Methodological Approaches in the Investigation of Sex & Gender in Cardiovascular Disease

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Abstract

Background: Differences exist in the presentation, pathophysiology, management, and outcomes of cardiovascular conditions in men and women. These differences may arise from sex-dependent factors such as chromosomal complement, regulation of sex hormones, and sex-specific factors like pregnancy. Beyond sex, gender, a multifaceted psychosocial concept, also has an impact on cardiovascular health and disease. Transgender individuals experience incongruence between the sex they were assigned at birth and their gender identity. These individuals may engage with gender-affirming hormone therapy (GAHT), such as oestrogen or testosterone, and the effects of such treatments upon cardiovascular health have yet to be determined, and may provide insight into cardiovascular pathophysiology.

Aims: This thesis aims to enhance our understanding of the role of sex and gender in cardiovascular disease, including transgender cardiovascular health, through a range of methodological approaches.

Methods: Chapter 3) A systematic review assessing the influence of GAHT upon the blood pressure of transgender individuals is undertaken; Chapter 4) The Gender and Sex Determinants of Cardiovascular Disease: From Bench to Beyond-Premature Acute Coronary Syndrome (GENESIS-PRAXY) gender stratification questionnaire is adapted and applied to a UK sample of cisgender individuals (n=446) to construct a gender score via principal component analysis (PCA); Chapter 5) A bioinformatic analysis of sex and gender stratified differentially expressed microRNA (miRNAs) in human plasma of individuals (n=36), derived from the original GENESIS-PRAXY study, who have experienced acute coronary syndrome (ACS) is undertaken; Chapter 6) A descriptive analysis of the Vascular Effects of Sex Steroids in Transgender Adults (VESSEL) study, which utilises a range of vascular phenotyping procedures (e.g. flow-mediated dilatation, peripheral artery tonometry, and pulse wave analysis (PWA) and velocity (PWV)) in transgender individuals using long-term GAHT compared to cisgender individuals is presented.

Results: Chapter 3) The systematic review identified 14 studies including 1,309 transgender individuals, which demonstrated broadly no change in blood pressure in transmasculine individuals using testosterone. Both increases and decreases were observed within the transfeminine population using oestrogen therapy. These studies were of limited quality due to their uncontrolled pre-post design, lack of intervention and blood pressure measurement standardisation, inadequate follow up and small sample sizes; Chapter 4) The gender stratification analysis demonstrated a continuum of gender scores in this population derived from five gender-related questionnaire instruments. Gender score distributions were distinct from the GENESIS-PRAXY analysis, highlighting that gender and its related factors are dynamic and context dependent; Chapter 5) miR-664a-5p, miR-3613-5p, miR-382-5p, miR-134-5p, miR-10b-5p, miR-885-5p, miR-206, and miR-32-5p were found to be differentially expressed in females versus males in ACS. Many of these miRNA and associated gene networks demonstrate a number of roles important to ACS pathophysiology including the regulation of vascular smooth muscle cell proliferation, endothelial injury and inflammation, atherosclerosis progression. miR-3605-5p and miR-4467 were differentially expressed in males with feminine versus masculine gender characteristics; Chapter 6) Due to the impact of coronavirus disease 2019 (COVID-19), the VESSEL study was discontinued prematurely, however, the feasibility of local recruitment of transgender participants is demonstrated.

Discussion: This thesis expands our appreciation of the means by which gender can be measured and its potential influence, in addition to sex, upon epigenomic regulation in cardiovascular disease. Moreover, it improves our understanding of limitations and barriers in conducting research in transgender populations. Overall, this thesis provides valuable insight into the methodological approaches used the investigation of sex and gender in cardiovascular disease, which can be applied in future cardiovascular research in cisgender and transgender populations.

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Author's Declaration

I declare that the work presented in this thesis has been completed by myself, unless otherwise stated. Sections of this thesis have informed a BSc (Med Sci) and MSc (Med Sci) dissertation under my supervision. I have undertaken the analyses included in this thesis, wrote thesis chapters independently and put this work into the context of the wider topic of this thesis. Elements of this thesis have also informed publications, which I have cited within this thesis and acknowledge support from the coauthors on these articles. I acknowledge the support from Anna Clark for performing second abstract screening and contributing to the quality assessment in Chapter 3. I acknowledge Yatheesh Kodigehalli Nandakumar for undertaking preliminary microRNA regulatory network analysis relating to Chapter 5, which is not included within this thesis. I acknowledge the GENESIS-PRAXY research group for providing samples used in Chapter 5, Glasgow Polyomics for performing next generation sequencing, and Dr Sheon Samji for her assistance in microRNA extraction. I acknowledge Joanne Flynn for her support in performing the vascular phenotyping studies in Chapter 6, and Dr Katriona Brooksbank for her assistance in obtaining ethical approval for this study.

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Abbreviations

ACS	Acute coronary syndrome
AIx	Augmentation index
AIx@75	AIx corrected to heart rate of 75 beats per minute
AR	Androgen receptor
ARE	Androgen response element
AUC	Area under curve
BRFSS	Behavioural risk factor surveillance system
BSRI	Bem sex role inventory
COVID-19	Coronavirus disease 2019
eNOS	Endothelial nitric oxide synthase
ER	Oestrogen receptor
FDR	False discovery rate
FMD	Flow mediated dilatation
GAHT	Gender-affirming hormone therapy
GENESIS-PRAXY	Gender and Sex Determinants of Cardiovascular Disease: From Bench to Beyond-Premature Acute Coronary Syndrome
GGI	GENESIS-PRAXY Gender Index
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
GO-BP	Gene ontology biological processes
GO-CC	Gene ontology cellular component

GO-MF	Gene ontology molecular function
GPER	G protein coupled oestrogen receptor
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
HPG	Hypothalamic-pituitary-gonadal
HR	Hazard Ratio
IM	Intramuscular
KEGG	Kyoto encyclopaedia of genes and genomes
LnRHI	Logarithmic transformation of reactive hyperaemia index
MACE	Major adverse cardiovascular events
MGPQ	Modified GENESIS-PRAXY questionnaire
MI	Myocardial infarction
miRNA	MicroRNA
NGS	Next generation sequencing
NSTEMI	Non-ST elevation myocardial infarction
OR	Odds ratio
PAT	Peripheral arterial tonometry
PCA	Principal component analysis
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
PWA	Pulse wave analysis
PWV	Pulse wave velocity

RAAS	Renin-angiotensin-aldosterone system
RCT	Randomized controlled trial
RHI	Reactive hyperaemia index
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
STEMI	ST elevation myocardial infarction
TIA	Transient ischaemic attack
VESSEL	Vascular Effects of Sex Steroids in Transgender Adults
VIRGO	Variation In Recovery: Role of Gender on Outcomes in Acute Myocardial Infarction Patients
WPATH	World Professional Association for Transgender Health

Chapter 1 Introduction

1.1 Chapter overview

In this introductory chapter, the definitions used in this thesis in relation to sex, gender, and transgender terminology are outlined. Next, the relationship between sex, gender and cardiovascular disease in cisgender and transgender populations are briefly discussed. This chapter concludes with an outline of the thesis structure and provides an overview of each chapter and the aims and research objectives addressed.

1.2 Background

1.2.1 Sex & gender terminology

Although the terms ‘sex’ and ‘gender’ are often used interchangeably in everyday language, and even in clinical research, their meanings and use are not equivalent (Rioux *et al.*, 2022). This thesis will aim to use these terms appropriately and provides their definitions below in order to facilitate a clearer understanding of the important, and scientifically relevant, differences in these terms.

Sex refers to the biological characteristics of an individual. This is often assigned at birth and is broadly based on the appearance of external genitalia (Mauvais-Jarvis *et al.*, 2020). Largely, these factors are determined by an individual’s chromosomal complement and exposure and response to sex hormones. This relationship is not wholly binary and there are many disorders of sex development (i.e. intersex) resulting from gonadal, chromosomal and anatomical abnormalities that demonstrate this (Ahmed *et al.*, 2016), as outlined in Chapter 2.

Sex is therefore considered as a physiological component comprising from the presence and function of chromosomal complement, sex hormones and secondary sex characteristics (Clayton & Tannenbaum, 2016). Consequently, the terms ‘male’, ‘female’ and ‘intersex’ are used when reporting either the sex of a participant or biological sex-related factors.

Conversely, gender is derived from social, cultural and behavioural factors (Tannenbaum *et al.*, 2016). It is a multidimensional concept that incorporates many factors including: gender identity (i.e. one's internal sense of masculine, feminine or alternative gender); gender roles (i.e. societal expectations of a particular gender); gender relations (i.e. the interpersonal interactions that occur between and within genders); and institutionalised gender (i.e. distribution of power across society, as seen by differences in educational attainment, income, political representation) (Connelly *et al.*, 2021). Therefore, when discussing gender, the terms 'men', 'masculine', 'women', and 'feminine' are applied.

With respect to gender modalities (i.e. an individual's experience of gender in relation to the sex they were assigned at birth), a cisgender person is someone who's gender identity and natal sex is congruent (Rioux *et al.*, 2022). Conversely, transgender is an umbrella term to describe individuals whose gender identity is not congruent with the sex they were assigned at birth (Figure 1-1).

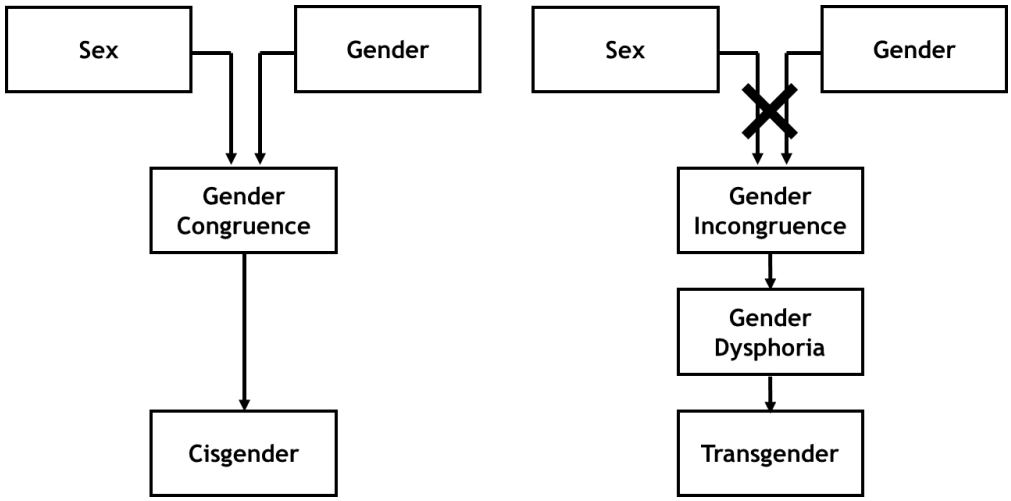

Figure 1-1. Model of gender congruence and incongruence.

Transgender people may experience distress or discomfort as a consequence of their gender identity differing from physical or social attributes typically ascribed to their sex assigned at birth (i.e. gender dysphoria) (Dhejne *et al.*, 2016). Gender dysphoria may result in adverse effects on an individual's psychological, physical and social well-being. The intensity and frequency of gender dysphoria is not uniform in this population and may be alleviated by a range of measures. This may

include social changes, e.g. modifying their gender expression to match their identity to gender-affirming medical and surgical interventions (T'Sjoen *et al.*, 2019).

Terms	Definition
Sex	The classification of a person as male or female, typically occurring at birth, based upon biological characteristics such as genitalia, reproductive organs, chromosomes and hormones
Gender identity	A person's intrinsic sense of being a man, woman or an alternative gender
Cisgender	People whose gender identity aligns with the sex they were assigned at birth.
Gender dysphoria	Distress or discomfort that may be experienced because a person's gender identity differs from that which is physically and/or socially attributed to their sex assigned at birth.
Transgender	An umbrella term for people whose gender identity differs from their natal sex
Transgender man	A person whose sex was assigned female who identifies as a man
Transgender woman	A person whose sex was assigned male who identifies as a woman
Gender-affirming hormone therapy	Hormonal therapy aiming to align the physical characteristics of an individual with their gender identity

Table 1-1. Transgender terminology definitions

Importantly, the term 'transgender' should be used as an adjective (e.g. transgender people or individuals) and not a noun (e.g. transgenders) or verb (e.g. transgendered) (Rioux *et al.*, 2022). A transgender man is an individual who was assigned female sex at birth and identifies as a man. A transgender woman is therefore a natal male who identifies as a woman. Terms such as 'Male-to-Female' are no longer considered acceptable in reference to transgender individuals (Coleman *et al.*, 2022).

Transgender individuals may also identify as non-binary, and have gender identities that do not conform to gender associated with binary gender systems (i.e. man or woman) (Liszewski *et al.*, 2018). Non-binary individuals may have a neutral gender (i.e. neutrois), no gender (i.e. agender), masculine and feminine gender (i.e. bigender), or two or more genders (i.e. paragender). (Reisner & Hughto, 2019). Importantly, these gender identities may also change over time (i.e. they are gender fluid).

These terminologies will continue to evolve as our understanding and appreciation of the intricacies of gender deepens. The use of appropriate sex and gender terminology has several advantages. It provides an accurate description of populations being studied, it allows stratification between biological and psychosocial systems, and prevents careless conflation (e.g. describing gender in a rat model). Importantly, it is respectful to the individuals being researched and has the potential to facilitate clinical research that is reflective of the complex bio-sociocultural interactions of the human condition (Rioux *et al.*, 2022).

1.2.2 Sex, gender, & cardiovascular Disease

Cardiovascular disease is a major contributor to worldwide mortality in both cisgender men and women (Roth *et al.*, 2017). The differences evident in the epidemiology, pathology, clinical presentation and outcomes of cardiovascular disease, such as hypertension, between men and women is being increasingly acknowledged (Colafella & Denton, 2018; Virani *et al.*, 2020). Importantly, an appreciation of the role of sex and gender promoting these disparate cardiovascular mechanisms and outcomes, has improved our understanding of both sex-related pathophysiology and psychosocial mechanisms by which gender-specific inequalities and stressors act (Mauvais-Jarvis *et al.*, 2020).

Ischaemic heart disease is a prime example of this relationship. This condition occurs more frequently and in a much younger age demographic in men compared to women (George *et al.*, 2015). Many biological mechanisms may be responsible for these differences including pro-inflammatory and dyslipidaemic Y chromosome

haplogroups (Charchar *et al.*, 2004, 2012), or the numerous cardioprotective effects of oestrogen in vascular smooth muscle cells (e.g. promoting vasodilatation via inhibiting calcium influx) (Pabbidi *et al.*, 2018). Moreover, significant anatomical differences, such as smaller epicardial coronary arteries or atherosclerotic plaque composition evident in females, may promote sex-dependent effects in this condition (Lansky *et al.*, 2012; Patel *et al.*, 2016).

However, these sex-related pathophysiological mechanism may not fully explain the differences observed between men and women, and the integration of social determinants of disease are crucial to understand these variances. For instance, in the Variation In Recovery: Role of Gender on Outcomes in Young Acute Myocardial Infarction Patients (VIRGO) study, women with acute myocardial infarction (MI) had lower socioeconomic status and quality of life, and higher levels of psychological stressors compared to men (Bucholz *et al.*, 2017). Addressing such factors in younger women with ischaemic heart disease, who demonstrate higher rates of comorbidities, hospitalisation and mortality following acute coronary syndrome (ACS) compared to men, are imperative to improving patient outcomes (Clemmensen *et al.*, 2015; Alabas *et al.*, 2017; Dreyer *et al.*, 2017).

Importantly, the pathological mechanisms responsible for mediating adverse gender-related effects are unknown. It has been demonstrated that in young women who have experienced an acute coronary event, mental stress-induced myocardial ischaemia is twice that of men, despite eliciting equivalent exercise or pharmacological-evoked ischaemia (Vaccarino *et al.*, 2018). Similarly, upregulated amygdalar metabolism, a neural centre associated with stress responses and emotional processing, promotes an inflammatory state in women with impaired cardiological function (Fiechter *et al.*, 2019). Potential mediators responsible for this relationship may include epigenetic modification, which may modulate gene regulation in response to environmental stimuli. Multiple epigenetic modifications contributing to atherosclerosis have been described in vascular structures (Rizzacasa *et al.*, 2019). Consequently, this process may

provide a link between adverse gender-related psychosocial influences and the vascular health of individuals.

These influences are undoubtedly important to the cardiovascular health of transgender individuals, where there is emerging evidence of increased cardiovascular risk (Caceres & Streed, 2021). People who are transgender may engage with gender-affirming healthcare and receive sex hormone therapy such as oestrogen or testosterone (T'Sjoen *et al.*, 2019). The influence of these hormones in the context of natal sex chromosomes is unclear, although data from conditions of chromosomal aneuploidies and excess cross-sex hormone exposure, such as Klinefelter's or polycystic ovarian syndrome, suggest that transgender individuals may experience increased cardiovascular risk as a result of this interaction (Gravholt *et al.*, 2018; Zhang *et al.*, 2020a).

Moreover, non-traditional risk factors such as the Gender Minority Stress, may exacerbate this risk further. Within this model, distal stressors, such as gender non-affirmation (i.e. misgendering), stigma, discrimination, and proximal stressors, including internalised stigma, and negative expectations, contribute to enhanced levels of stress (Streed *et al.*, 2021). These may promote adverse behavioural traits, and promote poorer health outcomes. Factors such as mental health disorders, substance misuse, smoking, and health inequalities undoubtedly contribute to the burden of cardiovascular risk in the transgender population (Reisner *et al.*, 2016; Kcomt *et al.*, 2020).

Consequently, this thesis sets out to explore the relationship between sex, gender and the development of cardiovascular disease in cisgender and transgender individuals. To accomplish this a variety of methodological approaches are undertaken including: a systematic review of the effect of gender-affirming hormone therapy (GAHT) upon the blood pressure of transgender individuals; the application of a gender questionnaire to generate gender scores for use in cardiovascular research; the bioinformatic analysis of differentially expressed microRNA (miRNA) in ACS stratified by sex and gender; and the vascular phenotyping of transgender individuals on long-term GAHT.

1.3 Thesis aim & objectives

1.3.1 Aim

This PhD commenced in February 2019 and as a consequence there was significant impact of the Coronavirus disease 2019 (COVID-19) pandemic upon my studies. In particular, non-COVID-19 related studies were halted at the Glasgow Clinical Research Facility for a considerable period following the national lockdown. Furthermore, I was redeployed back to the NHS for a period of 3 months. As a consequence there was significant disruption relating to the studies included within this thesis resulting in the themes of this work being shifted.

Consequently, this thesis pragmatically aims to enhance our understanding of the role of sex and gender in cardiovascular disease, including transgender cardiovascular health, through a range of methodological approaches. These include literature review; systematic review; the application of a gender questionnaire to generate gender scores via principal component analysis (PCA); the bioinformatic analysis of differentially expressed microRNA (miRNA) in ACS stratified by sex and gender; and the vascular phenotyping of transgender individuals on long-term GAHT.

1.3.2 Objectives

1) To review the literature relating to the mechanisms by which sex and gender may influence cardiovascular health of cisgender and transgender populations (Chapter 2).

2) To systematically review the influence of GAHT upon the blood pressure of transgender individuals (Chapter 3).

