-9. PLS-DA biplots showing the magnitude and direction of markers/genes responsible for group separation in males (A) and females (B) respectively. C. VIP scores showing the top genes differentially expressed in males. D. VIP scores showing the top genes differentially expressed in females.

5.4.5 Plasma lipids

No significant effect of exposure or exposure-sex interaction was seen in plasma triglycerides concentration (P>0.05) but an apparent pattern towards a sex difference (P=0.08) was noted where females exhibited higher plasma triglycerides concentrations compared to males. Significant sex differences were observed in total cholesterol concentrations which were significantly higher in males compared to females but were not affected by B exposure (Fig. 5-10 A-B).

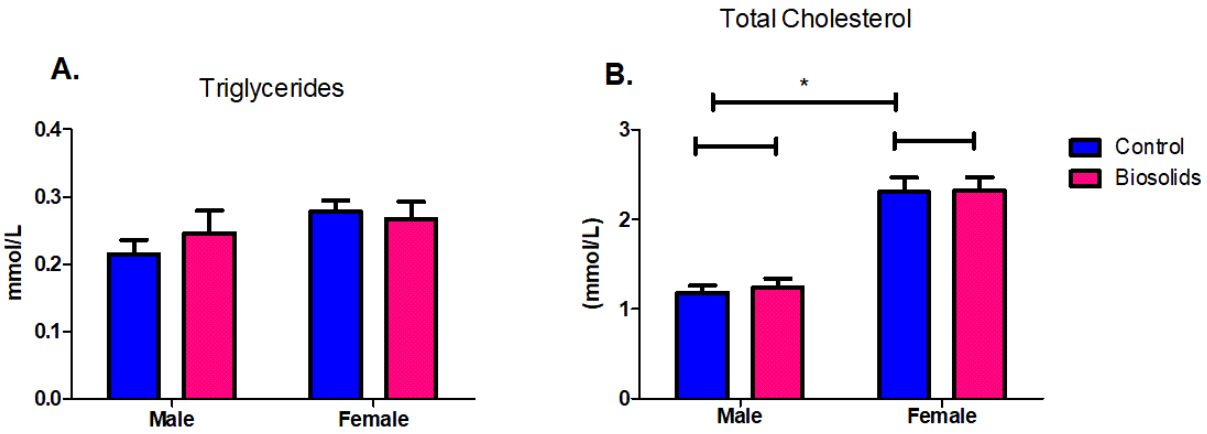

Figure 5-10. Plasma triglycerides and total cholesterol levels. A. Plasma triglycerides showing no significant exposure, sex or exposure sex interaction; P-value Exposure = 0.68, Sex = 0.08, Interaction = 0.39. B. Plasma total cholesterol levels showing significant sex differences; P-value Exposure = 0.77, Sex = < 0.0001, Interaction = 0.89. Males (Cn = 10, Bn = 9), Females (Cn = 12, Bn = 12). Data analysed by Two-way ANOVA and expressed as Mean ± SEM. * indicate significant (P < 0.05) differences between groups (control vs biosolids) and sex (male vs female).

5.5 Discussion

In the present study, transgenerational effects of developmental EC mixture exposure (biosolids) on histological and molecular markers of cardiac function were evaluated in sheep left ventricular tissue. Major findings included significantly increased mRNA expression of genes associated with cardiac stress (NPPB), inflammation (MHC-DYA), fibrosis (COL3A1), cardiac hypertrophy (AKT1) and insulin signalling (IGF1 and IGF1-R) in males whose mothers had been developmentally exposed to a mixture of ECs. In comparison, females in the B group exhibited an increase in expression of mRNA for the fibrosis marker (COL1A1). Despite these changes in the expression of key molecular markers, no significant transgenerational effects of EC exposure were seen in histological measures such as left ventricular cardiomyocyte number and collagen levels in either sex.

We have previously reported sex-specific histological and molecular changes in the left ventricles of sheep (F1 generation) developmentally exposed to a mixture of ECs (Khan et al., 2025b) which could indicate that exposed F1 animals are predisposed to changes in CV health. It has been reported that exposure to ECs during early embryonic or gestational development , particularly, during germ cell migration (Huang et al., 2012) or gonadal determination (Huang et al., 2012) may induce permanent changes in primordial germ cells, enabling inheritance of health effects to the next generation. To address whether this occurred in our model we examined the hearts of the F2 generation formed as a result of within group matings of the F1 animals. Consistent with findings in the F1 males, changes such as an increase in the molecular markers of cardiac inflammation and insulin signalling were also seen in the F2 B males in the present study. This demonstrates that components of the cardiac phenotype that results from developmental EC exposure in the F1 generation may be transmitted through their germline to the subsequent generation. The possibility of germline transmission of cardiac effects of mixed EC exposure is supported by the results of a study conducted in male zebrafish which reported paternal