3) To adapt and utilise a gender stratification questionnaire in a UK population (Chapter 4).

4) To investigate the differential expression of miRNA in the plasma of individuals who have experienced ACS by sex and gender (Chapter 5).

5) To demonstrate the feasibility of the Vascular Effects of Sex Steroids in Transgender Adults (VESSELS) study, and describe the vascular function of transgender people using long-term GAHT(Chapter 6).

1.4 Thesis structure

Chapter 2 of this thesis provides an overview of relevant literature. This chapter discusses the process of sex determination and preservation, and how factors related to this process may modulate the development of cardiovascular disease. This chapter then describes the concept of gender as a psychosocial construct, the effects of gender upon cardiovascular risk, and the means by which gender is measured in clinical research, and potential biological mediators. Lastly this chapter provides a background to gender-affirming healthcare and the potential associations with cardiovascular disease. This provides background to the methodological approaches undertaken in subsequent chapters.

Chapter 3 assesses the associations between masculinising (i.e. testosterone) and feminising (i.e. oestrogen) GAHT and blood pressure in transgender individuals through a systematic review of the literature.

Chapter 4 adapts and utilises the GENESIS-PRAXY questionnaire in a UK population. Principal component analysis is then used to generate gender scores within this population, which are then compared to a simple masculinity-femininity score.

Chapter 5 provides a bioinformatic analysis of differentially expressed miRNAs in human plasma in ACS stratified according to sex and gender. Regulatory network analysis was then used to predict sex and gender-dependent miRNA-gene interactions in this condition.

Chapter 6 demonstrates the feasibility of the VESSEL study along with a descriptive analysis vascular function of transgender people using long-term GAHT.

Chapter 7 discusses the overall findings of the thesis and compares these findings to the existing literature. The general strengths and limitations of this thesis are discussed in addition to directions for future research.

Chapter 2 Literature Review

2.1 Chapter overview

The aim of this chapter is to provide context for the means by which sex and gender may influence cardiovascular health of cisgender and transgender populations. This chapter outlines the processes involved in sex determination, differentiation and maturation, and variations that may occur in these processes. These factors are explored in order to demonstrate the complexity of these physiological functions, the potential for variation in aspects of sex biology, and how perturbations in factors relating to determining and maintaining sex-related traits may alter cardiovascular health. This chapter then describes the evidence for altered cardiovascular risk factors between males and females, and the mechanisms by which this altered risk occurs, and in particular the role of sex steroids and chromosomes. Additionally, this chapter describes the complex psychosocial construct of gender and its components, the effects of gender upon cardiovascular risk, the means by which gender is measured in clinical research, and potential biological mediators by which gender may act and alter cardiovascular outcomes. These sex and gender factors influence cardiovascular disease greatly, and are of huge relevance to both the study of individuals who are transgender and more broadly in cisgender populations also. This chapter then shifts its focus to transgender gender-affirming healthcare and its potential associations with cardiovascular disease. Taken together, this chapter aims to provide an overview of sex and gender, and how variations in these traits may facilitate the development of cardiovascular disease to provide a foundation of understanding that underpin the methodological approaches undertaken in subsequent chapters.

2.2 Sex differences in cardiovascular disease

2.2.1 Sex determination, differentiation & maturation

There are distinct epidemiological, pathophysiological and therapeutic differences between males and females with respect to health and disease. These

biological differences are attributed to chromosomal complement, functional gene expression, and sex hormone secretion and action. Sex refers to the biological characteristics of an individual and is determined by these factors (O'Neil *et al.*, 2018). Sex differentiation incorporates these physiological processes that result in divergence in characteristics between sexes. Understanding sex, and the factors that differ between males and females, may fundamentally improve our appreciation of sex-related differences in health and disease. This section will describe the process by which sex differentiation occurs, matures and is maintained, before describing how variation of these processes may facilitate the development of cardiovascular disease. This section is of particular relevance to Chapter 3 and Chapter 6, where the influence of exogenous sex hormones upon transgender vascular health is explored, and Chapter 5 in which an analysis of sex-dependent differentially expressed microRNA (miRNA) in acute coronary syndrome (ACS) is undertaken.

2.2.1.1 Sex determination & differentiation

Sex chromosomes are the predominant factor in determining sex. Sex determination commences at conception when the sperm, carrying an X or Y chromosome, fuses with the X chromosome containing ovum. Multiple genes are involved in gonadal determination and in turn sex differentiation. However, the predominant initiator in testicular development, and consequently sex differentiation, is the Sex-Determining Region Y (*SRY*) gene located on the Y chromosome (Nagahama *et al.*, 2021). This encodes the SRY protein, also known as testes-determining factor. This binds DNA via its high-mobility group (HMG) box, thereby acting as a transcription factor that regulates a series of genes important to sex-determination. In particular, upregulation of SOX9, a member of the SRY-related HMG—box genes, is believed to be pivotal to testis cord formation (Li *et al.*, 2014b).

This promotes the undifferentiated, and bipotential, gonadal tissue to virilize, secrete testosterone and anti-Müllerian hormone, thereby promoting the respective development and regression of Wolffian and Müllerian ducts, and

instigating the development of the male phenotype (Lucas-Herald & Mitchell, 2022). Conversely, in females the absence of *SRY*, X-linked or autosomal genes, promote ovarian development and the pre-pubertal female phenotype (Arnold, 2017).

The importance of the *SRY* gene in sex determination can be exemplified by phenotypic males with de la Chapelle syndrome (i.e. testicular disorder of sex development) and a 46(XX) karyotype, which occurs in 1 in 20,000 male new-borns (de la Chapelle, 1981; Vorona *et al.*, 2007). This condition results in a male phenotype, despite 'female' chromosomal complement, and arises as a consequence of the translocation of the *SRY* gene to the X chromosome during paternal meiosis (Lambert *et al.*, 2021). *SRY* resides in close proximity to pseudoautosomal region 1 (PAR1), which undergoes recombination with the homologous X chromosome PAR1 region during meiosis (Helena Mangs & Morris, 2007). PAR1 genes are inherited in an autosomal rather than sex-linked fashion, therefore unequal PAR1 recombination including the *SRY* region, permits the translocation of *SRY* to the X chromosome.

Males with this 46(XX) karyotype develop with typical male genitalia, however, can develop ambiguous genitalia or be born with both testicular or ovarian tissues (de la Chapelle, 1987). Although the *SRY* gene in these individuals promotes the gonadal primordia to form testis, they lack the azoospermia factor region (AZF) in the long arm of the Y chromosome, which is essential for spermatogenesis, and are therefore infertile (Dominguez & Reijo Pera, 2013).

Interestingly, this 46(XX) male syndrome can also occur in individuals lacking the *SRY* gene. Such circumstances may arise from the overexpression of genes responsible for testicular differentiation (*SOX* family genes) or the repression of pro-ovarian/anti-testis genes (e.g. *WNT4, RSPO1*), thereby highlighting the potential influences of downstream modifiers of the *SRY* gene (Grinspon & Rey, 2016). As a result, the presence or absence of several genetic components, but predominantly the *SRY* gene, promotes gonadal differentiation and fetal exposure to sex hormones (e.g. androgens or oestrogens), which consequently determines

phenotypic sex. Other factors that promote genetic differences between male and female cells include functional differences between Y and X homologous genes (e.g. *ZFX* vs *ZFY*), and random X chromosome inactivation and escape in female cells, which can result in higher gene expression in females than males (Mauvais-Jarvis *et al.*, 2020). Similarly, loss of function of *SRY* via deletion or mutation, or dysregulation related sex-determining effectors may mediate the development of Swyer syndrome. This condition comprises of a 46 XY karyotype with unambiguously female genitalia, the presence of mullerian structures but the absence of functioning gonads (King & Conway, 2014).

Therefore, in the context of sex chromosome aneuploidies, where individuals are born with abnormal numbers of sex chromosomes, the sex of these individuals is determined by the Y chromosome, and the presence of *SRY* and its downstream effectors (Mcelreavey & Fellous, 1999). Therefore, individuals with 47 (XXY; Klinefelter syndrome & XYY; XYY syndrome) are male, whereas individuals with 45 (XO; Turner syndrome) or 47 (XXX; Trisomy X) karyotypes are females. The tolerance of excess sex chromosome complement can be attributed to the process of X chromosomal inactivation and the relatively limited number of functional genes encoded in the Y chromosome.

2.2.1.2 Sex & gonadal maturation

The second phase responsible for promoting sex differentiation, which occurs in genetically and hormonally primed individuals, arises with the advent of puberty. Puberty is characterised by the development of secondary sex characteristics, gonadal maturation and reproductive capability (Abreu & Kaiser, 2016). The hypothalamic-pituitary-gonadal (HPG) axis regulates this process and ongoing adult sex hormone secretion in adulthood. This axis is typically supressed during childhood but reactivates at the advent of puberty with the sustained increase in gonadotropin-releasing hormone (GnRH) secretion from hypothalamic neurones. GnRH in turn acts upon the anterior pituitary gonadotrope GnRH receptors to promote the secretion of gonadotropins, luteinising hormone (LH) and follicular stimulating hormone (FSH). These gonadotropins sequentially promote gonadal

maturation and sex-steroid secretion and provide negative feedback along this axis (Tena-Sempere, 2012).

Following puberty, cells that express androgen and oestrogen receptors (ERs) may demonstrate sex-specific traits, including secondary sex characteristics. However there are a number of conditions where this relationship is more complex. Androgen insensitivity syndrome, when present in its complete form, is a disorder of androgen resistance characterised by a female phenotype in individuals with a XY karyotype (Hughes *et al.*, 2012). This occurs as a result of missense mutations distributed throughout the eight exons of the androgen receptor (AR) gene, which are typically localised in regions that encode deoxyribonucleic acid (DNA)-binding and ligand-binding domains. Testosterone concentrations are maintained within or above the male reference ranges, while excess testosterone is peripherally aromatised to oestrogen, which in combination with testicular produced oestrogen has concentrations lower than expected in females but higher than typically evident in males.

Similarly, natal males (46, XY) with mutations in 5α-reductase type 2 (*SRD5A2*), which is responsible for the conversion of testosterone to dihydrotestosterone (DHT), experience fetal androgen deficiency (Cheon, 2011). DHT is a potent androgen and plays a vital role in fetal male sex development. Consequently, these individuals will develop external genitalia, which may appear ambiguous or female. Internal genitalia include seminal vesicles, epididymis, vas deferens, and ejaculatory duct and the testes may be present in the inguinal sac or abdomen.

These children are typically raised as girls, however, following puberty they virilise and develop male secondary sex characteristics as a consequence of testosterone, rather than DHT, being the essential androgen in this process. In geographical regions with a high prevalence of this condition the transition from a female to male phenotype has been accepted culturally (Loughlin, 2021). In the Dominican Republic, this process is termed ‘Guevedoce’, whereas in Papua New Guinea it is known as ‘kwolu-aatmwol’. Interestingly, this process results in many individuals adopting a masculine gender identity despite being raised as girls.

Variation in these sex-related factors may alter the cardiovascular risk of individuals. UK cohort studies have demonstrated that individuals with sex chromosomal aneuploidies, such as Klinefelter and Turner syndrome, experience higher cardiovascular mortality than the general population (Swerdlow *et al.*, 2005; Schoemaker *et al.*, 2008). Moreover, Klinefelter syndrome is associated with substantive increases in the risk of venous thromboembolism in addition to increased cardiovascular risk (Bojesen *et al.*, 2006; Zöller *et al.*, 2016). The exact mechanisms underpinning this increased risk is unclear but may represent a complex interplay between increased X chromosome gene dosages (e.g. excess X-linked coagulation factor VIII levels), the presence of hypergonadotropic hypogonadism or testosterone use, but also the neurocognitive morbidity associated with this condition, which may impede healthcare engagement and access and promote adverse health behaviours (Gravholt *et al.*, 2018).

The synthesis and function of sex hormones promote secondary sex characteristics, but also distinct differences and responses in a number of organ systems, such as the vasculature (Connelly *et al.*, 2020). This has also been demonstrated in the autosomal recessive disease, congenital adrenal hyperplasia (CAH), which may result from mutations in *CYP21A2*, which encodes the 21-hydroxylase enzyme (Merke & Auchus, 2020). Females with this condition are virilised with ambiguous genitalia due to excess cortisol precursors being diverted through androgen biosynthetic pathways in response to cortisol deficiency mediated adrenocorticotropic hormone (ACTH) release. Results from a recent meta-analysis of 20 studies demonstrate that individuals with this condition have elevated blood pressure, carotid intima thickness and insulin resistance and therefore demonstrate elevated cardiometabolic risk compared to the general population, albeit in a complex and heterogenous phenotype where glucocorticoid exposure may contribute to such findings (Tamhane *et al.*, 2018). Similarly, women with polycystic ovarian disorder, a condition defined as the presence of androgen excess and oligo-anovulation, demonstrate an increased risk of myocardial infarction (MI), ischaemic heart disease and stroke (Zhang *et al.*, 2020a).

Therefore, although sex is typically represented as a binary trait, it is in fact a much more complex interplay between sex-related chromosomes, genes and hormones, which through numerous variations promote the development of secondary sex characteristics that exist along a continuum. Disruption in these factors may modulate the development of cardiovascular disease, which may be of particular relevance to transgender individuals where there may be interplay between natal sex chromosomes and GAHT. This relationship is explored further in Chapter 3 and Chapter 6.

2.2.2 Sex & cardiovascular disease

The role of sex in influencing the epidemiology, pathophysiology, clinical presentation and outcomes in cardiovascular disease is being increasingly recognised (Roth *et al.*, 2017; Colafella & Denton, 2018). Cardiovascular disease comprises many distinct conditions, and to review all sex-related factors would be beyond the scope of this thesis. Consequently, the influence of sex will be discussed in relation to hypertension, ischaemic heart disease and heart failure as exemplars of this relationship. In particular hypertension will form the focus of this section as it remains the most researched model of the impact of sex on cardiovascular outcomes.

2.2.2.1 Hypertension

Hypertension is one of the most important modifiable risk factors for cardiovascular disease and mortality worldwide (Yusuf *et al.*, 2020). Blood pressure is a sex-dependent trait as demonstrated in a meta-analysis of 3,476 people without hypertension, systolic and diastolic blood pressure was 6 mmHg and 4 mmHg higher in males than females, respectively (Staessen *et al.*, 1991). In the 2021 update of the US Heart Disease and Stroke Statistics, the age-adjusted prevalence of hypertension between 2015 to 2018 in those over the age of 20 years was 51.7% in males and 42.8% in females (Virani *et al.*, 2021).

The differences in blood pressure and the development of hypertension between males and females is influenced significantly by age. In childhood the blood pressures of males and females are comparable. However, following puberty and the increased secretion of sex steroids, blood pressure rises and exhibits sex-specific differences, with males exhibiting higher blood pressure than their female counterparts (Jackson *et al.*, 2007).

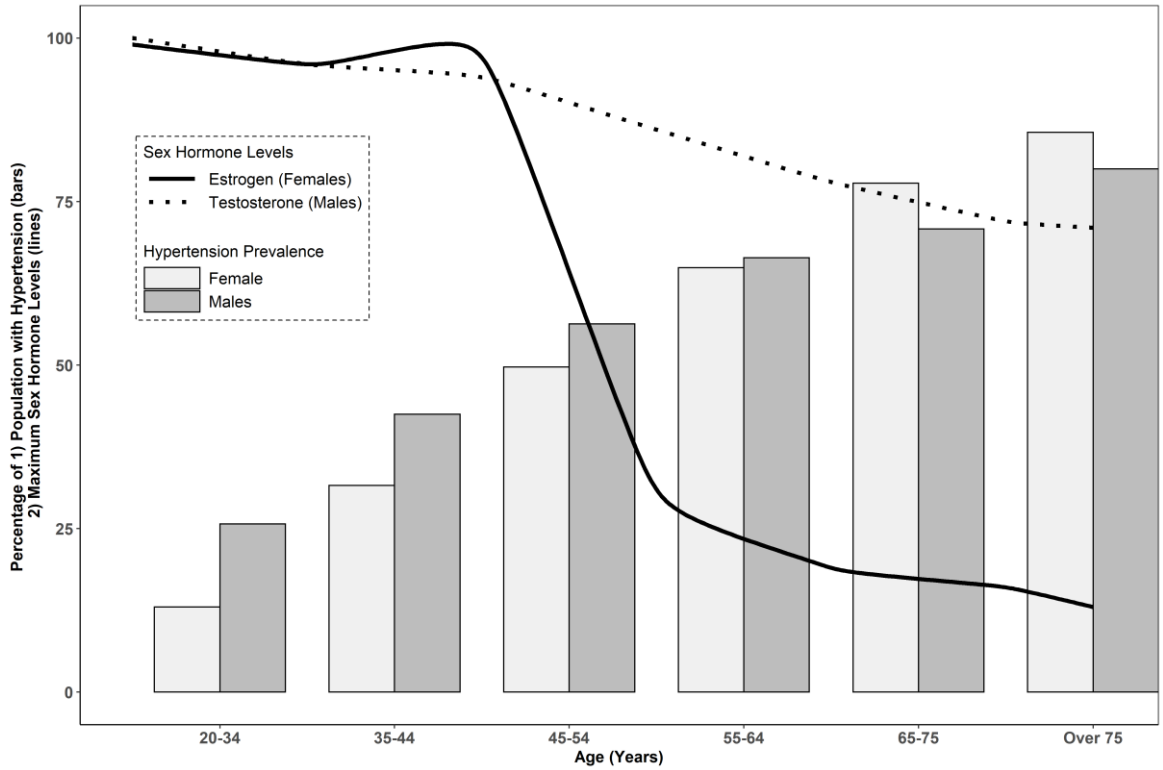

Figure 2-1. Hypertension prevalence, sex hormone levels and ageing.
Hypertension prevalence for males (dark grey) and females (light grey) are depicted alongside relative male testosterone (solid line) and female oestrogen levels (dotted line). Figure published under a Creative Commons License, no permission necessary for use (Connelly et al., 2021).

Data from the US between 2013 and 2016 obtained from the National Health and Nutrition Examination Survey (NHANES), demonstrated that the hypertension prevalence in females and males per age grouping was 13% versus 25.7% (20-34 years), 31.6% versus 42.5% (35-44 years), 49.7% versus 56.3% (45-54 years), and 63.9% versus 66.4% (55-64 years) (Virani *et al.*, 2020). At ages beyond 64 years, females demonstrate a higher prevalence of hypertension than males. This rise coincides with the fall in endogenous oestradiol levels occurring following the

menopause, thereby suggesting a significant role of sex steroids in this relationship (Figure 2-1)(Connelly *et al.*, 2021). Importantly, body mass index, smoking and socioeconomic status may also facilitate increases in hypertension within postmenopausal women, and this association may represent a complex interplay between sex steroids and behavioural risk factors (Tikhonoff *et al.*, 2019).

The steep upwards trajectory evident in blood pressure has also been demonstrated in a longitudinal analysis of 32,833 individuals examined over 40 years (Ji *et al.*, 2020). In this cohort, females demonstrated a sharper increase in blood pressure from the third decade of life. Consequently, fundamental differences in the development of blood pressure dynamics may exist between males and females. These factors, which may be either sex (e.g. sex hormones, chromosomal complement, pregnancy or epigenetic changes) or gender (e.g. psychosocial traits such as relative economic deprivation) mediated ultimately ameliorate the cardioprotective blood pressure advantages evident in females in their youth and promote the sex-convergence of hypertension prevalence later with age (Connelly *et al.*, 2021).

The impact of hypertension on the development of adverse cardiovascular outcomes is also inequitable between sexes. In the INTERHEART cohort, which studies the effect of potentially modifiable risk factors associated with MI in 52 countries, blood pressure was a more potent risk factor for MI in females than males (Yusuf *et al.*, 2004). Recently, in a prospective study from Tromsø, Norway, including 33,859 individuals, although the risk of MI was greater in males, this risk was influenced more significantly by blood pressure in females (Albrektsen *et al.*, 2017). Similarly, in a UK Biobank study of 471,998 people, the relative risk of MI in those with elevated blood pressure was over 80% higher in females compared to males, although males had an overall higher risk (Millett *et al.*, 2018). The stronger association of hypertension and adverse cardiovascular outcomes in females is not limited to MI. In the prospective Reasons for Geographic and Racial Differences in Stroke (REGARDS) study, the association of hypertension and ischaemic stroke was twice that in females compared to males, even following adjustment for risk factors associated with this disease (Madsen *et al.*, 2019).

Despite hypertension occurring more commonly in males, females develop this condition at an accelerated rate and are exposed to sex-specific risk factors such as hypertensive disorders of pregnancy and the menopause (Ostchega *et al.*, 2020; Ji *et al.*, 2021; Virani *et al.*, 2021). In a pooled analysis of 27,542 individuals from established cohorts (i.e. Framingham Heart Study, Multi-Ethnic Study of Atherosclerosis, Atherosclerosis Risk in Communities Study, and Coronary Artery Risk Development in Young Adults Study) hypertension-related organ damage (e.g. the relative risk of MI, heart failure and stroke) increased at lower blood pressure thresholds in females compared to males (Figure 2-2) (Ji *et al.*, 2021).

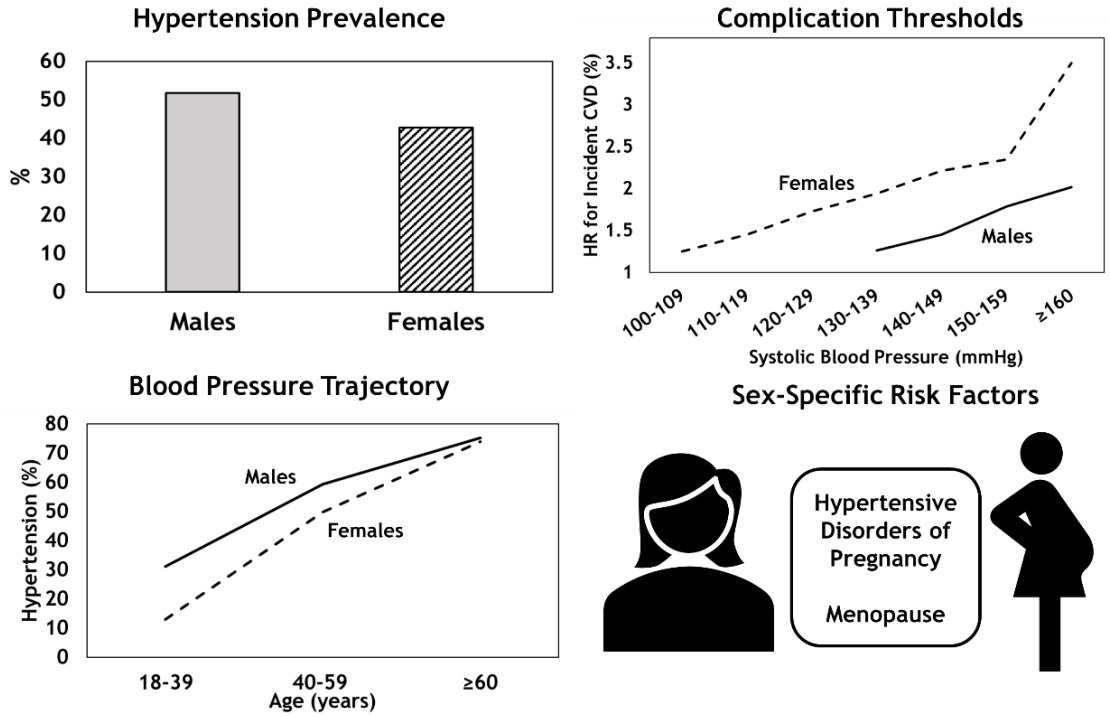

Figure 2-2. Sex differences in hypertension.

This figure demonstrates the overall hypertension prevalence (top left), complication thresholds per blood pressure category (top right), blood pressure trajectories across the lifespan (bottom left) and hypertension sex-specific risk factors in females (bottom right). HR: hazard ratio; CVD: cardiovascular disease. Figure published under a Creative Commons License, no permission necessary for use (Connelly et al., 2022).

2.2.2.2 Ischaemic heart disease

Sex-specific differences are also evident in ischaemic heart disease. Overall, this condition is more prevalent and occurs earlier in life in males compared to females (George *et al.*, 2015). Young to middle aged women typically are afforded protection against atherosclerosis and the development of this condition.

In a UK Biobank study of 471,998 individuals with no history of cardiovascular disease, women who smoked or had evidence of hypertension had a significantly higher relative risk of developing a MI compared to men (Millett *et al.*, 2018). Importantly, these sex-specific associations reduced with age, suggesting that as the population ages and the prevalence of these risk factors increase, the incidence of MI in women may become equal to men. It should be noted that the incidence of MI-related hospitalisation has increased in young women despite decreasing in men (Arora *et al.*, 2019).

Ischaemic heart disease phenotypes also appear to be sex-specific. In younger females, MI appears to be driven by plaque erosion rather than rupture, which is more common in males and older females (Wang *et al.*, 2016b). Whereas overall, females demonstrate more coronary microvascular dysfunction and impaired coronary flow reserve compared to males (Taqueti *et al.*, 2017). Consequently, females presenting with ACS demonstrate less obstructive disease on angiography, however, they experience higher mortality in the following year compared to males (Shaw, *et al.*, 2008; Kunadian *et al.*, 2017). Similarly, in the National Hospital Discharge Survey 1979–2005, the in-hospital mortality relating to MI was 14.9% in females and 10.2% in males (Fang *et al.*, 2010).

Sex differences are also evident in the management of this condition. In an analysis of 13,193 patients who received coronary artery bypass grafting, females, who comprised only ~20% of this cohort, had higher risk of major adverse cardiac and cerebrovascular events in the five year follow up (Gaudino *et al.*, 2022). A large component of this increased risk was attributed to a higher rate of perioperative MI. This may be a consequence of smaller native coronary arteries

and bypass conduits and increased tendency to vasospasm in females, resulting in a technically more complex procedure.

Therefore, although the absolute risk of MI is elevated in males compared to females, the pathophysiology underlying established ischaemic heart disease, risks associated with the development of ischaemic heart disease, and management modalities and mortality demonstrate a number of sex-dependent differences. Mechanisms responsible for these differences are likely to be diverse. The role of sex-specific differentially expressed miRNA and related gene networks in ACS are investigated as a potential mediators of these differences are investigated in Chapter 5.

2.2.2.3 Heart failure

Interestingly, the lifetime risk for the development of heart failure appears comparable between sexes. In the Framingham Heart Study this was estimated as 21% for males and 20% for females (Lloyd-Jones *et al.*, 2002). However, heart failure subtypes differ greatly between sexes. In a study of 28,820 participants from four community-based cohorts and followed up for incident heart failure over 12 years, males experienced a twofold higher risk of heart failure with reduced ejection fraction (HFrEF) compared to females (Ho *et al.*, 2016). In the Swedish Heart Failure Registry, females accounted for 55% of all patients with heart failure with preserved ejection fraction (HFpEF) and only 29% of HFrEF patients (Stolfo *et al.*, 2019). Obesity is a major risk factor in the development of HFpEF and this relationship appears to be particularly pronounced in females (Di Cesare *et al.*, 2016; Savji *et al.*, 2018).

Nevertheless, the higher proportion of females with HFpEF may be due to the age distribution of the population at risk as females continue to exhibit a higher life expectancy (Groenewegen *et al.*, 2020). In a pooled analysis from the Cardiovascular Health Study and the Multi-Ethnic Atherosclerosis Study, at the index age of 45 years the lifetime of HFpEF was similar in males and females,

however, the risk of HFrEF was twice as high in males (10.6% versus 5.8%) (Pandey *et al.*, 2018).

Taken together, there are significant differences between males and females in relation to the prevalence of these cardiovascular conditions, the mechanisms by which they occur, the thresholds by which the develop, and disease subtypes and phenotypes. These sex differences are not uniform and must be considered in the context of a particular cardiovascular condition. Only through investigation of these disparities through a range of methodological approaches can we expand our understanding of the role of sex in the development of cardiovascular disease and improve sex-based clinical pathways.

2.2.3 Sex-related mechanisms in vascular pathophysiology

In combination with sex-chromosome derived gene expression, sex hormone secretion and function shape the basis for distinct biological systems, which with respect to disease are divergent in their epidemiology, pathophysiology and management (Mauvais-Jarvis *et al.*, 2020). Therefore sex-related mechanisms of cardiovascular disease can generally be influenced by three main factors: sex hormones, sex chromosomes, and differentially expressed sex-regulated genes. This is important to both the understanding of sex and gender differences in cisgender populations, but also in transgender populations whereby there may be interactions between natal sex chromosomes and exogenous sex steroids. In this section, the mechanisms and evidence by which sex chromosomes hormones mediate cardiovascular risk in cisgender populations will be reviewed. Describing the role of each of these factors in the pathophysiology of every disease of the cardiovascular system is beyond the scope of this thesis, and consequently some exemplars have been identified of particular interest. This section introduces concepts important to Chapter 5, where an analysis of sex-dependent differentially expressed microRNA (miRNA) in acute coronary syndrome (ACS) is undertaken, and Chapter 3 and Chapter 6, where the influence of GAHT upon transgender vascular health is investigated.

2.2.3.1 Sex chromosomes: Y chromosome

It has been demonstrated that a degree of the increased risk of cardiovascular disease in males is inherited via the sex-determining Y chromosome. Y chromosome haplogroups represent sequences of this chromosome that are closely related and share a common ancestor (Kivisild, 2017). In an analysis of Y chromosome lineage, males with Y chromosome haplotype I, which represents one of the most common Y chromosome haplotypes, demonstrated a higher age-adjusted risk of ischaemic heart disease in the British Heart Foundation Family Heart Study (odds ratio (OR) 1.75. 95% CI 1.2, 2.5) and West of Scotland Coronary Prevention Study (OR 1.45, 95% CI 1.2,1·9) than males with other haplogroups (Charchar *et al.*, 2012). This increased risk was independent of traditional and socioeconomic cardiovascular risk factors. However, macrophage transcriptome analysis demonstrated this haplogroup to be associated with the downregulation and adaptive immunity and upregulation of macrophagic inflammatory response pathways. This suggests modulation of the immune response, and in particular vascular inflammation, may in turn promote the development of ischaemic heart disease in recipients of this Y chromosomal lineage. More recently, in an analysis of 129,133 male UK Biobank participants, the Y chromosome haplogroup I1 was found to be associated with ischaemic heart disease, where carriers demonstrated an 11% increases in the risk of ischaemic heart disease compared to all other haplogroups (Eales *et al.*, 2019). Likewise, this haplogroup was associated with alterations in atherogenic pathways including immunity, oxidative stress, coagulation and extracellular matrix remodelling.

Similarly, in a study of 1,288 men, the *HindIII*(-) polymorphism within the non-recombining region of the Y chromosome demonstrated elevated total and low-density lipoprotein (LDL) cholesterol levels compared to subjects with the *HindIII*(+) genotype (Charchar *et al.*, 2004). These effects appear to be independent of testosterone hormonal concentrations and suggest that the Y chromosome loci may influence LDL levels, and potentially modulate the development of ischaemic heart disease.

The Y chromosome has also been found to modulate blood pressure in humans and animal models of hypertension. In particular, the origin of the Y chromosome (i.e. where it has been inherited from a hypertensive or normotensive male parent) modulates levels of circulating angiotensin II, angiotensin (1-7), renal renin–aldosterone angiotensin system (RAAS) receptor expression, and renal responsiveness to RAAS stimulation in stroke-prone spontaneously hypertensive rats (Sampson *et al.*, 2014).

Loss of the Y chromosome in leukocytes affects ~15% of the male population of older age and has been independently associated with secondary major cardiovascular events in an atherosclerotic population (Haitjema *et al.*, 2017). This suggests that the Y chromosome is likely essential in maintaining cardiovascular health in males. However, it has not yet been demonstrated whether the loss of the Y chromosome in these cells promotes disease progression or is a marker for genomic instability in a pro-atherogenic phenotype (Lau, 2020).

During pregnancy, cells may be exchanged between mother and fetus, and these cells may persevere in the mother as microchimerism (Khosrotehrani *et al.*, 2004). Many of these females carry male cells which are believed to have originated from the male fetus (i.e. male-origin microchimerism). In a population of 766 Danish female participants in the Diet, Cancer and Health cohort between 1993–1997, 71.2% were found to demonstrate male-origin microchimerism. This was identified via sequencing the Y chromosome, and in particular the DYS14 DNA, in their blood. In this analysis, male-origin microchimerism was associated with a reduced rate of ischaemic heart disease (hazard ratio (HR) 0.44; 95% CI 0.23, 0.83). However, there was no improvement in cardiovascular mortality (Hallum *et al.*, 2021). Importantly, this was also associated with lower rates of all-cause and cancer-specific mortality. The mechanism responsible for the relationship is not clear. However, it does suggest a potential interaction between the female phenotype and introduction of male cells and requires further investigation.

Consequently, Y chromosomal gene expression may facilitate altered cardiovascular risk via these inherited or transmitted mechanisms, and directly

contribute to sex differences in the susceptibility to disease. However, more research is required to fully ascertain the overall contribution of this chromosome in the development of cardiovascular disease. This may be of particular relevance to transgender women, where the interaction between the inherited Y chromosome, the introduction of feminising sex hormones, and cardiovascular risk merits further investigation. The influence of GAHT on blood pressure and vascular function in this population is investigated in Chapter 3 and Chapter 6 of this thesis, respectively.

2.2.3.2 Sex chromosomes: X chromosome

Sex differences in gene expression influencing cardiovascular risk may occur as a consequence of the escape of X chromosomal genes from X inactivation. Typically, the imbalance of gene dosages that occurs in the 46 XX karyotype, compared to 46 XY, is addressed through the random inactivation of a single X chromosome within a given cell achieved via chromosome-wide transcriptional silencing (Regitz-Zagrosek & Kararigas, 2017). However, disruption of this mechanism and the escape of X inactivation permits unbalanced gene expression. It is anticipated to occur in ~15% of X-linked genes and is believed to contribute to sex differences between males and females (Berletch *et al.*, 2011). Recently, X-linked genes that escape this process have been implicated in regulating sex-specific myofibroblast activation pathways and aortic value stenosis progression, which may contribute to the increased fibrosis observed in females with this condition (Aguado *et al.*, 2022).

However, the wider role of the X chromosome in sex differences in vascular pathophysiology is not well studied. This is in part due to the relative complexity of this allosome compared to the gene poor Y chromosome (Clarke & Assimes, 2018). Evolutionary degeneration of the Y chromosome has resulted in it containing few genes, whereas comparatively the X chromosome contains as much as 5% of the total human genome. Comparatively, the Y chromosome expresses ~70 protein-encoding genes, whereas ~800 are expressed by the X chromosome.

Consequently, the X allosome and its incumbent genes have significant potential to modulate sex differences in cardiovascular disease.

Due to the complexity of X-linked genes expression, this X chromosome has been broadly excluded from genome-wide association studies, and the contribution of this sex chromosome towards the development of cardiovascular disease is poorly understood. In a recent comprehensive X chromosome wide meta-analysis including ~43,000 cases of coronary arterial disease and ~58,000 controls from 35 international study cohorts, no associations between X chromosomal variants were discovered (Loley *et al.*, 2016). However, this neutral result may indicate that the genetics of the X chromosome is more complex than anticipated (e.g. incomplete understanding of X chromosome inactivation patterns or X chromosomal gene coverage).

In murine models, autosomal translocation of the *SRY* gene permits the investigation of sex chromosomal influence in cardiovascular disease in XX and XY mice that may have either male or female gonads. This four core genotype murine model (i.e. XY males, XY females, XX males, XX females) permits the assessment of the relative effects of sex hormones versus chromosomes within a phenotype. Using these methods, XX mice have demonstrated higher vulnerability to myocardial ischaemic reperfusion injury compared to XY mice, which was determined a consequence of X chromosomal dosage rather Y chromosome absence (Li *et al.*, 2014a). Similarly, dyslipidaemia and the development of atherosclerosis was greater in XX than XY mice and these effects were largely independent of sex hormones (AlSiraj *et al.*, 2019). These findings are of particular relevance in postmenopausal females and transgender men (Chapter 6), where the XX genotype may contribute to the accelerated development of atherosclerotic disease in the absence of the cardioprotective effects of oestrogen.

2.2.3.3 Sex hormones: testosterone & the vasculature

Testosterone is secreted from Leydig cells in the testis. This is governed by the hypothalamus–pituitary–testes axis, whereby GnRH is secreted from the hypothalamus thereby stimulating the anterior pituitary to produce LH. This gonadotroph then binds to its G-protein coupled receptor on gonadal Leydig cells (Isidori *et al.*, 2008). This promotes the transport of cholesterol into mitochondria by the carrier protein StAR (steroidogenic acute carrier regulatory protein) where it is synthesised into testosterone via a series of cytochrome p450 (CYP) enzymes (Figure 2-3).