inheritance of BPA induced changes in insulin signalling pathways, cardiac epicardial oedema and cardiac malformation (Lombó et al., 2015). In contrast to what we have reported previously in the F1 males where there was an increased expression of cardiomyocyte apoptosis marker CASP3, in the F2 males there was an increased expression of the mRNA for NPPB. NPPB is a major component of natriuretic peptide system which is involved in cardiac protection via reducing biochemical stress and preserving anti-ischemic, antiproliferative, and antiapoptotic functions (Berezin and Berezin, 2020) and its increased expression in the F2 males could be a compensatory mechanism to attenuate hypertrophic and apoptotic changes. In addition to changes in the expression of BNP mRNA, the current study also documented an increase in expression of mRNA for markers of the AKT1 signalling pathway that had not been seen in the F1 generation. Given the role played by AKT1 in cardio-protection via promoting cardiomyocyte growth and function (Wardman and Heineke, 2025) this could suggest an effect on the regulation of cardiomyocyte growth and survival in the F2 generation. The increase in AKT1 signalling may also indicate that concentric cardiac remodelling has occurred in the F2 males which would be supported by the observation of increased wall thicknesses in these animals without any proportional enlargement of left ventricular chamber size (Chapter 4). This phenotype contrasts with that seen in the F1 males who had increased left ventricular dimensions without any changes in wall thicknesses (Khan et al., 2025a). These parameters were unaffected by B exposure in the females of either the F2 (present study) or the F1 generation (Khan et al., 2025b). This difference in the effects of developmental EC exposure between the male and female animals highlights that ECs induced transgenerational CV changes could be sex dependent.

In the current study, no significant effect of grand maternal B exposure was seen on the left ventricular cardiomyocyte number in either sex and are in agreement with the findings in F1 animals (Khan et al., 2025b). A sex difference was observed where males had higher number of ventricular myocytes compared to females which could likely be explained by the greater heart weights in males

compared to females. The lack of changes in CM number could be interpreted as surprising given the high expression of apoptosis marker in B group, particularly in B males, and the fact that postnatal cardiomyocyte proliferation is not substantial (van Amerongen and Engel, 2008). However, there is compelling evidence that terminally differentiated cardiomyocytes can re-enter the cell cycle and manipulate the cell cycle signalling pathways to compensate for the functional loss of cardiomyocytes (Ko and Nomura, 2022) and by so doing a functional cell population could be perpetuated. The histological analysis did not indicate any effect of ancestral EC mixture on either the amount or distribution of collagen within the F2 ventricular tissue of both males and females. However, when compared to their respective controls, there was a significant increase in the expression of mRNA for the fibrosis markers COL3A1 and COL1A1 in males and females respectively. The increase in COL3 in B males and COL1 in females is of interest as it can strongly influence the development and progression of cardiac pathology. An increase in COL1 is common during cardiac remodelling phase and particularly important because it can raise myocardial stiffness, impairing both diastolic filling and systolic contraction whereas COL3 is increased during early phase of cardiac injury which forms a more elastic network that stores and releases energy during cardiac contractions (Ricard-Blum, 2011; Singh et al., 2023). The absence of discernible differences in the level of fibrosis with an increase in COL3A1 in males and COL1A1 in females may also represent subclinical extracellular matrix (ECM) remodelling occurring at early phases of cardiac injury.

Another important finding from this study was the reversal of sex specific differences in the expression of sex steroid receptor ESR2 also known as ESRß irrespective of generational B exposure. The ESR2 expression in the current study was significantly higher in males relatives to females which is the opposite of what we have seen in the F1(Khan et al., 2025b). Cardio protection has been shown to be mediated by ESRß predominantly in females compared to males (Fliegner et al., 2010; Gabel et al., 2005; Skavdahl et al., 2005; Wang et al., 2008). The functional upregulation of ESRß can be associated with oestrogen

aromatization in males and has paramount significance particularly the observation of a parallel increase in fibrosis marker Col1 in males compared to females. It has been shown that only ESRß signalling can promote the expression of collagens in males while in females both ERα and ESRß are involved (Dworatzek et al., 2019; Medzikovic et al., 2019). Elevated ESR2 in male myocardium may also represent an adaptive response to stress or remodelling, facilitating oestrogen-mediated signalling despite lower systemic estrogen compared to females.

In addition to sex differences in oestrogen receptor signalling, sex differences observed in lipid profile of F1 animals (Khan et al., 2025b) were also reversed in the F2. Females in the F2 demonstrated significantly elevated plasma triglycerides and an apparent pattern for higher total cholesterol level compared to males. While sex steroids are thought to be involved in driving sex differences in lipid homeostasis and beneficial effects (Wang et al., 2011), the finding presented here in is one example of current debate on the role of oestrogens on favourable outcomes on lipid levels and the emergence of CVDs in females. While the higher expression of ESR2 seen in males might explain the favourable lipid profile observed in the current study, this may also be due to confounding factors such as total body fat composition, fat distribution and insulin sensitivity all of which can influence lipid homeostasis. Consistent with the findings of (Khan et al., 2025b), we did not see any significant effect of EC exposure in either sex. Studies investigating the transgenerational effect of ECs on lipid homeostasis are lacking, however certain prenatal ECs exposure studies in F1 have shown that lipid homeostasis can be altered in a sex-specific manner. For example, prenatal BPA exposure in rats has been reported to compromise lipid metabolism in females but not males (Tonini et al., 2021). In another study on developmental exposure to BPA, male rats had altered fat gene expression and higher triglycerides, while females showed changes in fat cell density (Lejonklou et al., 2017) indicating that ECs can have sexually dimorphic effects on lipid metabolism. Specifically in sheep, developmental exposure to mixture of ECs i.e. biosolids has shown adverse effects on triglycerides levels in prepubertal