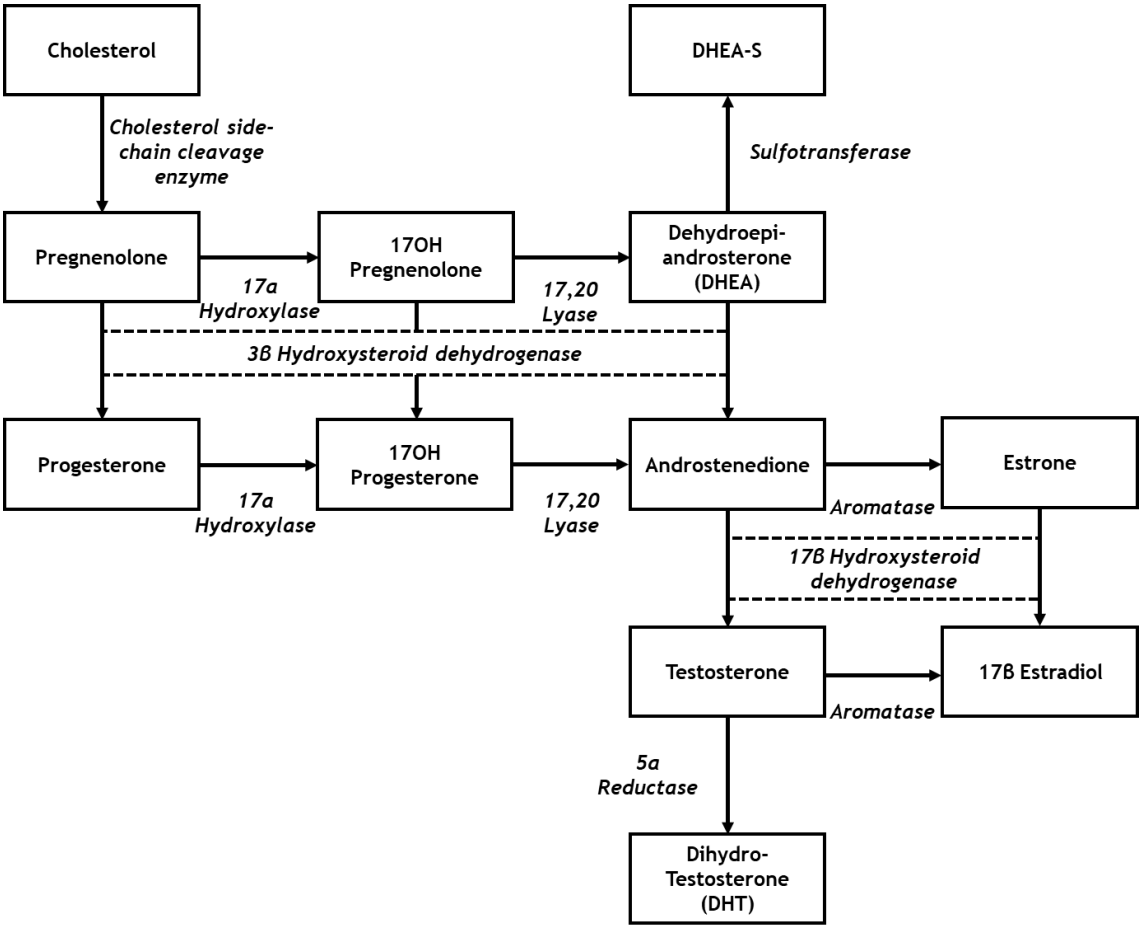

Figure 2-3. Sex steroid synthesis.

This figure demonstrates the biochemical pathway required for testosterone and oestrogen synthesis. Adapted from Shea *et al* (Shea *et al.*, 2014). Reproduced with permissions.

Once released, the majority of testosterone is bound with high affinity to sex hormone-binding globulin (SHBG) and to a lesser extent albumin, corticosteroid-binding globulin (Goldman *et al.*, 2017). Unbound free testosterone accounts for 1–4% of total testosterone. Binding to these proteins is important to the bioactivity of testosterone, where SHBG modulates the bioactivity of sex steroids by limiting their diffusion into target tissues. Consequently, bioavailable testosterone refers to the fraction of circulating testosterone that is not bound to SHBG and reflects the sum of free and albumin bound testosterone.

Testosterone is peripherally aromatised to estradiol and converted to DHT via 5α-reductase. Both testosterone and DHT activate the AR, where the latter has a relative affinity approximately four times that of the former (Swerdloff *et al.*, 2017). However, as DHT synthesis relies on the action of 5α-reductase, the action of this androgen is typically most physiologically relevant in tissues where this enzyme is highly expressed, such as the fetal genitalia and the adult prostate, skin, and liver.

Importantly, the AR is expressed throughout the cardiovascular system on vascular endothelial and smooth muscle cells (Lucas-Herald *et al.*, 2017). This receptor is comprised of a 110 kDa protein receptor that contains three major functional regions: the N-terminal transcriptional regulation domain, the DNA binding domain and the ligand binding domain (Heinlein & Chang, 2002). Two AR variants, AR-A and AR-B, have been identified, however, the functional role of these receptor subtypes is unclear.

After binding to the cytosolic AR, a conformational change takes place, whereby co-localised chaperone proteins (e.g. heat shock proteins and cytoskeletal elements) are dissociated and the AR dimerises and undergoes nuclear translocation (Boese *et al.*, 2017). Once this occurs, the AR interacts with the androgen response elements (ARE) to regulate a multitude of genomic responses (Chistiakov *et al.*, 2018). Conversely, testosterone may modulate non-genomic effects via membrane bound ARs. These act via multiple intracellular pathways involving PKA, PKC, and MAPK (Lopes *et al.*, 2012).

Through these mechanisms, testosterone can mediate vasodilatation via endothelium dependent mechanisms through the release of vasodilating factors into the vascular smooth muscle cells, such as increasing nitric oxide bioavailability via AR facilitated endothelial nitric oxide synthase (eNOS) activation (Figure 2-4) (Moreau *et al.*, 2020). Endothelium independent mechanisms of vasodilation may also occur, which is in part mediated by inhibition of voltage-operated calcium channels and the activation of potassium channels on vascular smooth muscle cells (Tambo *et al.*, 2016).

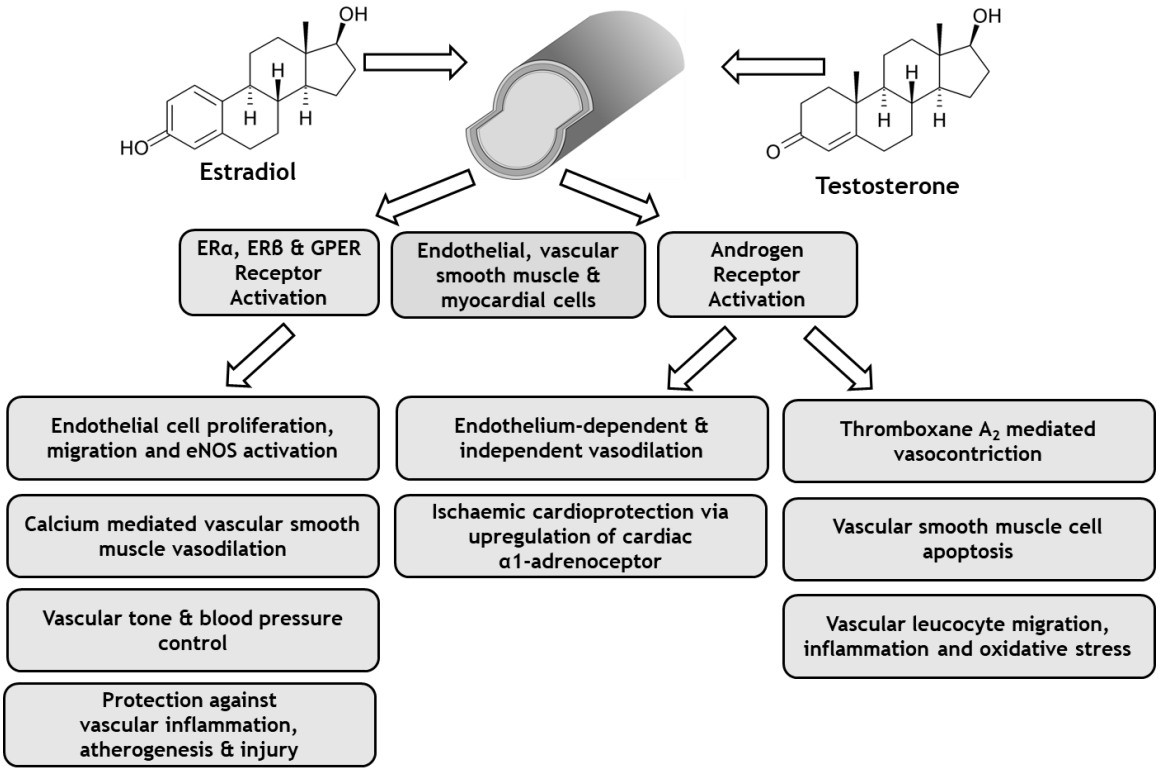

Figure 2-4. Vascular effects of sex hormones.

This figure demonstrates the vascular expression of sex steroid receptors and the effects by which these hormones modulate vascular function in health and disease. Figure published under a Creative Commons License, no permission necessary for use (Connelly *et al.*, 2019).

Testosterone may also facilitate ischaemic cardioprotection via the upregulation of the cardiac α1-adrenoceptor, which has been demonstrated in isolated perfused hearts and ventricular myocytes from orchiectomised rats (Tsang *et al.*, 2008). Recently, testosterone has been shown to improve vascular remodelling through the growth arrest-specific protein 6/Axl pathway, which plays a role in cell

survival, migration and inflammation (Chen *et al.*, 2020). In a randomised control trial, testosterone supplementation was found to reduce inflammatory mediators in hypogonadal men, such as TNFα, IL-1ß and increase IL-10 (Malkin *et al.*, 2004). This evidence supports the immunomodulatory role of this sex hormone.

The vasoconstrictive actions of testosterone have also been observed (Ceballos *et al.*, 1999). In young male spontaneously hypertensive rats, testosterone supplementation increases blood pressure, which is mediated by the RAAS (Dalmasso *et al.*, 2017). The pressor response promoted by testosterone may be modulated via angiotensin II (Ang-II) and altered Ang-II receptor (AT1R/AT2R) ratios (Mishra *et al.*, 2019). By influencing vascular Ang-II receptor subtypes, testosterone may modulate Ang-II responses thereby increasing the risk of hypertension in males.

In rat models it has also been demonstrated that testosterone promotes leucocyte migration via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase cyclooxygenase (COX)-dependent mechanisms. This promotes oxidative stress and inflammation, which may in turn increase the risk of vascular injury and disease (Chignalia *et al.*, 2015). The AR may promote reactive oxygen species generation via increasing the expression a number of pro-oxidants, such as NAD(P)H oxidases, xanthine oxidases and COX-2, and the transcription of factors important to the c-Src and PI3K/Akt pathways (Cruz-Topete *et al.*, 2020). Additionally, testosterone promotes AR-mediated mitochondrial-associated reactive oxygen species generation and apoptosis in vascular smooth muscle cells (Lopes *et al.*, 2014). Consequently, testosterone may contribute to both vascular health and disease. The influence of testosterone on blood pressure and vascular function of transgender men is explored in Chapter 3 and Chapter 6, respectively.

2.2.3.4 Sex hormones: testosterone, males & cardiovascular risk

Levels of testosterone decline with age in healthy men (Harman *et al.*, 2001; Yeap *et al.*, 2012). In elderly men low testosterone predicts increased risk of cardiovascular disease and mortality (Araujo *et al.*, 2011; Corona *et al.*, 2011;

Ruige *et al.*, 2011). In an observational study of 1,031 men with low testosterone receiving replacement therapy mortality was significantly reduced (HR 0.61; 95% CI 0.42,0.88) (Shores *et al.*, 2012). Similarly, in a retrospective cohort study of 8,808 men with evidence of androgen deficiency dispensed testosterone was associated with lower cardiovascular risk over a median follow-up of 3.4 years (HR 0.67, 95% CI 0.62,0.73) (Cheetham *et al.*, 2017).

To date, no prospective interventional studies investigating the impact of testosterone replacement upon cardiovascular events or mortality as primary endpoints have been reported. In a double-blind placebo randomised controlled trial, the use of testogel for one year in 138 hypogonadal men was associated with an increase in non-calcified coronary artery plaque volume compared with the control group (Budoff *et al.*, 2017). These results should be interpreted with caution as the testosterone cohort demonstrated overall less plaque at study completion, which may have resulted from disparate baseline plaque burdens between study groups (Yeap *et al.*, 2018).

A randomised controlled trial of testosterone in 209 men with mean age of 74 years, limited mobility and a high prevalence of comorbid disease (e.g. hypertension, diabetes, hyperlipidaemia and obesity) demonstrated an increased risk of cardiovascular adverse events resulting in the premature discontinuation of the trial (Basaria *et al.*, 2010). These results must again be carefully interpreted as cardiovascular risk was not the primary outcome of this trial and the small number of events that occurred did not undergo structured evaluation.

In the Testosterone's Effects on Atherosclerosis Progression in Aging Men (TEAM) trial, which utilised a parallel-group randomised trial design, the effects of testosterone therapy on atherosclerosis progression assessed by carotid intima media thickness and CT coronary artery calcium scores was studied (Basaria *et al.*, 2015). Three hundred and eight hypogonadal males aged over 60 years were treated with either 7.5 g of testosterone gel or placebo gel for a total of three years. In this trial, testosterone was not associated with either of these outcomes.

However, this short follow up period may not have been sufficient to ascertain any clinically significant differences between these groups.

A meta-analysis of 27 placebo-controlled randomised trials demonstrated that testosterone therapy increased cardiovascular-related events (OR 1.54, 95% CI 1.09,2.18) in men (Xu *et al.*, 2013). However, more recent meta-analyses have not demonstrated testosterone to be associated with increased risk of cardiovascular events (Onasanya *et al.*, 2016; Alexander *et al.*, 2017; Corona *et al.*, 2018). Given these conflicting results, it is unsurprising that no consensus exists regarding the overall impact of testosterone therapy on vascular health in men.

In response to this controversy, the Testosterone Efficacy and Safety (TestES) consortium undertook an individual patient data meta-analysis of 17 trials encompassing 3,431 participants (Hudson *et al.*, 2022). The analysis demonstrated no evidence that testosterone therapy increased short or medium term cardiovascular risk in hypogonadal men, however, could not confirm the long-term safety of this sex hormone. However, the testosterone replacement therapy for assessment of long-term vascular events and efficacy response in hypogonadal men (TRAVERSE) trial (NCT03518034) commenced in 2018 and aims to assess the effect of testosterone on major adverse cardiovascular events (MACE). This trial is powered to assess cardiovascular events, will enrol 6,000 males and aims provide a definitive answer to whether longer-term testosterone therapy modulates cardiovascular outcomes in this population (US National Library of Medicine, 2019). The influence of testosterone upon the cardiovascular risk of transgender men is discussed further in section 2.4 of this chapter. Additionally, the effects of this sex hormone on blood pressure and vascular function of transgender men is investigated in Chapter 3 and Chapter 6, respectively.

2.2.3.5 Sex hormones oestrogen & the vasculature

Oestrogens are the predominant feminising sex hormones in females and have important roles in both reproductive and non-reproductive systems. Oestrogen

consists of three main subtypes: 17β-estradiol (E2; estradiol), estrone (E1) and estriol (E3) (Figure 2-5). 17β-estradiol is the most abundant and physiological significant of these hormones in premenopausal females (Cui *et al.*, 2013). Estrone predominates following the menopause and is synthesised in adipose tissue from adrenal dehydroepiandrosterone (DHEA). Estriol is the least potent oestrogen is synthesised in large quantities by the placenta through 16α-hydroxylase. Lastly, estetrol (E4) is only produced during pregnancy as a consequence of the fetal liver exclusively facilitating 15α- and 16α-hydroxylation of this sex steroid (Holinka *et al.*, 2008).

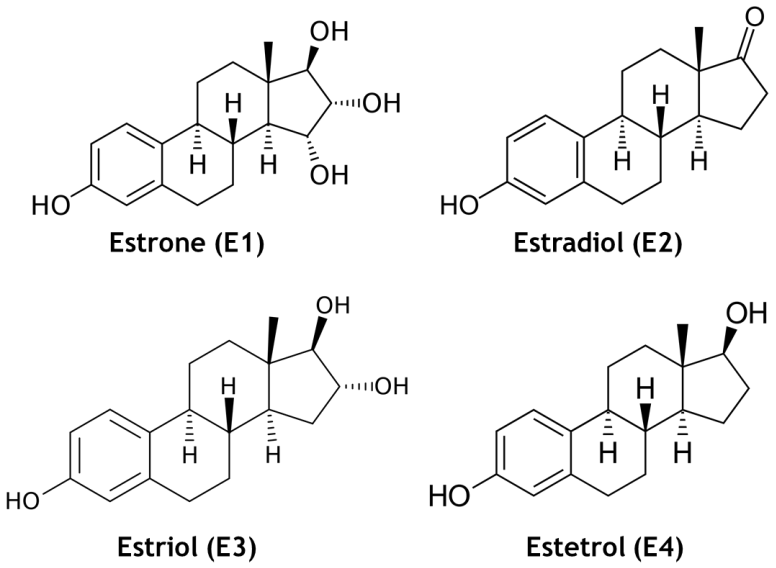

Figure 2-5. Structural biology of oestrogen subtypes.

In premenopausal women, oestrogens are synthesised from cholesterol primarily in the ovaries. Theca cells produce androgens, which are then converted to oestrogens via the aromatase enzyme in granulosa cells (Figure 2-3). In males, gonadal oestrogen synthesis occurs Leydig cells, Sertoli cells and in spermatocytes (Hess *et al.*, 1997). Physiologically significant concentrations of oestrogens may also be synthesised at extra-gonadal sites including the adrenal glands, brain and adipose tissue (Barakat *et al.*, 2016).

The functions of oestrogens are diverse. Their primary role is to facilitate the development and maintenance of reproductive organs and secondary sex

characteristics. The effect of oestrogens in males is less well understood, however, it has been suggested that they play a role in reproduction health (Cooke *et al.*, 2017). Importantly, ERs are ubiquitously expressed and therefore offer a diverse range of physiological functions.

In the vasculature, ERs are expressed within vascular smooth muscle and endothelial cells (Khalil, 2013). Estradiol may bind to the cytosolic ERs, ERα or ERß. These receptors then dimerise and undergo nuclear translocation where they regulate oestrogen response elements (ERE). In addition, interaction with activator protein-1 (AP1) and specificity protein-1 (Sp1), which are located on the promoter of oestrogen responsive genes, facilitate transcriptional regulation (Iorga *et al.*, 2017). In addition, estradiol may also bind to membrane bound ERs (ERα, ERß and G protein coupled oestrogen receptor (GPER)). Their activation may stimulate MAPK/ERK/PI3K/cAMP second messenger signalling that facilitate rapid non-genomic responses.

Endothelial ERα has been shown to promote endothelium-dependent vasodilatation through eNOS, endothelial proliferation and migration, and promotes carotid artery re-endothelialisation (Chambliss *et al.*, 2010; Kypreos *et al.*, 2014). In murine models, this receptor subtype mediates the cardioprotective effects of oestrogen in response to vascular injury and atherosclerosis (Hodgin *et al.*, 2001; Pare *et al.*, 2002).

The RAAS is an important component of cardiovascular health and its mediators, such as plasma renin, fluctuate in response to oestrogen levels throughout the menstrual cycle (Chidambaram *et al.*, 2002). These actions are mediated via ERα activation of juxtaglomerular nuclear ERE of renin expressing cells, which is a necessary component of basal renin expression (Lu *et al.*, 2016). Moreover, in premenopausal women an ERα mediated increases in activity of angiotensin-converting enzyme (ACE) 2 and angiotensin (1-7), a bioactive peptide that opposes the vaso-injurious effects of Ang II and promotes vasodilatation. Consequently this leads to the development of a vasodilatory phenotype (Medina *et al.*, 2020).

ERβ may also facilitate alteration in cardiovascular risk in females. Vascular smooth muscle cell ion channel aberrations and hypertension have been observed in mice deficient in ERβ (Zhu *et al.*, 2002). ERβ activation has also been demonstrated to reduce blood pressure in spontaneously hypertensive rats (Jazbutyte *et al.*, 2007). In oestrogen replete premenopausal females *ERβ* gene variant rs10144225 minor alleles are associated with the development of salt-sensitivity of blood pressure, which is believed to be mediated by an increased aldosterone/renin ratio (Manosroi *et al.*, 2017). This again demonstrates the reciprocity between sex hormones and RAAS mediators.

Lastly, loss of GPER action promotes endothelium-dependent vasoconstriction, atherosclerosis and vascular inflammation (Barton & Prossnitz, 2015). Direct activation of this receptor via the GPER G1 agonist promotes coronary vessel relaxation in a concentration and sex-dependent manner in rats (Debortoli *et al.*, 2017). Consequently, selective targeting of this membrane bound receptor may be considered in the future as a potential mediator of cardiovascular risk (Dinh *et al.*, 2021).

2.2.3.6 Sex hormones: oestrogen, females & cardiovascular risk

Premenopausal females are protected from cardiovascular disease in an oestrogen-dependent manner relative to age-matched males. Low levels of oestrogen in younger females and premature menopause are associated with an increased risk of cardiovascular disease (Jacobsen *et al.*, 1997; Morselli *et al.*, 2017a). Moreover, declining oestrogen availability following the menopause is associated with dyslipidaemia, increases in blood pressure and an elevated risk of cardiovascular disease (Anderson *et al.*, 2013; Kilim, 2013).

Observational studies initially suggested a cardioprotective effect of exogenous oestrogen therapy in postmenopausal females (Hale & Shufelt, 2015; Keck & Taylor, 2018). However, despite this randomised trials such as the Heart and Estrogen–Progestin Replacement Study (HERS) and Women's Health Initiative (WHI)

study refuted the cardiovascular safety of this recommendation (Stampfer & Colditz, 1991; Manson *et al.*, 2003; Wassertheil-smoller *et al.*, 2014).

Subsequent trials and secondary analyses have demonstrated that early introduction of oestrogen therapy (<10 years from menopause onset) in younger (<60 years) postmenopausal women is either neutral or beneficial with respect to cardiovascular risk. Indeed, the lower absolute risk of adverse events among women 50 through 59 years of age suggests that hormone therapy may be beneficial (Pinkerton, 2020). Conversely, initiation in older women with longer periods of oestrogen deprivation may in fact be harmful, particularly in those with established cardiovascular disease. This phenomenon has been termed the 'timing hypothesis' (Hale & Shufelt, 2015; Lobo, 2017; Marjoribanks *et al.*, 2017; Keck & Taylor, 2018). The influence of oestrogen upon the cardiovascular risk of transgender women is discussed further in section 2.4 of this chapter. Moreover, the relationship between this sex hormone, blood pressure and vascular function of transgender women is investigated in Chapter 3 and Chapter 6, respectively.

2.2.3.7 Sex-specific risk factors

Sex differences, as a consequence of chromosomal or sex hormone mediated mechanisms, result in a number of sex-specific factors that modulate cardiovascular disease. For instance, anatomically females have smaller epicardial coronary arteries and higher baseline myocardial blood flow. As a consequence, endothelial shear stress is increased, which influences endothelial function and the propensity to develop atherosclerosis (Patel *et al.*, 2016). Even the pathophysiology of atherosclerosis differs between sexes, which is evident in the composition of plaque in those presenting with ACS. In patients presenting with this acute ischaemic event, females demonstrated higher levels of comorbid disease. However, plaques were less prone to rupture, they did not contain a necrotic score and overall there was less extensive coronary calcium and coronary artery disease (Lansky *et al.*, 2012).

One of the major differences between males and females that arises from these sex-related mediators is the capacity to become pregnant, which may have profound effects in the development of cardiovascular disease. Hypertensive disorders in pregnancy may play a pivotal role in the upward trajectory in blood pressure evident in females from third decade, and may facilitate the loss of female cardioprotection later in life (Benschop *et al.*, 2019; Ji *et al.*, 2020). Transient periods of elevated blood pressure during pregnancy may facilitate the development of a sustained hypertensive phenotype. In the Nurses' Health Study II, which included 58,671 females participants without a history of hypertension or cardiovascular disease, hypertensive disorders of pregnancy doubled the rate of self-reported chronic hypertension with a mean follow up of 25-32 years (Stuart *et al.*, 2018). Furthermore, in the Rochester Epidemiology Project medical record-linkage system, based on 9,862 pregnancies between 1976 and 1982, the development of hypertensive disorders of pregnancy and pre-eclampsia substantially increased the prevalence of hypertension and doubled the risk of stroke and ischaemic heart disease in a median follow-up of 36.2 years (Garovic *et al.*, 2020). Consequently, these studies suggest there is strong association between the development of gestational hypertension and long-term cardiovascular vulnerability, which is specific to the female sex.

2.2.4 Limitations of sex in cardiovascular risk prediction

The Framingham Heart Study provided seminal research in the definition of cardiovascular risk factors, which have subsequently influenced clinical guidelines and public health policy in relation to cardiovascular disease prevention for decades (Tsao & Vasan, 2015). This research coined the term ‘coronary risk factors’ as major determinants of cardiovascular risk. These would later be described as ‘traditional risk factors’ and comprise hypertension, smoking, diabetes and dyslipidaemia (Mahmood *et al.*, 2014). As demonstrated throughout this section, although males and females are exposed to these risk factors, the prevalence and processes by which they occur, and the influence they have upon the cardiovascular risk of an individual differs significantly between sexes.

Sex is incorporated in approximately one third of cardiovascular prediction models and risk assessment tools, such as the Framingham Heart Score, that only utilise these traditional risk factors (Paulus *et al.*, 2016). However, these may underestimate cardiovascular risk in women. This may be of consequence of the lack of psychosocial assessment and the estimation of short-term cardiovascular risk in a population that typically experiences an extended lifespan compared to males (Lakoski *et al.*, 2007). Moreover, there has been insufficient representation of women in cardiovascular research, and inadequate conduct of sex- and gender-based analyses, which prevents gender equity in cardiovascular health and hinders these predictive assessments (Scott *et al.*, 2018; Jin *et al.*, 2020).

Ultimately, sex unilaterally, as a biological concept, cannot help us understand why women experience disparities in referrals and timeliness of coronary revascularization, or enrolment and engagement in cardiac rehabilitation following ischaemic events compared to men and consequently experience worse outcomes (Colella *et al.*, 2015; D'Onofrio *et al.*, 2015; Roswell *et al.*, 2017; Hyun *et al.*, 2021). Only through gender-based analyses and incorporation of 'non-traditional' risk factors can we expand our understanding of how these facilitate the development of cardiovascular disease and the differences evident in cardiovascular event outcomes between men and women (Azizi *et al.*, 2020; Connelly *et al.*, 2021a).

2.3 Gender & cardiovascular disease

This chapter section will provide context for the concept of gender and its contributing dimensions, the means by which gender is measured in clinical research and lastly, by what mechanisms gender-related factors may alter the cardiovascular risk of an individual. This section also presents a background to the gender stratification analysis undertaken in Chapter 4, and the investigation of gender-specific differentially expressed miRNA and regulatory gene networks within Chapter 5 of this thesis.

2.3.1 What is gender?

Gender is a multi-factorial concept that is derived from psychosocial, behavioural and cultural components that may modulate health (Clayton & Tannenbaum, 2016; Schiebinger & Stefanick, 2016). Gender incorporates numerous dimensions including gender identity (i.e. personal sense of masculinity, femininity, alternative or lack of gender), gender roles (i.e. societal and cultural expectations of gender), gender relations (i.e. interpersonal dynamic between genders), and institutionalized gender (i.e. political, educational, social distribution of power in society) (Figure 2-6) (Connelly *et al.*, 2021). These gender norms impact the behaviour of individuals, how they perceive themselves and others, and how they interact with one another and society (Tannenbaum *et al.*, 2016).

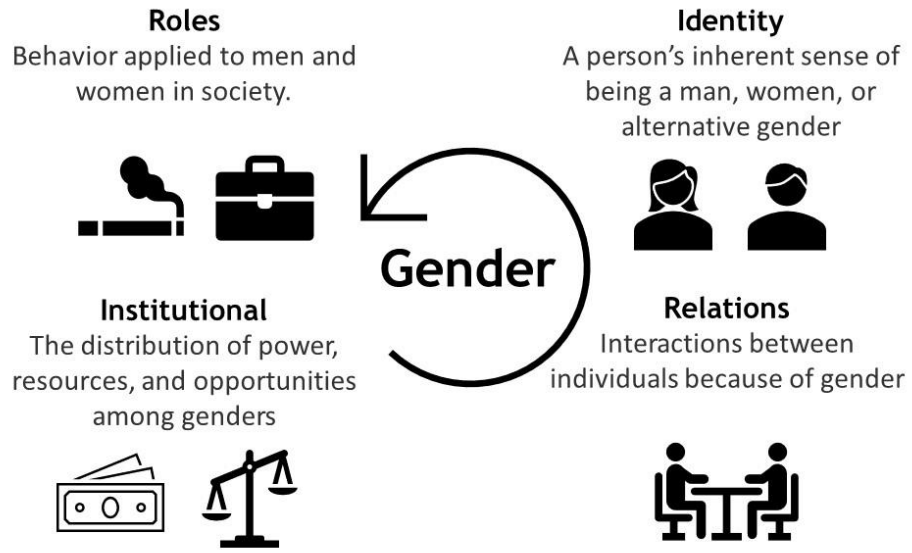

Figure 2-6. Gender component framework.

2.3.1.1 Gender identity

The concept of gender identity was first published in 1968 by Dr Robert Stoller, who hypothesised that sex and gender were not interchangeable but independent and interacting concepts (Calvo *et al.*, 1985). The American Psychology Association defines this as 'A person's deeply-felt, inherent sense of being a boy,

a man, or male; a girl, a women, or female; or an alternative gender (e.g. genderqueer, gender nonconforming, gender neutral) (American Psychological Association, 2015). Consequently, a person's gender identity refers to their intrinsic sense of self (e.g. 'I am a man', 'I am a woman', 'I am an alternative gender', 'I lack gender'). This may be congruent with a person's assigned sex, resulting in an individual being cisgender. Conversely, if conflict exists between a person's sex and gender, the individual is transgender.

Traditionally, gender has been considered dichotomous, where individuals would classify themselves as either masculine or feminine. However, contemporary definitions allow for a greater conceptual range, whereby individuals may identify along a spectrum of gender identities, that may be fluid and dynamic. Importantly, both masculine and feminine traits may co-exist and interact with environmental and social factors.

The majority of individuals are assigned a 'male' or 'female' sex at birth and subsequently a congruent gender identity and gender role forms as a 'boy' or 'girl', respectively (Joseph *et al.*, 2017). These gender identities are consolidated in adolescence and adulthood, where an individual adopts gender roles based on stereotypical social and behavioural norms considered appropriate for that culture and society. Gender identity is not the same as gender roles or even expression although these may be correlated. An individual may identity as a cisgender woman but may not comply with stereotypical gender roles considered typical for that gender (Polderman *et al.*, 2018).

It is unclear whether such psychosocial behaviours are influenced wholly environmentally, or whether biological or genetic factors are contributory. Recently, theories of gender identity have been developed, however, demonstrate significant limitations as these cannot be studied in animal models (Roselli, 2018). Although broadly considered psychosocial in nature, emerging evidence suggests that a significant proportion of gender identity is inherited and polygenic in aetiology.

In a study of gender diagnosticity (i.e. the Bayesian probability that an individual is predicted to be male or female on the basis of gender-related characteristics) in 839 monozygotic and dizygotic same-sex twin pairs, a heritability of 53% was demonstrated (Lippa & Hershberger, 1999). Heritability estimates for masculinity and femininity were 36% and 38%, respectively. This hypothesis is supported by data from older (n=2,647 pairs, mean age 41.2 years) and younger (n=1,503, mean age 23.2) cohorts from the Australian Twin Registry, where heritability estimates for masculinity and femininity were approximately one third with slightly higher estimates in the older cohort (Loehlin & Martin, 2000).

In a multi-national and generational cohort, a significant proportion (~40%) of gender diagnosticity is heritable (Loehlin *et al.*, 2005). As demonstrated in meta-analysis of twin correlations and reported variance components for 17,804 traits from 2,748 publications including 14,558,903 partly dependent twin pairs, estimated heritability of behavioural and personality traits are approximately 49% (Polderman *et al.*, 2015). The heritability of gender identity is in keeping with these estimates although clearly interaction with societal norms and gender expectations is the primary component in establishing individual gender identity.

The gender of an individual can have profound effects on cardiovascular health. In the Gender and Sex Determinants of Cardiovascular Disease: From Bench to Beyond-Premature Acute Coronary Syndrome (GENESIS-PRAXY) study, the cardiovascular morbidity and mortality of participants following ACS was associated more strongly with gender than sex (Pelletier *et al.*, 2015). In particular, individuals with ischaemic heart disease who demonstrate feminine personality traits, in addition to those with feminine gender roles, are at increased risk of recurrent ischaemic heart disease (Pelletier *et al.*, 2016). Importantly, these associations were independent of sex, thereby demonstrating that gender traits alone may modulate cardiovascular risk and outcomes.

Similar effects were apparent in the Variation In Recovery: Role of Gender on Outcomes in Young Acute Myocardial Infarction Patients (VIRGO) study. In this study, women with acute MI had lower socioeconomic status and higher levels of

psychosocial stressors than men (Bucholz *et al.*, 2017). They were also found to have lower quality of life at the time of MI. Consequently, these gender-mediated factors may significantly increase the risk of cardiovascular disease in young women.

2.3.1.2 Gender roles

Gender roles are societally imposed behavioural norms applied to men and women (Mauvais-Jarvis *et al.*, 2020). These regulate a multitude of routine health behaviours and expectations that may affect cardiovascular disease susceptibility and management. These factors include primary earner status, employment, type of occupation, and carer and parental responsibilities (Tadiri *et al.*, 2021). Moreover, the means by which we interact with our environment and undertake lifestyle choices can be modulated by this gender component. Gender roles may also be influenced by culture, whereby expectations may differ between urban and rural communities in addition to developed and developing societies.

In a pooled cross-sectional, individual-level data analysis comprising 47,045 participants, job strain was associated with a number of cardiovascular risk factors (e.g. diabetes, smoking, physical inactivity, obesity) (Nyberg *et al.*, 2013). Shift work and longer-working hours may potentiate this risk (Kang *et al.*, 2012; Torquati *et al.*, 2018). In a multi-cohort study of 102,633 individuals with a mean follow up of 13.9 years a higher mortality was observed in men with cardiometabolic risk factors compared to healthy men or all women. Importantly, the effect of job strain on mortality was independent of the presence or management of traditional risk factors (Kivimäki *et al.*, 2018). Similarly, in the Copenhagen City Heart Study and the INTERHEART study, job insecurity and permanent work stress were respectively associated with at least a two-fold higher odds of MI in men only (Rosengren *et al.*, 2004; Netterstrøm *et al.*, 2010). Conversely, marital stress but not work stress has been shown to increase risk of recurrent cardiac events in women with established ischaemic heart disease (Orth-Gomér *et al.*, 2000).

The effects of gender roles on the development of cardiovascular disease may intersect with sex. Smoking remains a leading risk for early death and disability worldwide and elicits profound effects of the risk of developing cardiovascular disease (Huxley & Woodward, 2011; Reitsma *et al.*, 2017). The interactions between sex, gender and smoking in cardiovascular disease first became evident in a prospective study of ~25,000 individuals, where it was demonstrated that the relative risk of MI in smoking women exceeded that of men by over 50%, even after adjustment for major cardiovascular risk factors (Prescott *et al.*, 1998). Similarly, in a recent meta-analysis of over 2.4 million individuals and more than 44,000 ischaemic heart disease events, women who smoke have a 25% higher relative risk for ischaemic heart disease compared to men (Huxley & Woodward, 2011).

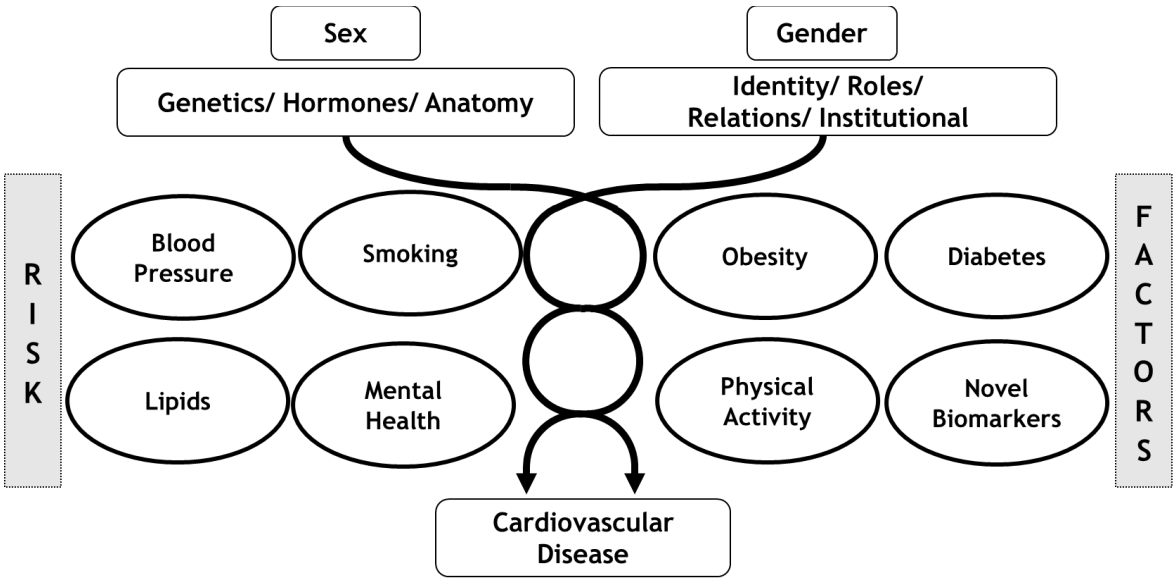

Figure 2-7. Sex and gender as modifiers of cardiovascular risk.

Biological sex and sociocultural gender influence cardiovascular risk by interacting with cardiovascular risk factors. Reproduced with permission (Connelly *et al.*, 2021a).

However, gender roles modulate smoking prevalence and consumption, which are higher in men compared to women (Woodward *et al.*, 2005; Maurice *et al.*, 2006; Reitsma *et al.*, 2017). It is therefore unclear whether gender-related differences in smoking mediates this increased risk, or whether established smoking gender norms in combination with a sex-mediated predisposition increases this risk

(Peters *et al.*, 2015). The intersection and interaction between sex and gender and a multitude of cardiovascular risk factors occurs commonly (Figure 2-7).

2.3.1.3 Gender relations

Gender relations reflects the interactions between individuals as a consequence of their gender identity and/or expression (Tadiri *et al.*, 2021). Examples of this include relationships, marital status, family dynamics and local networks of psychosocial support.

In a prospective cohort study of 90,987 people aged 40–69 years in Japan without a diagnosis of cardiovascular disease, women inhabiting households with their spouse and children had a greater than two fold higher risk of ischaemic heart disease than women living with spouses only (Ikeda *et al.*, 2009). Conversely, in a population-based cohort study of 302,885 people aged 40-60 in Finland, men who were married had a lower risk of MI incidence and fatality compared to single or cohabiting men, even following adjustment of socioeconomic factors including education, occupation, income and employment status (Kilpi *et al.*, 2015). This association was not apparent in women, however, cohabitation was associated with greater fatality following MI.