lambs as well as sex-specific changes in the lipid profile of F1 offsprings (Thangaraj et al., 2025). However, as we mentioned previously (Khan et al., 2025b), the lack of a transgenerational impact of B exposure on plasma lipid levels in the present study is limited by the fact that the observations were based on a single plasma sample which may not be able to detect any subtle changes in lipid homeostasis.

5.6 Conclusion

It can be concluded from this study that developmental exposure to mixture of ECs in the form of biosolids can result in transgenerational sex-specific changes in markers of CV function. In the present study, offsprings from EC-exposed sheep showed changes in molecular markers of cardiac stress, hypertrophy and inflammation, with these effects being markedly greater in males. In contrast, female offspring exhibited a lesser effect, except for an increased expression of COL1A1, a marker of fibrosis. The cardiac phenotype observed in F2 males aligns with our previously reported effects in the F1 generation (Khan et al., 2025b). Although the present findings support inheritance of cardiac molecular changes, the underlying mechanisms remain unclear. Future studies using environmentally realistic EC mixture models, larger sample sizes, and targeted investigation of sex-specific epigenetic and molecular pathways are needed to understand how developmental EC exposure may contribute to inherited CVD risk, particularly in males.

Chapter 6 Discussion

6.1 Overview

Cardiovascular diseases (CVD) persist to be the leading cause of death worldwide even though significant progress has been made in cardiac diagnostics and therapeutics over the last few decades. While multiple risk factors are involved in CVD, exposure to complex anthropogenic chemicals during development constitute one of the risk factors for CVD in later life. The present thesis utilized the biosolids treated pasture (BTP) sheep model to address the growing concern of developmental programming of CVD as a result of low-level environmental chemical (EC) mixture exposure. Developmental programming of CV impacts were investigated in both sexes of sheep to gain an understanding of the sexually dimorphic nature of adult CVD. Moreover, transgenerational investigation of cardiac effects was also focus of current thesis. Following gestational exposure to mixture of chemicals, cardiovascular function in adult offspring was assessed by techniques including blood pressure measurements, heart rate variability and echocardiography. Traditional molecular techniques including histology and gene expression using qPCR were applied to investigate the cardiac changes in left ventricle tissues. These investigations revealed adverse CV changes which differed by sex and indicates that EC mixture exposure during gestation may perturb the adult CV function in a sex-specific manner. This work provides important leads to future research on mechanistic insights through which EC exposure can induce sex-specific CV remodelling in adult offspring.

Chapter 1 detailed the introduction of CVD as a leading cause of death and the implications of ECs exposure as a contributing factor for CVD. In the subsequent sections, importance of sex specific nature of CVD was highlighted and evidence of later life CVD in the context of developmental origins of health and disease (DOHaD) was discussed. Separately, literature on EC exposure and CVD from human, animal and invitro cardiomyocyte studies was outlined. The subsequent 4 chapters, 2 of which have been published, present primary research conducted on sexually dimorphic effects of developmental EC exposure and adult CV function assessed via in vivo and invitro studies in the F1 and F2 generations,

respectively. These chapters documented sex-specific cardiac changes in the F1 and F2 adult male and female offspring following in vivo analysis of cardiac function in live animals and histological and molecular analysis of left ventricular tissues collected at postmortem.

Developmental EC exposure induced sexually dimorphic CV changes in adult F1 offsprings. These changes were observed in the form of increased left ventricular dimensions, end-diastolic and systolic volumes, stroke volume and cardiac output in EC exposed males while these parameters were not affected in females. The cardiac phenotype seen in males corroborated with eccentric left ventricular hypertrophy (Carabello, 2002; Devereux and Roman, 1999). Eccentric hypertrophy is differentiated from concentric hypertrophy by the lack of changes in wall thicknesses accompanied by an increase in the left ventricular dimensions which was also observed in EC exposed males only. Eccentric hypertrophy is also characteristic of athlete's heart undergoing exercise endurance training (Scharf et al., 2010), however, given these animals were not subjected to any postnatal exercise interventions, the possibility of eccentric LVH due to exercise endurance is unlikely. EC exposed phenotype in males also exhibited significantly lower heart rates (HR) compared to controls. This can be taken as a consequence of direct autonomic regulation by EC exposure or a compensatory mechanism to counter volume overload in the form of high end-diastolic and systolic volumes as well as greater stroke volume and cardiac output in these animals. On the other hand, EC exposed females in the F1 exhibited lower heart rate variability as shown by lower root mean square of successive differences (RMSSD), and the standard deviation of the IBI of normal sinus beats (SDNN) which again shows altered autonomic regulation or sympathetic dominance in EC exposed females. This observation in EC exposed females is of significance as a lower HRV due to autonomic imbalance is a risk factor for CVD (Thayer et al., 2010). It has been demonstrated that an increase in sympathetic activity can lead to an increased risk of ventricular arrhythmias during myocardial ischaemia (Balanescu et al., 2004). In the F1 offsprings, there was no significant effect of developmental EC exposure on blood pressure parameters in either sex. However, systolic, diastolic and mean blood pressures

were higher in males compared to females which could be a result of sex steroid hormone differences regulating blood pressure and adds to the evidence that males are at increased risk of CVD development.

EC exposed F1 offsprings also displayed sexually dimorphic cardiac changes in the histological and molecular analysis of left ventricle tissues. EC exposed males had higher levels of interstitial, perivascular and replacement fibrosis which suggested increased extracellular matrix remodelling due to developmental EC mixture exposure. It is believed that both interstitial and replacement fibrosis play an important role in the progressive decompensation of LVH (Lazzeroni et al., 2016). While this increase in fibrosis is essential to maintain normal structural integrity of myocardium, in excess it can lead to impaired diastolic and systolic function of the heart (Segura et al., 2014). These changes were also accompanied by higher expressions of inflammatory (DRB1, DYA) and apoptosis marker (CASP3) in the EC exposed males. This indicates that EC exposure can result in sexually differentiated cardiac pathology involving extracellular matrix remodelling, inflammatory and apoptotic pathways which can culminate in cardiac dysfunction. Except for an increased apparent pattern in NPPA, B exposed phenotype in females appeared to be preserved compared to males. NPPA encodes for ANP which is a natriuretic peptide and play a role in cardio protection due to its anti-inflammatory, anti-apoptotic and anti-fibrotic potential (Kasama et al., 2008). The upregulated ANP can also be interpreted as a result of increased sympathetic activity observed in EC exposed females in the form of low HRV (IMAIZUMI and TAKESHITA, 1993).

Intergenerational assessment of cardiac function in F2 revealed sex-specific impact of developmental EC exposure. As revealed by echocardiography, male offsprings from the B group whose germ cells were exposed to ECs during *in utero* development showed significantly lower HR and an increase in left ventricular wall thicknesses. The lower HR in F2 males shows the intergenerational programming of ANS regulation of HR as this was also observed in the F1 B males. In contrast to F1, wall thicknesses were increased in the F2 B males which may indicate the development of concentric left ventricular

remodelling/hypertrophy. However, in the F1 EC exposed male phenotype, due to an increase in left ventricular dimensions without any evidence of changes in wall thicknesses, and the presence of higher LVDd, LVDs and CO, an eccentric left ventricular hypertrophy was proposed. Left ventricular hypertrophy is an important compensatory mechanism to meet the hemodynamic stress and maintain ventricular integrity however; an individual with pathological LVH is at greater risk of heart failure, arrythmias and death (Bornstein et al., 2023). The possibility of concentric LVH phenotype in the F2 EC exposed males implies that EC exposure could lead to sex dependent transgenerational cardiac remodelling. In comparison to males, the F2 females exhibited significantly smaller PA diameter compared to controls, a finding which was also seen in the F1 females and signals that developmental EC exposure might have sexually dimorphic intergenerational impact on pulmonary vasculature.

Sex stratified differences were also detected in the molecular parameters in F2 adult LV. It was interesting to witness the increased expression of markers related to cardiac stress (BNP), inflammation (DYA), and hypertrophy (AKT1) as except for DYA, these markers were not affected in the F1 EC exposed males. These observations were also accompanied by an increase in fibrosis marker COL3A1 in B males. Collectively, the increased expression of BNP and COL3A1, together with increased AKT1 signalling and the observation of increased wall thicknesses in B males observed earlier indicates concentric hypertrophy/remodelling. The upregulated AKT1 signalling might suggest a compensatory mechanism towards hypertrophic changes as AKT1 has been implicated in the regulation of cardiac hypertrophy (Oudit et al., 2004). It was interesting to see that while COL3A1 was increased in F2 EC exposed males, COL1A1 was increased in females; however, when analysed histologically, no significant difference in collagen scoring was detected which indicates subclinical extracellular matrix remodelling. A summary table with key findings from the F1 and F2 are presented in table 6-1.

Table 6-1. Comparison table outlining key findings from F1 and F2 generations.