In a meta-analysis of marital status and risk of cardiovascular diseases in 34 prospective studies including more than 2 million subjects, in comparison to married individuals those who are unmarried demonstrated an increased risk of all-cause mortality, ischaemic heart disease and mortality and stroke mortality (Wong *et al.*, 2018). A greater risk from death from ischaemic heart disease and stroke was also observed in divorced compared to married individuals. In this analysis, there was increased all-cause mortality and ischaemic events in unmarried men that was not evident in unmarried women when compared to married individuals.

Married individuals may benefit with respect to these outcomes due to social spousal support, which may reduce the time to seek medical assistance and

improve adherence to treatment and lifestyle behavioural changes (Wu *et al.*, 2012; Austin *et al.*, 2014). Spousal support may also potentiate participation in cardiac rehabilitation, which would in turn improve survival outcomes (Kachur *et al.*, 2017). In addition to these examples of social causation theory, the stress hypothesis may also contribute to the positive effects of marriage (Quinones *et al.*, 2014). Marriage, or an equivalent close spousal relationships, may broadly have beneficial influences on economic, behavioural and emotional welfare thereby promoting the recognition of disease and ensuring treatment adherence. Lastly, sustained emotional spousal support may ameliorate maladaptive neuroendocrine responses to stressors that may contribute to the pathophysiological processes responsible for adverse cardiovascular outcomes such as atherosclerosis (Wirtz & von Känel, 2017).

This association was also explored in a further meta-analysis of 21 studies with 7,891,623 individuals and 1,888,752 deaths, which assessed sex differences in the association between marital status and the risk of cardiovascular, cancer, and all-cause mortality (Wang *et al.*, 2020). In this analysis, unmarried individuals were at a higher risk of all-cause and cardiovascular mortality. However, this association was again stronger in men than women. Similarly, in those who were divorced or separated, men had significantly higher cardiovascular and all-cause mortality compared to women. Consequently, it is evident that women and men do not benefit equally from stable spousal relationships with respect to cardiovascular health.

2.3.1.4 Institutionalised gender

Institutionalised gender refers to the distribution of power, resources, and opportunities among genders. Consideration of institutional gender is of particular importance when undertaking cross-national, cultural or even when examining rural-urban differences as gender equity may differ significantly. Moreover, these differences may be apparent within the microcosm of a particular field or institution where there is an imbalanced gender dynamic and equity (Tadiri *et al.*, 2021).

Socioeconomic deprivation is often a consequence of institutionalised gender and the sequalae of this may modulate the health differentially amongst genders. In high-income countries socioeconomic deprivation is associated with the risk of cardiovascular disease (Hippisley-Cox *et al.*, 2008; Manrique-Garcia *et al.*, 2011). In a recent meta-analysis of low socioeconomic status and cardiovascular disease, comprising of over 22 million individuals and over 1 million cardiovascular events, indices of socioeconomic status were associated greater risk of cardiovascular disease in both men and women (Backholer *et al.*, 2017). However, the association of socioeconomic deprivation and cardiovascular disease was stronger in women compared to men. The excess risk of cardiovascular disease associated with the lowest versus highest educational attainment was 18% higher for women compared with men. Indeed, income is a strong predicter of cardiovascular morbidity and mortality regardless of angiographic coronary disease extent or other traditional risk factors (Shaw *et al.*, 2008a).

Differences between men and women in hypertension are believed to be broadly driven by sex-determined factors (Colafella & Denton, 2018). However, data from the Cohorte des Consultants des Centres d'examens de santé (CONSTANCES) have demonstrated the role of gender-mediated inequalities. In this cohort, that comprises of 59,805 individuals between the ages of 25-69 years, hypertension prevalence was higher in men than women (Neufcourt *et al.*, 2020). However, the relationship between the development of this condition and socioeconomic deprivation was more potent in women. This was most apparent younger individuals (ages 25 to 34 years) and was especially associated with education and demonstrates the disproportionate impact of social inequalities in women.

This association appears to hold true for an individual's perception of their value in society. In a meta-analysis of the association between subjective social status and cardiovascular disease, lower subjective social status (i.e. an individual's perception of their position in social hierarchy) significantly increased the risk of ischaemic heart disease, hypertension and diabetes (Tang *et al.*, 2016). Consequently, an individual's cardiovascular risk appears to be altered by their

own perception of their position in society, which ultimately reflects a consequence of complex factors, including gender.

We must also consider the equitable access and delivery of health care between men and women as a potential risk factor for poorer cardiovascular outcomes. Several studies have demonstrated that women experience different, and often less aggressive treatment trajectories, than men. In a survey of US cardiologists, most participants exhibited implicit gender bias in relation to the theoretical management of women with ischaemic heart disease (Daugherty *et al.*, 2017). In an Australian study of patients attending healthcare services, the odds of a woman having all necessary cardiovascular risk factors recorded was 12% lower than men (Hyun *et al.*, 2017). In a study of 10,112 patients with ischaemic heart disease recruited across Europe, Asia and the Middle East, women were also less likely to achieve risk factor treatment targets as a consequence of less aggressive treatment trajectories for cardiovascular risk factors (Zhao *et al.*, 2017). Similarly, there continues to be significant barriers to the inclusion and representation of women in cardiovascular trials, thereby impeding the exploration of gender differences in treatment responses and deriving research outcomes potentially predominantly relevant to men (Scott *et al.*, 2018).

These differences in approach also occur in the acute management of patients. In acute heart failure, women receive less intensive diuresis following hospitalisation compared to men (Meyer *et al.*, 2013). In a UK study of over 500,000 patients with a diagnosis of acute MI, almost one in three patients received an initial diagnosis other than ST elevation MI (STEMI) or non-ST elevation MI (NSTEMI) at first medical contact, and this was more likely to occur in women (OR 0.63 for men compared to women) (Wu *et al.*, 2018). Incorrect initial diagnoses inevitably results in delays to the management of women, which can impact mortality (Melberg *et al.*, 2013; Bugiardini *et al.*, 2017). This was exemplified in an analysis of audio logs and medical records of 244 consecutive STEMI patients who contacted an emergency communication service in Norway, akin to contacting 999 ambulance services in the UK. Despite demonstrating similar clinical presentations, women were not prioritised by ambulance services, with only 78.7% being classified as most urgent

(i.e. red alert priority) compared to 89.4% in men. This ultimately resulted in delays in care and increased periods of myocardial ischaemia comparted to men (Melberg *et al.*, 2013). Consequently, gender may influence the equitable research, access and delivery of healthcare, which ultimately influences clinical outcomes.

2.3.2 Measurement of gender in cardiovascular research

Historically, the incorporation of gender measurement has been disparate as clinical research did not have the quantitative tools for assessing the impact of gender on health outcomes (Nielsen *et al.*, 2021). However, as this field has evolved, the inclusion of gender stratification in clinical research is viable and has demonstrated utility (Pelletier *et al.*, 2014).

In the 1970s, Dr Sandra Bem set forth a framework of gender theory whereby masculine and feminine traits were not considered opposing concepts but were distinct (Bem, 1974). Using a sample of 561 male and 356 female University students the Bem sex role inventory (BSRI) was devised. This quantifies the self-attribution of traits that are representative of gender roles. In the BSRI, masculine traits are categorised as taking the lead, being aggressive, competitive, dominant, self-reliant, and athletic, whereas compassion, affection, sympathy, warmth, and being yielding are considered feminine. This BSRI conceptualises androgyny, which combines masculine and feminine traits, and individuals who are undifferentiated that do not score highly for masculine or feminine traits. This remains the most commonly used measure of gender roles, and has been incorporated into other gender measures (Pelletier *et al.*, 2015), including the questionnaire used in Chapter 4 of this thesis. However, it does not assess additional gender dimensions but rather focuses broadly on personality traits.

In a cross-sectional study in Scotland utilising the BSRI, masculinity but not femininity was associated with smoking in men and women (Emslie *et al.*, 2002). Similarly, in a study conducted in Glasgow, Scotland, in 704 men and 847 women, lower femininity scores were associated with a higher risk of ischaemic heart

disease death in men but not women (Hunt *et al.*, 2007). Conversely, masculine scores were not associated with this outcome.

Gender diagnosticity was introduced by Lippa and Connelly in the 1990s (Lippa & Connelly, 1990). This approach was built upon the Bayesian probability that an individual is male or female based on gender-related indicators. This method assesses gender-related differences in behaviours that discriminate between gender within a given population. This was constructed based upon occupational preferences and past times. In a sample of 654 men and 210 women, masculine individuals from either male or female sex demonstrated higher mortality at any given age compared to their feminine counterparts (Lippa *et al.*, 2000).

More recently Pelletier *et al* developed a multidimensional composite gender score, the GENESIS-PRAXY Gender Index (GGI), from a range of psychosocial gender-related variables, which would permit discrimination between men and women (Pelletier *et al.*, 2015). Through the construction of a propensity score derived from coefficient estimates in the logistic regression model with biological sex as dependent variable and gender variables as covariates, the GGI was generated.

These gender-related characteristics included: 1) primary earner status; 2) personal income; 3) hours per week completing housework; 4) primary person responsible for doing housework; 5) stress at home; 6) masculinity traits; and 7) femininity traits (Pelletier *et al.*, 2016). These factors were used to produce a propensity score based on the probability of the individual being female. The GGI ranges from 0-100 with higher scores relating to characteristics traditionally ascribed to women and lower scores being ascribed to men. Importantly, this score demonstrated a spectrum of masculine and feminine score in both males and females (Figure 2-8).

In GENESIS-PRAXY higher scores, which denoted feminine characteristics (i.e. higher number of hours per week doing housework, primary responsibility doing housework, higher level of stress at home, BSR femininity score, lower personal

income, not being primary earner) increased the risk of multiple adverse outcomes including hypertension, diabetes and depression. Moreover, feminine individuals were at greater risk of recurrent ACS over 12 months, which was independent of sex (Pelletier *et al.*, 2016). When this methodology was applied to the Canadian Community Health Survey (CCHS) (n = 63,522) and Austrian Health Interview Survey (n = 15,771), individuals with traits ascribed to women demonstrated more adverse cardiovascular health and higher risk of cardiovascular disease, independently from biological risk factors (Azizi *et al.*, 2021). This gender stratification questionnaire is adapted and applied in a UK population for future use in cardiovascular research in Chapter 4 of this thesis. Moreover, by utilising samples obtained from the original GENESIS-PRAXY study, multiple circulating miRNA were found to be differentially expressed in ACS by sex and gender in Chapter 5 of this thesis.

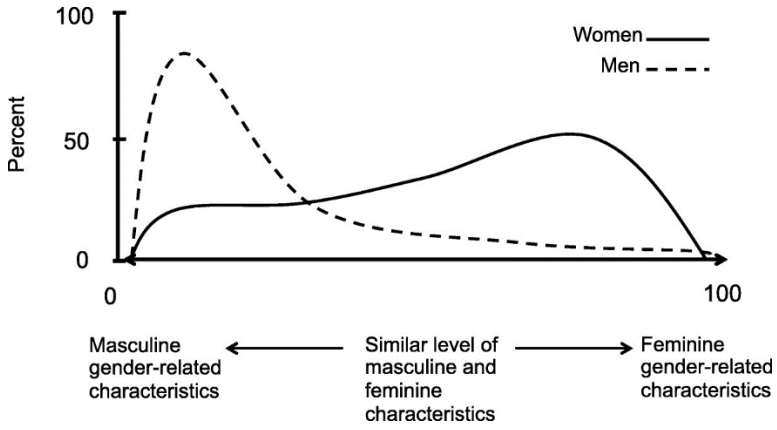

Figure 2-8. Gender Score Distribution in the GENESIS-PRAXY study.
Reproduced with permission (Pelletier *et al.*, 2015).

Recently, the Stanford Gender-Related Variables for Health Research assessment tool has been developed for clinical and population research (Nielsen *et al.*, 2021). Following a comprehensive review of English language measures of gender from 1975 to 2015, exploratory and confirmatory factor analyses identified 7 gender-related variables. These included discrimination, independence, work strain, caregiver strain, risk-taking, emotional intelligence, and social support. These were tested against self-rated health outcomes, including mental health.

Importantly, this study does not rate these factors as either ‘masculine’ or ‘feminine’ with the view that this does not provide sufficient or useful detail, which could be applied to behavioural interventions. Analysing each gender-related factor separately allows for that specific factor to be associated with poorer health outcomes, for instance work strain and the incidence of MI in men, to be targeted directly (Kilpi *et al.*, 2015).

This disaggregated means of measuring gender has also been explored in the HEalthy LIfe in an Urban Setting (HELIUS) study (Bolijn *et al.*, 2021). In this population-based analysis of 9,185 participants of six ethnic groups, three variables collected in the original HELIUS questionnaire including weekly hours spent on household chores, primary earner status and sex-predominant occupations were utilised as a surrogate for gender characteristics. Masculine traits, such as being the primary earner and performing male-dominated occupations, were associated with higher cardiovascular risk and this association was stronger in women. However, it is unclear whether the results of these studies truly reflect the effect of gender or alternatively social stratification. It is nevertheless difficult to disentangle these factors in the context of gender-related social inequalities.

It is clear a greater understanding of gender factors, as a source of differential health outcomes, is therefore urgently required and will offer opportunities to improve equitable healthcare access and delivery.

2.3.3 Biological sequalae of gender

If gender-related psychosocial traits facilitate the development of cardiovascular disease there must be a mechanism by which this is achieved. Two putative processes by which gender may mediate adverse pathophysiological consequences have recently emerged.

2.3.3.1 Psychosocial stressors & myocardial perfusion

The influence of psychosocial stressors has emerged as a potential deleterious gender mediator. Mental stress and depression are established as non-traditional risk factors that contribute to the development of ischaemic heart disease (Shah *et al.*, 2011; Rich-Edwards *et al.*, 2012). Women are twice as likely to develop depression compared to men and therefore the effects of this cardiovascular modifier may be more prevalent in this population (Kuehner, 2017). In the context of MI, increased baseline stress levels were associated with worse recovery in angina-related and overall quality of life (Xu *et al.*, 2015). Importantly, stress levels did not vary between men and women in this study, however, this trait was more common in women who subsequently experienced worse outcomes.

One mechanism that may be responsible for this is the myocardial response to mental stress. In young women who have suffered a MI there is a comparable rise in ischaemia induced by exercise and pharmacological-induced stress (Vaccarino *et al.*, 2018). However, these women experienced a two-fold rise in mental stress-induced myocardial ischaemia compared with men. In addition, microvascular dysfunction and peripheral vasoconstriction were also observed in women but not men, thereby demonstrating that feminine stressors may evoke ischaemia via autonomic microvascular disruption (Sullivan *et al.*, 2018). It is possible that high emotional stress can provoke sympathetic activation and inflammation thereby impeding cardiac perfusion.

The amygdala plays a key role in neural responses to stress and emotions (Lagraauw *et al.*, 2015). Elevated basal metabolic amygdala activity, which was determined by 18F-fluorodexoyglucose PET/CT, predicts major adverse cardiovascular events (Tawakol *et al.*, 2017). Importantly, this occurs independently of established cardiovascular risk factors. In concert with increased amygdala stimulation there is evidence of elevated haemopoietic activity and vascular inflammation. Moreover, elevated amygdalar function is associated with decreased left ventricular ejection fraction and fixed perfusion defects in women and not men (Fiechter *et al.*, 2019). These findings suggest that there may be a

neural–haemopoietic–arterial axis that modulates cardiovascular risk and function in women. Consequently, it is probable that psychosocial stressors modulated by gender may provoke a biologically sex-based predisposition to this vascular insult.

2.3.3.2 Gender-mediated epigenomic modification

Social determinants of health can be viewed as 'conditions in the environments in which people are born, live, learn, work, play, worship, and age' (Mancilla *et al.*, 2020). They are influenced by factors such as political, socioeconomic, and cultural constructs and may modulate health outcomes throughout life through a multitude of potential mechanisms. Gender may be considered a social determinant of cardiovascular health in this context (O'Neil *et al.*, 2018).

Epigenetic mechanisms & gender

Social epigenomics investigates the means by which social experiences promote epigenetic gene modification and regulation (Mancilla *et al.*, 2020). These social and environmental factors may alter the epigenome and regulate gene expression with potentially adverse downstream molecular consequences, which modulate the development of disease. Epigenetic modifications consist of three main categories: 1) DNA methylation; 2) post-translational histone modification; and 3) regulation of gene expression by non-coding ribonucleic acid (RNAs), such as miRNA (Costantino *et al.*, 2018).

DNA methylation is achieved through the addition of a methyl group to the fifth position carbon in cytosine-paired-with-guanine (CpG) dinucleotide sequences (Notterman & Mitchell, 2015). Promotor regions are enriched with these sequences, therefore CpG methylation typically results in the suppression of gene transcription via obstructing transcription factor binding to DNA or though the recognition of methylated sites by chromatin modifying enzymes. This methylation is enduring and is present in newly synthesised DNA.

A further means of epigenetic modification is the post-translation modification of histone proteins. Histones package DNA in nucleosomes and these proteins can be post-translationally modified via methylation, acetylation, ubiquitination and phosphorylation (Costantino *et al.*, 2018). Modifications to the N-terminal histone tail of nucleosomes regulate their chromatin state and mediate accessibility of DNA by transcriptive proteins (Rizzacasa *et al.*, 2019). Consequently, these post-translational modifications may promote transcriptional repression and activation by regulating DNA supercoiling (Mancilla *et al.*, 2020).

Lastly, regulatory non-coding RNA such as miRNA and long non-coding RNAs play a vital role in the regulation of gene expression. In Chapter 5 of this thesis a bioinformatic analysis of sex and gender stratified differentially expressed miRNAs in human plasma of individuals who have experienced ACS is undertaken. miRNAs are endogenous, single-stranded, short non-coding RNA sequences that range from 22 to 26 nucleotides and regulate gene expression at the post-transcriptional level. Since miRNA were first identified in *Caenorhabditis elegans* in 1993, thousands of miRNA have been identified both in humans and across a variety of organisms (Lee *et al.*, 1993; Hammond, 2015).

Canonical miRNAs are encoded in the genome as individual genes or as gene clusters, which contain a few to several hundred distinct miRNA (Treiber *et al.*, 2019). Initially, miRNAs are transcribed by RNA polymerase II as primary miRNAs (pri-miRNAs), which consist of over 200 nucleotides and where 20–25 nucleotides of the mature miRNA are embedded in the stem of a pri-miRNA hairpin structure (Figure 2-9). Pri-miRNAs are then processed to ~70 nucleotide single hairpin precursor miRNAs (pre-miRNAs) via the nuclear microprocessor complex, which comprises the RNase III enzyme, Drosha, and the double-stranded RNA-binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) (O'Brien *et al.*, 2018). Additionally microprocessor-independent miRNA may be generated from non-canonical biogenesis involving mirtrons and tailed mirtrons. These are generated via splicing and successive lariat debranching and function as pre-miRNAs that do not require microprocessor cleavage (Treiber *et al.*, 2019).