Parameter/Variable	F1	F2
Left ventricular internal diameter in diastole and systole	↑ in B males No change in females	Not affected in either sex
Left ventricular pre wall thickness	Not affected in either sex.	↑ in B males
End-diastolic and systolic volumes	↑ in B males No change in females	Not affected in either sex
Heart rate	↓ in B males ↑ in B females	↓ in B males ↑ in B females
Cardiac output	↑ in B males No change in females	Not affected in either sex
NPPA	↑ in B females No change in males	Not affected in either sex
NPPB	Not affected in either sex	↑ in B males
Akt1	Not affected in either sex	↑ in B males No change in females
COL1A1	Not affected in either sex	↑ in B females No change in males
COL3A1	Not affected in either sex	↑ in B males No change in females
CASP3	↑ in B males	Not affected in either sex
MHCII-DRB1	↑ in B males	Overall ↑ in B group
MHCII-DYA	↑ in B males	↑ in B males No change in females
IGF1	↑ in B males	↑ in B males
IGF1-R	↑ in B males	↑ in B males

Taken together, findings from current research showed that exposure to low level chemicals during gestation can lead to sex-specific effects on cardiac function in adulthood some of which can persist into future generations. By using a translational animal model of EC mixture exposure, these findings suggest an adverse cardiac phenotype in males, with the female phenotype being comparatively better preserved and provides a field for future research directions on sex-specific developmental EC mixture exposure consequences of adult CV functioning, not only in the immediate but also in the future generations.

6.2 Strengths

The biosolids treated pasture (BTP) sheep model utilized in the current research is a unique model with high translatability into humans. Compared to traditional component-based chemical models, BTP model focuses on mixture of low-level chemicals which human beings are exposed to in their daily lives. These low-level mixture of chemicals are important as when presented in mixture form, they can lead to additive effects which can be far more severe than the individual chemical effect. Biosolids were used as source of chemicals in the form of fertilizer on pasture which provided an exposure medium. The main advantage associated with the use of biosolids is that biosolids contains a number of anthropogenic chemicals including BPA, PCBs, PFAS, DEHP, PBDE, steroids, antibiotics, veterinary medicine (Clarke and Smith, 2011) which otherwise would be difficult to test each chemical exposure toxicity individually. By allowing sheep to graze on pasture treated with biosolids is also unique in the sense that it does not incur any unnecessary risks associated with chemical exposure such as restraining the animal or administration via specific routes. Compared to the use of traditional rodent species which has a short lifespan, the use of large animal model i.e. sheep is another strength of present research. Sheep is an outbred specie with developmental pattern similar to humans and has an extended gestational period which is ideal for chronic chemical exposure studies. Sheep also has similarity in cardiac anatomy and physiology to those in

human. Adult sheep heart weights are comparable to human beings and several cardiac parameters such as ejection fraction, left ventricular end-diastolic and end-systolic dimension, aorta and pulmonary artery diameter have been reported to be closer to human beings (Rusakova and Zhuravleva, 2025). Another strength which brings uniqueness into this research comes from taking into consideration the sex-specific and transgenerational effects of developmental chemical mixture exposure. Developmental chemical exposure cardiac studies have predominantly been conducted in a single sex, which does not allow for the assessment of sex-specific differences in cardiac outcomes. The current research demonstrates that cardiac changes induced by developmental chemical exposure are sex-specific, with males and females being affected differently. With regards to current regulatory frameworks, sexual dimorphism in the effects of ECs on cardiac function parameters in the current study holds immense significance. While sexual dimorphism is an increasingly recognized factor in chemical testing, this largely remains under appreciated. Regulatory bodies however are now emphasizing on sex-specific testing strategies by recognizing sex as a biological variable (SABV) in chemical-based toxicity trials. The present research further adds to the importance of sexual dimorphism in chemicals related cardiac toxicity and recommends that future toxicity testing models should incorporate both sexes particularly in chronic low dose chemical exposure settings. The transgenerational assessment of cardiac function in adult male and female sheep is another important aspect in this research which has not been previously explored.

From a technical point of view, the use of specialized equipment such as polar belts with ACTi heart monitors, also used in humans for the assessment of heart rate variability is an added benefit. These are wearable devices which does not require any specialized settings and can be used for non-invasive measurements of inter-beat variability. The respective echocardiographic assessment in live animals and analysis of cardiac tissues at postmortem allowed for a more in-depth validation of EC related cardiac changes. Additionally, the disadvantage associated with short term blood pressure measurements was eliminated by an assessment of BP each day at the same time for an extended period of 15 days.

6.3 Limitations

As well as its strengths, there are also certain limitations associated with current research. Biosolids being the byproducts of wastewater treatment contains a mixture of chemicals including endocrine disruptors particularly BPA, PBDEs, parabens, triclosan, polyphenols and steroidal hormones such as oestradiol which can interfere with normal endocrine functioning. Biosolids also contains persistent chemicals such as PFAS and PFOA, veterinary and human medicine as well as heavy metals including Pb, As Hg, Cd and Cu. A major and recognized limitation of a mixture-based toxicity models such as BTP, is that it's difficult to ascribe the adverse CV effects to a particular class of chemicals as there is no precise knowledge on the number and or specific data on individual chemical concentrations. While chemical loads in biosolid have been detected in soil and tissues from sheep grazing on BTP (Rhind et al., 2013; Rhind et al., 2005a), there is uncertainty about how much chemical load each animal has been exposed to as this would largely be dependent on temporal changes in soil and batch variances in biosolids as well as the geographical location. This uncertainty prevents the proper interpretation of data where the focus is on individual chemical-based toxicity. While the use of sheep as large animal model for developmental cardiac studies has strengths (discussed above) it has certain limitations such as their outbred nature and the fact that a greater number of resources are needed in large animal husbandry, a limited sample number can be utilized which reduces the statistical power of the study. Moreover, since the present research assessed the cardiac function only in adulthood, whether any postnatal cardiometabolic changes contribute to the adult cardiovascular effects cannot be established.

6.4 Future directions

Future research on sex-specific developmental EC programming of adult cardiovascular function could investigate the role of epigenetic mechanisms such as DNA methylation in adult CVD susceptibility. High throughput techniques like whole genome bisulfide sequencing (WGBS), sanger sequencing and running methylation specific primers on PCR could identify specific DNA methylation

pattern in cardiac tissue. The differential expression of cardiac function genes particularly ANP, BNP, CASP3 and AKT1 signalling in male and female offsprings could be investigated further by advanced techniques such as RNA sequencing to gain an in depth understanding of the transcriptome. Traditional histological and molecular techniques can also be extended to other regions of the heart to investigate the changes seen in structures such as pulmonary artery, aorta, right ventricles and pericardial fat. While traditional echocardiography in present research has yielded useful results, advanced cardiac imaging such as cardiac magnetic resonance (CMR) would be helpful in characterization of precise volumes and advanced cardiac pathological changes such as myocardial fibrosis. Another avenue could be evaluating cardiac toxicity through EC mixture exposure (biosolids) in isolated sheep cardiomyocytes.

6.5 Conclusions

Cardiovascular diseases remain to be the leading cause of death worldwide which is also sexually differentiated. With multiple risk factors involved, the developmental programming of adult CVD by anthropogenic EC mixture exposure was investigated in this research. Findings revealed sex-specific differences in structural and functional parameters of adult CV functioning, some of which were also presented in the F2 generation. Echocardiography assessment in the F1 led to increased left ventricular dimensions, end-diastolic and systolic volume, stroke volume and cardiac output in F1 males while left ventricular wall thicknesses were increased in the F2 males which is concerning given that these changes corroborate with eccentric and concentric left ventricular hypertrophy, respectively and can lead to cardiac dysfunction. These changes were complimented by histological and molecular findings which also revealed increased expressions of genes implicated in wall stretch, hypertrophy, fibrosis, apoptosis and inflammation in males only. EC-exposed female phenotype on the other hand showed higher sympathetic dominance which can lead to CV health complications. These findings convincingly implicate the role of developmental EC mixture exposure in sex-specific and transgenerational adult cardiovascular remodelling which needs further exploration. Future research on epigenetic mechanisms and investigating the complex interplay between molecular

pathways would be helpful in identifying sex-specific CVD risk associated with developmental EC mixture exposure which could lead to targeted and timely interventions to address the growing burden of CVD around the world.