Pre-miRNA are then exported to the cytoplasm by the export receptor, exportin 5. Subsequently, the RNase III-type enzyme, Dicer, cleaves the pre-miRNA to generate a miRNA duplex intermediate consisting of a ~22 nucleotide miRNA (i.e. guide strand) and miRNA* (i.e. passenger strand). The miRNA guide strand of the duplex is incorporated into the RNA-induced silencing complex (RISC), while the miRNA* strand is released and degraded (Zhao *et al.*, 2019). The mature miRNA then guides the RISC-miRNA complex to complementary sequences predominantly in the 3'- untranslated regions of target mRNAs, although interactions with alternative regions do occur including 5'-untranslated regions and gene promoters (Broughton *et al.*, 2016; Zhou *et al.*, 2018). Nucleotides residing between position 2 and 7 (i.e. seed sequence) are responsible for this target-site recognition.

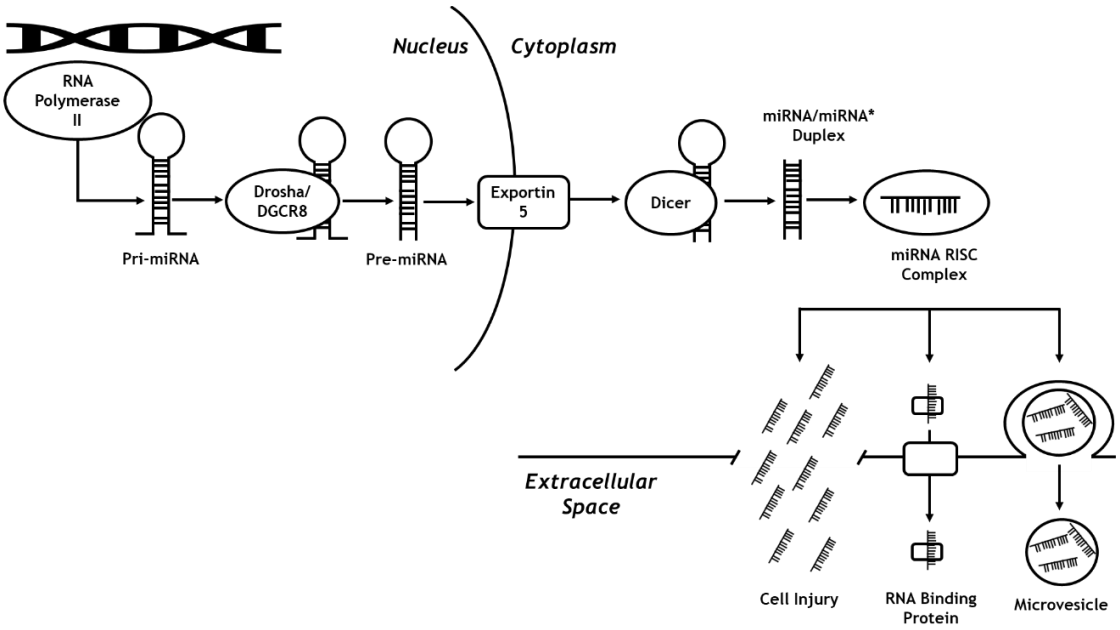

Figure 2-9. Canonical miRNA synthesis and extracellular release.

Subsequently, miRNA-mediated gene silencing is accomplished by base pairing of the 5´ region of miRNAs with the 3'-untranslated region of the target mRNA target sequence, resulting in translational repression and/or mRNA degradation, although gene upregulation has been also observed (Vasudevan, 2012). This permits miRNA-mediated gene regulation at the post-translational level, whereby

miRNA may target potentially hundreds of distinct mRNAs that are critical to a multitude of physiological processes.

Although miRNA function intracellularly, extracellular miRNA have been identified in circulating body fluids and tissue culture medium (Zhao *et al.*, 2019). miRNA may be released into the extracellular space via passive leakage in injured cells, active secretion in microvesicles or exosomes, or via RNA-binding proteins, such as high-density lipoprotein (HDL) or Argonaute 2 (Figure 2-9). These processes permit miRNA to travel to target cells, where they are internalised and modulate gene expression through aforementioned mechanisms. Circulating miRNAs demonstrate considerable stability and resistance to degradation by RNase activity, which highlight their potential utility for diagnostic and prognostic biomarkers, and putative therapeutic targets (Tsui *et al.*, 2002).

Epigenomic, Social Determinants & Cardiovascular Disease

Epigenomic modifications are of particular importance in the development of cardiovascular disease. For instance, during atherosclerosis extensive epigenetic modifications occur in endothelial cells, vascular smooth muscle cells, macrophages, inflammation and homocysteine homeostasis (Rizzacasa *et al.*, 2019). DNA methylation may also play a significant role in the regulation of blood pressure (Kato *et al.*, 2015). Histone modification, via triple methylation of lysine 27 in histone H3, has also been associated with progressive stages of atherosclerosis (Wierda *et al.*, 2015).

Alterations in the level of circulating miRNA have been identified in a number of cardiovascular conditions. In the plasma of patients following MI there were increased levels of miRNA, including miR-21-5p and miR-30d-5p, which correlated with left ventricular remodelling (Danielson *et al.*, 2018). Moreover, plasma miR-208a has been found to be elevated following MI and absent in healthy subjects (Wang *et al.*, 2010). Recently, differentially expressed circulating miR-21, miR-29a, and miR-126 have been found to be associated with a higher risk of premature death due to cardiovascular disease (Yamada *et al.*, 2021). This suggests that

serum levels of these circulating miRNA may be a novel risk factor for premature cardiovascular death and potentially be used as a biomarker to detect at risk individuals.

Importantly, these epigenetic modifications have also been associated with the presence of social and environmental deprivation. In a cohort of ~5,000 individuals, lower socioeconomic status was associated with accelerated epigenetic ageing (Fiorito *et al.*, 2017). Similarly, high neighbourhood socioeconomic deprivation, which is associated with age-related disease and mortality, is associated with DNA methylation-based epigenetic age acceleration (Lawrence *et al.*, 2020).

The association between deprivation and global DNA methylation content has also been linked to biomarkers of cardiovascular disease and inflammation (Mcguinness *et al.*, 2012). In the longitudinal Multi-Ethnic Study of Atherosclerosis study neighbourhood deprivation was associated with DNA methylation of genes related to stress and inflammation, which could contribute to the development of cardiovascular disease (Smith *et al.*, 2017). Recently, cardiac DNA methylation has also been implicated as a previously unrecognised biomarker of socioeconomic disparity in human heart failure outcomes. In this observational analysis a bimodal signature of cardiac DNA methylation in heart failure was observed and corresponded with racial and socioeconomic differences in all-cause mortality following mechanical circulatory support (Pepin *et al.*, 2021).

miRNA expression is also responsive to a variety of lifestyle exposures such as air pollution, smoking, physical activity and alcohol consumption, which in turn may be modulated by gender (Vrijens *et al.*, 2015; Panico *et al.*, 2021). In the recent coronary diet intervention with olive oil and cardiovascular prevention (CORDIOPREV) randomised control trial conducted in 805 individuals with ischaemic heart disease and evidence of endothelial dysfunction, the adoption of a Mediterranean diet improved endothelial function compared to low-fat diet. Several serum miRNAs, that are involved in gene regulation important to endothelial function, were also altered following the adoption of this diet

(Yubero-Serrano *et al.*, 2020). Consequently, environmental influences that may modulated by gender, such as nutritional exposure, may facilitate the modulation of miRNA levels that directly mediate gene processes responsible for vascular health and disease.

Importantly, miRNA regulation may also be sex dependent, which is driven by two predominant mechanisms. Firstly, EREs are common promotor elements and oestrogen binding may alter miRNA expression (Klinge, 2015). In a study of postmenopausal monozygotic twins discordant for hormonal replacement therapy usage, serum miR-21 and miR-146a levels, which are associated with inflammation, were lower in oestrogen users (Kangas *et al.*, 2014). Moreover, serum concentrations of miR-126-3p were higher at the ovulatory and luteal phases than in the follicular phase of the menstrual cycle in females (Li *et al.*, 2017). In endothelial cells, miR-126-3p supresses vascular cell adhesion molecule (VCAM1) and Spred1, a protein that inhibits mitogenic signalling. As a consequence endothelial proliferation, migration, and monocyte adhesion are promoted, which may contribute to the protective effects afforded by oestrogen against atherosclerosis. Many miRNA have now been identified that are responsive to oestrogen in terms of expression and influence vascular health (Pérez-Cremades *et al.*, 2018).

Additionally, the X chromosome encodes at least 118 miRNAs and ~15% of these are capable of escaping X-inactivation, which may result in elevated miRNA expression (Song *et al.*, 2009; Florijn *et al.*, 2018). Conversely, the Y chromosome contains only 4 annotated miRNAs, with 2 being identified within the pseudoautosomal PAR1 region, which can undergo X chromosome recombination (Kozomara *et al.*, 2019; Di Palo *et al.*, 2020).

Several X-chromosome encoded miRNA are dysregulated in cardiovascular disease. In a porcine model of MI, inhibition of the X chromosome miR-92 reduced infarct size and postischemic loss of function (Hinkel *et al.*, 2013). In humans, circulating X chromosome encoded miR-221 demonstrated a female-specific increase in individuals with metabolic syndrome, which acts as a precursor to cardiovascular

disease (Wang *et al.*, 2013). Consequently, it is possible that incomplete X chromosome inactivation may result in the augmented expression of miRNA deleterious towards the vasculature that are counteracted by the protective effect of oestrogen and oestrogen-sensitive miRNA (Florijn *et al.*, 2018). It is possible that perturbation of this balance, such as following the menopause, may enhance cardiovascular risk in females who experience oestrogen deprivation.

MiRNA, as mediators of epigenetic regulation, may become dysregulated in response to environmental exposures and are inherently modulated by sex. Importantly miRNAs are critical regulators of cardiovascular function and disease. Consequently, miRNA are prime candidates in the search of gender-mediated epigenetic modifiers and the pathophysiological sequalae to psychosocial gender. This hypothesis is consequently explored in Chapter 5 of this thesis.

2.4 Cardiovascular health in transgender people

Thus far, this thesis chapter has reviewed the relationship between sex and gender mechanisms, and cardiovascular risk in cisgender populations. This chapter section will now provide context for the association between being transgender and the development of cardiovascular disease. This section will begin by providing a background in terms of gender-affirming healthcare, the epidemiology of people who are transgender, and the evidence suggesting an increased risk of cardiovascular disease in transgender people. Consequently, this section aims to offer context to Chapter 3 and Chapter 6 of this thesis, where the influence of GAHT upon blood pressure and vascular function in transgender people are investigated, respectively.

2.4.1 Background

Transgender is an umbrella term to describe individuals whose gender identity differs from the assigned sex at birth (Coleman *et al.*, 2022). Transgender people identify with a gender that is not congruent with the sex they were assigned at birth. Transgender people may experience serious distress and dysphoria relating

to this gender incongruence that may adversely affect an individuals physical and mental wellbeing.

In many transgender individuals the impact of sex related factors, such as the utilisation of gender-affirming hormone (GAHT) therapy, and the interaction this elicits with natal chromosomal complement, may fundamentally alter individual's cardiovascular risk (Connelly *et al.*, 2019). Moreover, the psychosocial impact of gender and societal factors, including minority stressors, in these individuals in the context of this altered physiological state may further enhance this risk (Streed *et al.*, 2021b). The aforementioned sex and gender related mechanisms outlined in this thesis may modulate cardiovascular disease development in this population. Throughout the remaining sections of this chapter, the history of transgender healthcare, epidemiology, means of gender-affirmation and cardiovascular risk will be discussed.

2.4.2 History of transgender healthcare & terminology

Throughout recorded history individuals have existed who exhibit gender identities that were not congruent with their natal sex or did not conform to existing societal norms. For instance, the Roman emperor Elagabalus (204–222 AD) reportedly embraced feminine pronouns and gender expression, including feminine clothing, wigs and cosmetics. Moreover, Elagabalus offered a substantial reward to any physician that could provide them female genitalia, and therefore sought gender affirmation via surgical means (Bhinder & Upadhyaya, 2021). Additionally, the life of the spy, diplomat and soldier, Chevalier d'Éon de Beaumont, is well documented and many scholars consider her to be transgender (Rogister, 2018). In the 18th century d'Éon lived as a woman and attended the Russian court as such.

In the modern era, the first gender-affirming surgeries were undertaken in the 1920s at the Hirschfeld's Institut für Sexualwissenschaft in Berlin (Slagstad, 2021). Magnus Hirschfeld was a noted physician and sexologist who coined the term 'transvestite' in 1910, which is derived from Latin 'trans' (i.e. across) and 'vestis'

(i.e. clothing) (Beemyn, 2013). Hirschfeld identified that these individuals were not fetishists or suffering from psychopathy, and recognised that their sexuality was distinct from their gender identity. Hirschfeld collaborated closely with Eugen Steinach, an Austrian Endocrinologist, who is broadly consider the founder of the neuroendocrinology of sexual behaviour and played a significant role in identifying the impact of sex hormones on physical characteristics (Södersten *et al.*, 2014).

The first recorded transgender person to undergo gender-affirming surgery was Dora Richter. Under the care of Magnus Hirschfeld in 1922, she underwent an orchidectomy followed later by vaginoplasty. At this point in time, these surgeries were highly experimental and had a high risk of poor outcomes (Kiyar *et al.*, 2020). In May 1933, due to the emerging influence of the Nazi party in Germany, Hirschfeld's institute was attacked and Richter is not known to have survived. Hirschfeld had by this point been exiled to France, where he continued his research, writing and campaigning until his death in 1935 following a MI.

In 1939, Dr Michael Dillon, a British Physician, was the first documented person to be treated with oral testosterone for the purpose of gender-affirmation (Frey *et al.*, 2017). Diethylstilbestrol, a synthetic form of oestrogen, was also being utilised as GAHT in transgender women within this decade (Martinez *et al.*, 2020). In 1946, Dillon would be the first transgender person to undergo phalloplasty, which was overseen by Sir Harold Gillies, an eminent plastic surgeon who specialised in pioneering gender-affirming surgery.

The term transsexual was first coined by David Cauldwell, a US sexologist, in 1949 in his essay 'Psychopathia Transexualis', where he differentiated biological from psychological sex and determined the latter to be a consequence of social condition (Cauldwell, 2013). This remained a diagnostic term in the International Classification of Diseases and Related Health Problems (ICD-10) (World Health Organization, 2004), however, was removed in future revisions (World Health Organization, 2019).

The first high profile gender-affirming surgery to take place was undertaken in Denmark in 1952 on Christine Jorgensen, a transgender women who was a US citizen and World Ward II veteran (Frey *et al.*, 2017). This procedure was captured in international headlines and introduced the concept of being transgender and gender-affirmation to a global audience.

By 1966, Harry Benjamin, a German-American Endocrinologist, published 'The Transsexual Phenomenon', which articulated the concept of gender identity and argued against attempts to repress transgender identity via psychotherapy (Benjamin, 1967). In 1979, the Harry Benjamin International Gender Dysphoria Association was founded, offering standards of care in the clinical management of people who were transgender. This would later be renamed the World Professional Association for Transgender Health (WPATH), which continues to produce guidance on standard of medical and surgical care of transgender individuals (Frey *et al.*, 2017).

Finally, the term 'transgender' came to prominence in the 1990s coinciding with an increased frequency of studies of people who were transgender (Joseph *et al.*, 2017). This term arose in an effort of collective advocacy for freedom of gender expression, whereby it relies on self-identification rather than medical classification and continues to be the preferred terminology when describing this diverse population (Reicherzer, 2008).

2.4.3 Epidemiology

The overall size of the transgender population is not known due to a number of factors limiting the obtainment of accurate prevalence estimates. Transgender people are a heterogenous group where some will actively seek out gender-affirming therapy, while others make social transition only (Sineath *et al.*, 2016). A proportion of transgender individuals will live with gender incongruence as a personal choice or will fear external expression of this will result in adverse social, emotional and economic consequences (Winter *et al.*, 2016). Moreover, some individuals live in regions where stigma acts as a barrier to healthcare access or

where access to healthcare is not possible due the absence of gender identity services (Meerwijk & Sevelius, 2017). Furthermore, hormonal therapy may be sought from non-medical sources, such as the internet, rather than access healthcare directly. Lastly, most countries do not routinely collect census data relating to individuals identifying as transgender. As a consequence of these sampling biases, prevalence assessments may be difficult to interpret and may underestimate the true size of this population (Mueller *et al.*, 2017).

Arcelus *et al* have published a meta-analysis of reported prevalence rates in an attempt to overcome some of the biases inherent in previous epidemiological research (Arcelus *et al.*, 2015). They have estimated that the meta-analytical prevalence of transgender people to be 4.6 per 100,000, with transgender women and men demonstrating rates of 6.8 and 2.6 per 100,000, respectively. Importantly, a temporal analysis has demonstrated an increase in the reported prevalence of being transgender over the previous 50 years. However, this assessment is limited by the presumption that all transgender people engage with medical therapy, which is not the case, and therefore these data may represent increases in healthcare engagement rather than a true expansion in this population.

To ascertain prevalence estimates of the transgender people, population-based studies may also be utilised and provide a high degree of reliability. In a sample of community participants in Massachusetts, USA, the prevalence rate of identifying as transgender was found to be 0.5% (Conron *et al.*, 2012). This is consistent with data from the US 2014-2016 Behavioural Risk Factor Surveillance System (BRFSS), which suggests 0.48% of the US population identify as transgender. This amounts to 1.5 million US citizens, of which 770,000 identify as transgender women, 458,000 identify as transgender men and 332,000 as gender non-conforming (Downing & Przedworski, 2018).

A recent meta-regression analysis of population based surveys in the US estimated the transgender population to be 390 per 100,000 (Meerwijk & Sevelius, 2017). Similar estimates have been obtained in Flanders, Belgium where 0.7% of men and

0.6% of women identify as transgender (Van Caenegem *et al.*, 2015), and in the Netherlands, where this was reported as 1.1% of natal males and 0.8% of natal females (Kuyper & Wijsen, 2014).

The disparity between ascertainment of transgender status in those who have received treatment versus those who self-identify is best exemplified in a meta-analysis (Collin *et al.*, 2017). In this analysis, the meta-prevalence estimates were 9.2 per 100,000 for people who have undergone surgical or hormonal gender-affirmation therapy. This increased to 355 per 100,000 in studies of self-reported transgender identity. It is therefore evident that a significant disparity exists between the number of individuals who utilise gender-affirming therapy and those who identify as transgender, who have not or cannot engage with these services. When broader definitions of gender diversity are utilised in survey based research, higher prevalence ranging between 0.5-4.5% of the adult population have been observed (Zhang *et al.*, 2020b).

It is important to note that these prevalence estimates are not globally uniform. In China, for instance, there are estimated 400,000 transgender people, which accounts for ~0.03% of this population (Xie *et al.*, 2021). However, due to significant discrimination and lack of family, social and governmental support, this prevalence may be underestimated as individuals may not feel able to self-identify.