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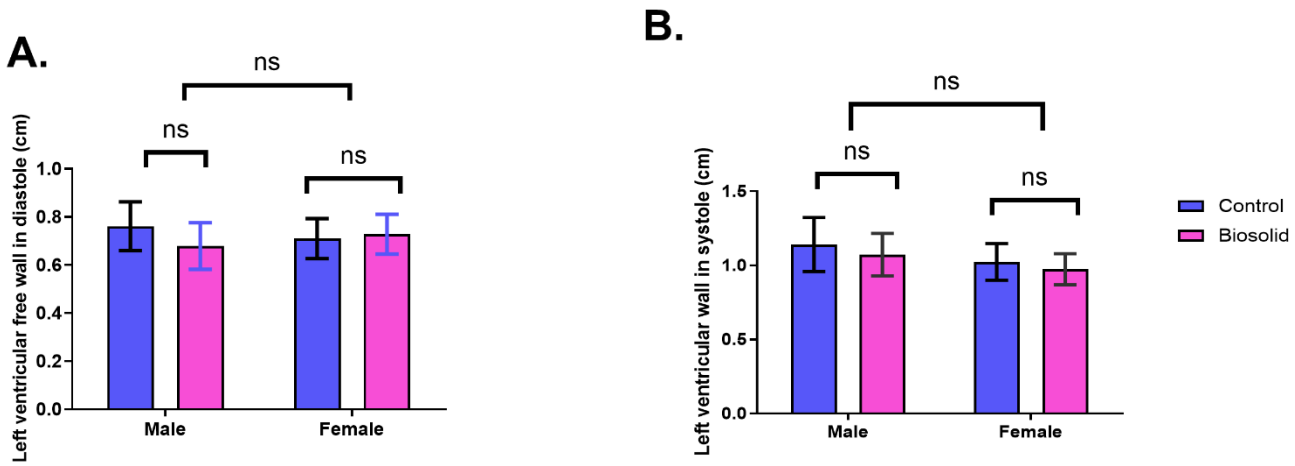
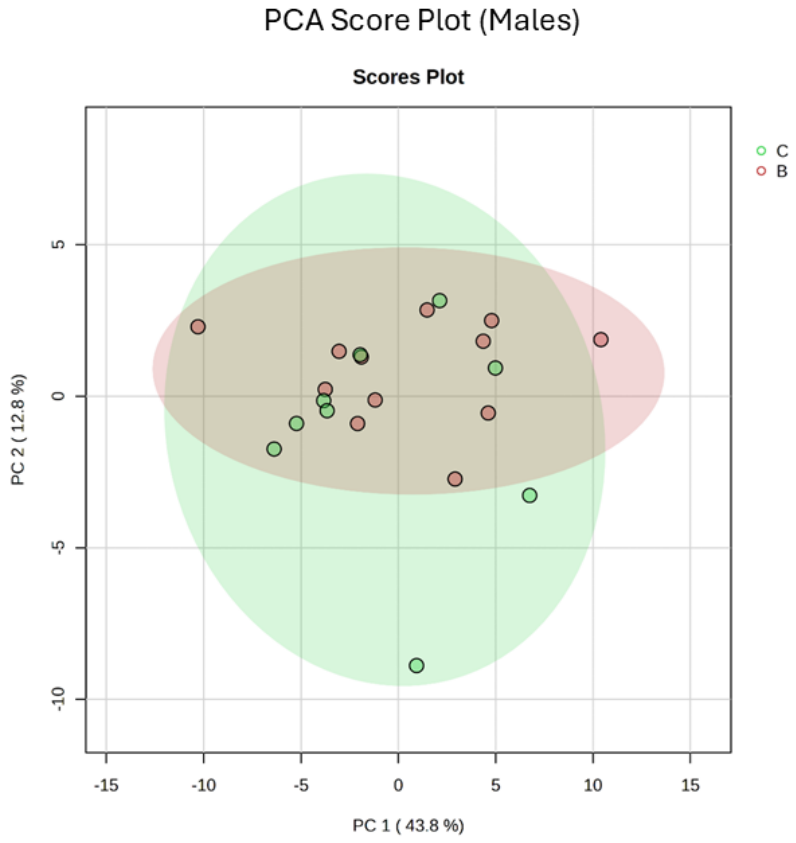
Appendices

Effect size	Sample size (n/group) for 80% power	Sample size (n/group) for 90% power
1.4	10	12
1.6	8	10
1.8	6	8

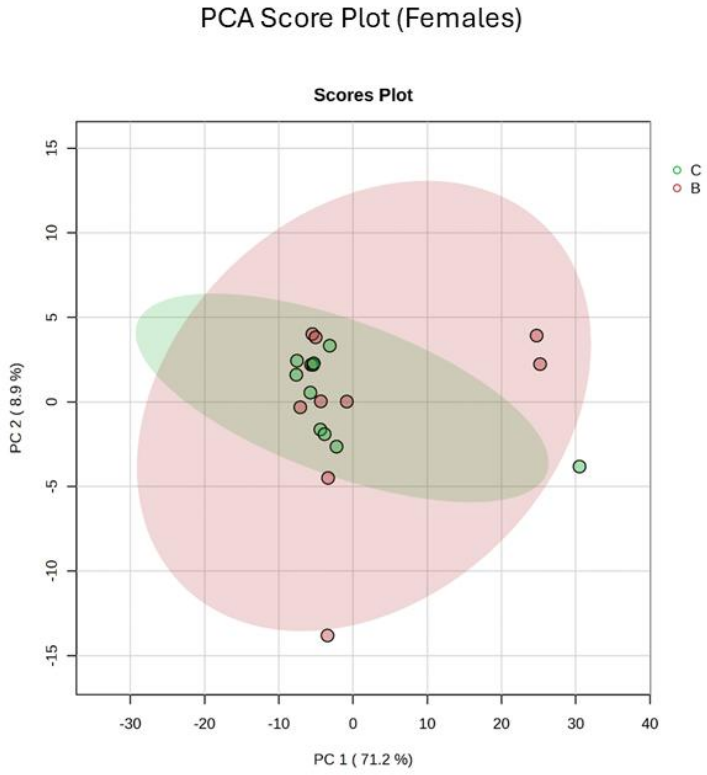
Supplementary Table 1: Presenting the sample size required for 80% and 90% power, at 5% significance level, for a two-tailed t test to detect a significant difference, given a series of effect sizes.



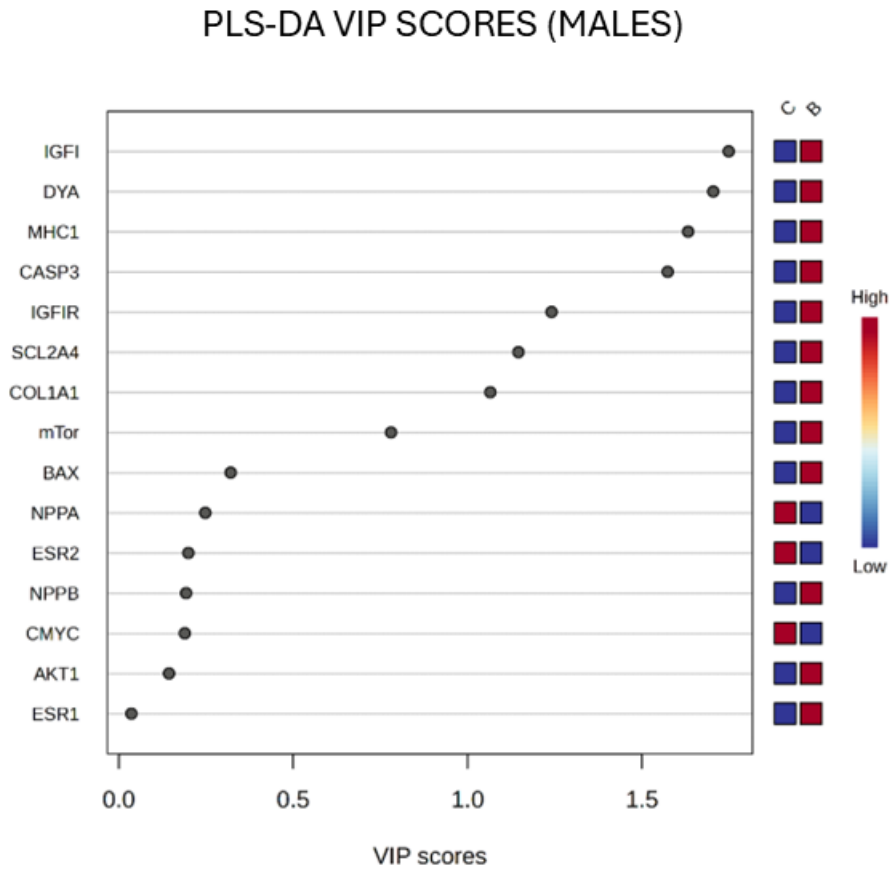
Supplementary Figure-1. (A) Left ventricular free wall thickness in diastole P-exposure = 0.73, Sex= 0.99, Interaction= 0.59 (B) left ventricular wall thickness in systole showing no significant effect of treatment (P=0.68), Sex=(P=0.45) or treatment~sex interaction (P=0.94).



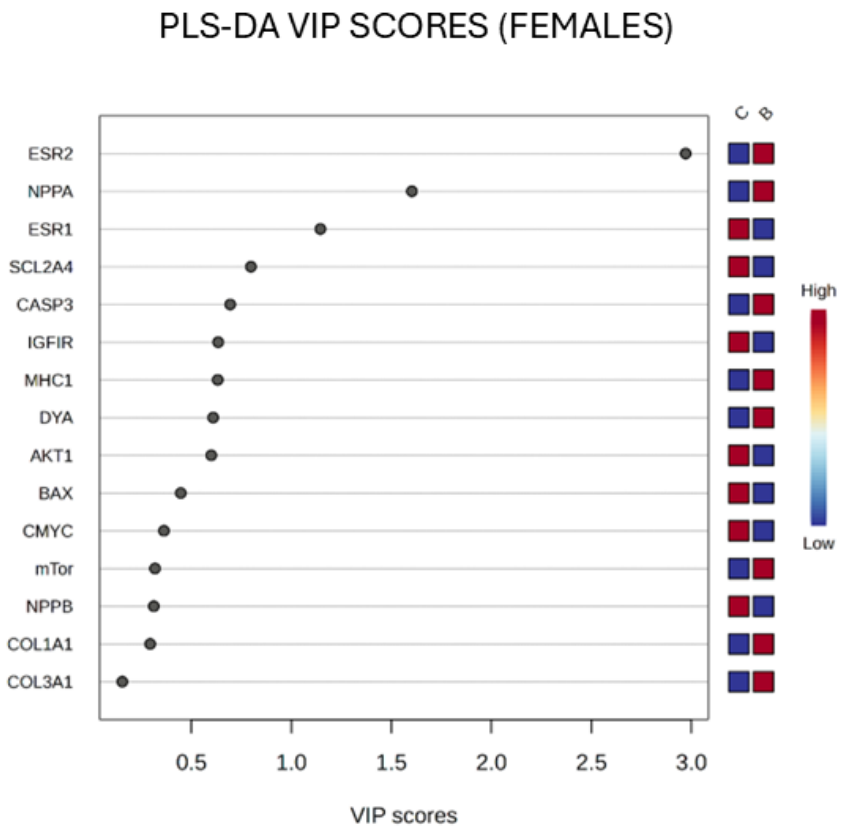
Supplementary Fig.2. PCA Score plot from males showing no distinct clustering of C and B groups while some animals are sitting outside the cluster formed by rest of the animals.



Supplementary Fig.3. PCA Score plot from females showing no distinct clustering of C and B groups and shows some animals sitting outside the cluster.



Supplementary Fig.4. PLS-DA VIP Scores in males showing top genes/markers differentially expressed between C and B groups.



Supplementary Fig.5.PLS-DA VIP Scores in females showing top genes/ markers differentially expressed between C and B groups.