In Iran, since 1987 a fatwa, decreed by Ayatollah Khomeini, has permitted the Department of Forensic Psychiatry, Tehran, to facilitate gender-affirming therapy (Mamoojee *et al.*, 2017). Following surgical therapy, an individual may be issued a new state identity card with that gender evident and procure hormonal therapy, which is state-subsidised. However, despite this governmental support, ‘public transvestitism’, same-sex relationships and gender fluidity are not permitted, thereby limiting the means by individuals can engage with their transgender identity, unless they are willing to undergo surgery. These issues are not limited to these regions and highlight global issues with acceptance and support with gender diverse individuals (Reisner *et al.*, 2016).

In terms of future healthcare delivery, it is apparent that the demand for gender identity services is increasing (Zucker, 2017). In Sweden, the incidence of applications for gender reassignment surgery rose significantly between the 1970s and 2000s (Dhejne *et al.*, 2014). The increasing prevalence of the transgender population has also been observed in recently meta-analyses (Arcelus *et al.*, 2015; Meerwijk & Sevelius, 2017). With growth of the transgender population and increasing demand for transgender health services, it is expected that the number of people receiving GAHT therapy will continue to increase, which may result in issues relating to equitable healthcare access and delivery.

2.4.4 Gender-affirming healthcare

The management of individuals who are transgender requires and multidisciplinary approach. This may include specialists such as psychiatrists, endocrinologists, sexual health specialists, urologists, gynaecologists, dermatologists, surgeons, and voice and communication therapists. Interventions are required to be person-specific as the goals of gender-affirming treatment should focus on the individual needs and expectations of a patient rather than prescriptive and dogmatic management guidelines (Coleman *et al.*, 2022). Although all these facets of transgender health are important, this section will focus on the use of GAHT and surgical therapies, as these factors are likely to be particularly important for physiological alterations that may modulate the development of cardiovascular disease.

The treatment goals of gender-affirming healthcare are to decrease endogenous sex hormone concentrations, thereby diminishing natal secondary sex characteristics. Concurrently, exogenous sex hormones facilitate the attainment of sex hormone levels consistent with the individual's gender identity (Hembree *et al.*, 2017). Two predominant means of gender-affirming interventions are offered to achieve this: medical and surgical therapies. Non-binary people, who may account for as much as 35% of the transgender population (James *et al.*, 2016), may require a different combinations and degrees of masculinisation, feminisation, demasculinisation and defeminisation that should be personalised to

that individual's needs and treatment goals (Cocchetti *et al.*, 2020). Evidence is sparse for this approach and therefore this thesis section will focus on predominantly on masculinising and feminising gender-affirmation.

2.4.4.1 Feminising gender-affirming hormone therapy

Transgender women utilise feminising hormone therapy to align their physical appearance to their gender-identity (T'Sjoen *et al.*, 2019). These changes include reduced facial and body hair growth, breast growth and the alteration of adipose tissue and muscle mass distribution.

In a multi-centre study of 229 transgender women utilising gender-affirming therapy, after one year, breast development was modest and typically occurred within the first six months of treatment (De Blok *et al.*, 2018). This was accompanied by substantive decrease in testicular volume (Fisher *et al.*, 2016). In a retrospective longitudinal study of 150 transgender women there was a significant increase in weight and body mass index (Quirós *et al.*, 2015).

Overall, data from the Kaiser care consortium has demonstrated that 58% of transgender women received hormonal therapy. However, this may be an underrepresentation as individuals may be utilising hormonal therapy from sources not supported by their insurance (Quinn *et al.*, 2017). Two predominant forms of feminising therapy are utilised by transgender women: oestrogen and androgen lowering therapies.

Oestrogen therapies

Previously, transgender women have been treated with either ethinyl estradiol, synthetic oestrogen, or conjugated equine oestrogens (Table 2-1). The latter mode of oestrogen delivery is extracted from pregnant mares' urine and contains a total of 10 different oestrogens including estrone, 17β estradiol, and a group of unique ring B unsaturated oestrogens (e.g. equilin and equilenin) that act predominantly via ERβ and not ERα (Bhavnani & Stanczyk, 2014). However, these

oestrogens are no longer recommended due to immunoassay interference in oestrogen measurement and their associated risk of thrombosis and potentially cardiovascular disease (Seal *et al.*, 2012; Nolan & Cheung, 2020).

Modern gender-affirming healthcare typically utilises bioidentical 17β-estradiol as the oestrogen of choice for feminising hormonal therapy in transgender women. The bioavailability of these agents has been improved through the formulation of ester prodrugs (oestrogen valerate or oestrogen cypionate), which are subsequently hydrolysed to 17β-estradiol (Cirrincione *et al.*, 2020). Estradiol hemihydrate is bioequivalent to estradiol and both this oestrogen formulation and estradiol valerate are dose equivalent (Banker *et al.*, 2021). Non-prodrug formulations of 17β-estradiol can also be utilised such as micronized estradiol.

Route	Formulation	Dose
Oral estradiol	Estradiol hemihydrate (Elleste Solo) or valerate (Progynova)	2-6 mg/day
Oral conjugated oestrogens	Sulfate esters of estrone, equilin sulfates, 17α-estradiol and 17β-estradiol (Premarin)	2.5 – 7.5 mg/day
Transdermal patches	Estradiol hemihydrate (e.g. estraderm, estradot patches)	0.025-0.2 mg/day (change patch every 3-7 days)
Intramuscular oestrogen	Estradiol valerate or cypionate	2-20 mg /1-2 weeks
Transdermal gel	Estradiol hemihydrate (e.g. Sandrena)	2 squirts (1.5mg) daily

Table 2-1. Feminising oestrogen therapies.

Oral oestrogen undergoes extensive first-pass metabolism where it is converted to estrone and estrone sulfate, which demonstrate less potent bioactivity. Following oral administration, oestrogen levels peak after a few hours and demonstrates sustained elevation for up to 12 hours (Kuhl, 2005). Transdermal preparations escape first-pass metabolism and typically reach maximum concentrations after several hours. Transdermal oestrogen patches are typically applied once or twice per week (Maheshwari *et al.*, 2021). With respect to the route of oestrogen delivery, data have emerged in postmenopausal cisgender women demonstrating that transdermal oestrogen is less thrombogenic than oral oestrogen therapy (Mohammed *et al.*, 2015; Bergendal *et al.*, 2016). These results have been extrapolated to transgender women, and it is recommended to utilise transdermal oestrogen therapy when individuals reach 40 years of age.

The median estradiol concentrations in healthy natal males in 150 pmol/L, which falls to 90 pmol/L in older men. Comparatively, premenopausal females exhibit estradiol levels of 400 pmol/L (Russell & Grossmann, 2019). To achieve these female concentrations, estradiol orally may be administered in doses of 2 to 6 mg per day typically as estradiol valerate or hemihydrate. Estradiol patches may be applied at doses of 0.025–0.2 mg per day, with patches changed every 3 to 5 days (Hembree *et al.*, 2017).

Androgen lowering therapies

The majority of transgender women require adjuvant therapy to facilitate the suppression of testosterone prior to gender-affirming orchidectomy as oestrogen monotherapy may not be sufficient to supress endogenous testosterone concentrations to female ranges (Leinung *et al.*, 2018). Following the commencement of oestrogen monotherapy, testosterone concentrations are expected to decrease towards the male hypogonadal range (6·9–10·4 nmol/L), however, this typically remains elevated when compared to the female concentrations (<2·6 nmol/L) (Tangpricha & den Heijer, 2017). Several classes of therapies may be utilised including cyproterone acetate, GnRH analogues and spironolactone (Table 2-2).

The most commonly utilised anti-androgen is cyproterone acetate. Cyproterone acetate is a steroidal anti-androgen that acts as a potent AR competitive antagonist (Sonneveld *et al.*, 2005). Furthermore, cyproterone acetate activates the progesterone receptor, which acts to supress GnRH and gonadotropins via central mechanisms to decrease testosterone production. This drug is not available in the US as it had never been licenced (Mamoojee *et al.*, 2017). Cyproterone acetate, in combination with oestrogen, is efficacious in lowering endogenous total testosterone concentrations in transgender women, when compared to oestrogen monotherapy (Angus *et al.*, 2019). Importantly, this anti-androgen has been associated with an increased risk of meningioma, depression and hyperprolactinaemia, and rarely fulminant hepatotoxicity (Bessone *et al.*, 2016).

Anti-androgen	Androgen Receptor Antagonist	Progesterone Receptor Agonist	Oestrogen Receptor Agonist	HPG Axis Suppression
Cyproterone acetate 25-50mg/day	X	X	-	X
GnRH analogues				
Leuprolide/ Triptorelin 11.25 mg IM / 12 weeks	-	-	-	X
Goserelin 10.8 mg SC/12 weeks				
Spironolactone 100-300/day	X	X	X	-

Table 2-2. Mechanisms of prominently utilised anti-androgen action.

Anti-androgen drug pharmacodynamic mechanisms are denoted by 'X'. HPG: Hypothalamic-pituitary-gonadal; IM: intramuscular; SC: subcutaneous. Reproduced with permission (Angus *et al.*, 2021).

As a consequence of these potential side effects, gender healthcare providers in the UK have adopted long-acting GnRH analogues as first-line therapy. The GnRH

agonists leuprorelin, triptorelin and goserelin are typically utilised for this purpose, and act to supress pituitary gonadotrophin secretion in order to reduce endogenous gonadal androgens. GnRH secretion is pulsatile and its activation of anterior pituitary gonadotrope GnRH receptors stimulate the synthesis and secretion of gonadotropins. GnRH agonists stimulate an initial increase in gonadotrophin secretion, which is known as the 'flare effect' (Kumar & Sharma, 2014). However, the chronic (i.e. tonic) activity of GnRH agonists promotes the downregulation of its receptor, thereby ameliorating gonadotropin secretion. GnRH analogues overcome the short 2–4 minutes half-life of the decapeptide GnRH through the substitution of Glycine residue in position 6, which is responsible for its degradation, with a D-Amino acid (Maggi *et al.*, 2016). In addition, the affinity of these molecules may be increased through the deletion of the position 10 glycine amide and the addition of an ethylamine residue to the position 9 proline, which is present in triptorelin.

Lastly, spironolactone is also used to ameliorate androgen levels and function though a multitude of actions. In addition to the classical antagonism of the mineralocorticoid receptor, spironolactone also acts a partial antagonist of the AR and partial agonist at the ER (Fagart *et al.*, 2010). Spironolactone, also acts as a weak inhibitor of 17 α-hydroxylase and 17,20-lysase, which are integral to the testosterone synthetic pathway. Finally, spironolactone demonstrates weak progestogenic activity, that ameliorates GnRH and gonadotrophin secretion, thereby lowering gonadal testosterone. Doses of 100-400 mg of this agent are typically required, which is much higher than is used in cardiac or hepatic failure. Compared with cyproterone acetate, spironolactone is associated with a greater requirement for surgical breast augmentation and is inferior with respect to reducing testosterone concentrations in transgender women (Seal *et al.*, 2012; Angus *et al.*, 2019).

Ultimately, due to the lack of prospective longitudinal studies, there is no strong evidence supporting the use of a particular anti-androgen therapy in order to improve clinical outcomes (Angus *et al.*, 2021). Studies broadly concentrate on

testosterone lowering ability, however, this may be an imperfect proxy for feminisation as many of these drugs act to directly inhibit the AR.

Adjunct therapies

5α-reductase inhibitors, such as finasteride or dutasteride, which inhibit the conversion of testosterone to DHT, may also be used. Other potential drugs include the nonsteroid AR antagonists, such as bicalutamide (Glintborg *et al.*, 2021). However, these are not recommended as they do not lower serum testosterone levels and there is limited data supporting their use.

Similarly, progesterone therapies, such as medroxyprogesterone, have been utilised in transgender women previously (Wierckx *et al.*, 2014a). Typically, these are considered a means to promote breast development. However, no study to date has demonstrated clinical efficacy of these drugs and they are not recommended. Moreover, there are data suggesting a potential increased risk of venous thromboembolism and stroke in cisgender women receiving progestogens, which would be a concern in this cohort (Manson *et al.*, 2003; Barsoum *et al.*, 2010).

2.4.4.2 Masculinising gender-affirming hormone therapy

Testosterone therapy is used to masculinise transgender men via facial and body hair growth, deepening of voice, cessation of menses, and increased musculature. Recent US health outcome data suggest as many as 52% of transgender men utilised hormone therapy (Quinn *et al.*, 2017). The aim of this therapy is to obtain these natal male secondary sex characteristics to allow these individuals to live their lives as men, reduce gender dysphoria and improve quality of life (Irwig, 2017). Long-term testosterone administration in transgender men promotes reduction in breast glandular tissue and substantive increases in fibrous connective tissue (Slagter *et al.*, 2006). In a longitudinal study of 11–21 months of masculinising hormone therapy in 223 transgender men, weight gain greater than 5 kg was observed in 30% of participants (Kyinn *et al.*, 2021).

Broadly, the treatments provided are akin to those of cisgender hypogonadal men, where the mainstay of treatment are injectable testosterone preparations (Table 2-3) (Unger, 2016). Testosterone has an approximate half life of 10 minutes following injection, however, this bioavailability is improved by 17β esterification. Esterification promotes testosterone oil solubility and renders this hormone inert, which permits the gradual absorption of testosterone following the cleavage of this ester.

Esterification of the 17β position of testosterone with undecanoic produces testosterone undecanoate. This hydrophobic side chain consists of the aliphatic fatty acid undecanoic acid, which contains 11 carbon atoms compared to testosterone enanthate that contains of 7 carbon atoms (Edelstein & Basaria, 2010). This alteration provides a more favourable pharmacological profile with a half-life of 34 days as a consequence of its solubility in a castor oil vehicle that decreases the rate of absorption (Corona *et al.*, 2014). This androgen prodrug is then steadily released from depot where it then enters the circulation and is cleaved by esterases to produce undecanoic acid and bioactive testosterone.

Formulation	Route	Dose
Testosterone enanthate or esters (Sustanon)	Intramuscular	125-250 mg / 3–6 weeks
Testosterone undecanoate (Nebido)		1000 mg /10-14 weeks
Testosterone gel (e.g. Testogel, Testim, Tostran)	Transdermal	50 -100mg / day
Testosterone patch (e.g. Testoderm)	Transdermal	2.5–7.5 mg / day
Testosterone undecanoate	Oral	40–120 mg /day
Crystalline testosterone pellets (Testopel)	Subcutaneous implant	2.5–7.5 mg/day (75 mg/pellet)

Table 2-3. Masculinising testosterone gender-affirming therapies.

Alternatives include intramuscular (IM) testosterone esters (i.e. Sustanon) and testosterone enanthate. Sustanon contains four testosterone esters with different durations of action where 1ml of this formulation contains testosterone decanoate (100 mg), isocaproate (60 mg), phenylpropionate (60 mg) and propionate (30 mg) (Edelstein & Basaria, 2010). These formulations exhibit significantly shorter half-lives than testosterone undecanoate and require injections every 3-6 weeks. IM testosterone may however be inconvenient and provide fluctuating testosterone levels, with supraphysiologic testosterone concentrations initially, which wane to the male hypogonadal range towards the end of the dosing interval (Shoskes *et al.*, 2016).

Transdermal delivery of testosterone represents an effective alternative to injectable androgens. Transdermal application of testosterone permits direct absorption into the systemic circulation. Testosterone may be applied as a hydroalcoholic gel, where typically <5% of testosterone applied is absorbed and forms secondary reservoir in the stratum corneum, which acts as a rate-controlling membrane of testosterone delivery (Basaria & Lakshman, 2009). One concern of testosterone gel is secondary transfer of testosterone to other individuals, including children, through touch that can promote virilisation (De Ronde, 2009). This may be circumvented by providing a physical barrier through the application of a testosterone transdermal patch.

Following oral administration testosterone undergoes extensive first-pass hepatic metabolism and therefore it is not a suitable means of administration (Barbonetti *et al.*, 2020). However, oral testosterone undecanoate is absorbed preferentially into the lymphatic system and subsequently hydrolysed to yield testosterone. The efficacy of this formulation is limited by its short half-life and erratic bioavailability, which results in fluctuating testosterone concentrations. Less commonly utilised formulations include testosterone nasal gel (i.e. Natesto), which is approved in several European countries and the US (Barbonetti *et al.*, 2020). Nasal mucosal absorption bypasses first-pass metabolism. However, this use of this formulation in transgender men remains uncertain.

Lastly, crystalline testosterone pellets containing 75mg of testosterone may be implanted subdermally. Testosterone pellets are formed via high-temperature moulding and provide a prolonged release of testosterone through the uniform erosion of the pellet surface (McCullough, 2014; Barbonetti *et al.*, 2020). Two pellets provide 25 mg of parenteral testosterone needed weekly and up to six pellets may be implanted with each pass of the insertion device (Unger, 2016). The pellets last for up to six months, however, most patients require a further insertion of pellets every 3 to 4 months. Testosterone pellets provide consistent testosterone concentrations for several months and may have pharmacokinetic advantages over IM testosterone preparations, however, their use is limited by the need for implantation (McMahon *et al.*, 2017).

2.4.4.3 Gender-affirming surgery

Some transgender individuals will undergo gender-affirming surgeries, which includes both genital and non-genital surgery. The aim of these surgeries is to align an individual's physical characteristics to their gender identity. Although overall outcomes may vary between individuals, these procedures have low rates of mortality associated with them (Neto *et al.*, 2012). Surgeries are typically offered for individuals who have received at least 12 months of hormonal therapy, however, international variation in practice exist (Mamoojee *et al.*, 2017).

Transgender women frequently receive mammary prosthesis as a means of breast reconstruction due to the limited capacity of oestrogen to promote breast growth (De Blok *et al.*, 2018). Similarly, facial feminisation surgery, such as forehead and hairline recontouring, rhinoplasty, eyebrow lift, vermilion reconstruction, jaw reduction and thyroid cartilage reduction, may also be undertaken (Wylie *et al.*, 2016). These procedures will be dependent on the baseline masculinisation of an individual and their overall treatment goals and expectations.

Transgender women may also undergo genital surgery with the aim of creating a perineogenital structure that is feminine in appearance and function. This may be obtained via a variety of surgical procedures including orchidectomy, penectomy,

meatus reconstruction, vaginoplasty, labiaplasty, and clitoroplasty (Wylie *et al.*, 2016). The latter is typically achieved via the construction of a clitoris from the dorsal neurovascular pedicle of the penile glans. Vaginoplasty is achieved via penile-scrotal skin flap inversion, however, other techniques such as sigmoid colon vaginoplasty may be used if primary attempts have failed. Regardless of the technique utilised, postoperative vaginal dilatation is required to avoid neo-vaginal stenosis.

Transgender men may undergo a series of surgeries. A mastectomy may be performed to promote a more masculine body image (Wylie *et al.*, 2016). As a consequence of the masculinising effects of testosterone therapy, facial surgery is rarely required.

Genital surgeries consist of hysterectomy, oophorectomy, vaginectomy, phallic construction via phalloplasty or metoidioplasty, and scrotoplasty. Despite genital constructive surgery being performed for over 50 years and in multiple countries, no gold standard procedure exists and techniques may vary. The preferred procedure of phalloplasty utilises a free vascularised forearm flap. A phallus is formed from cutaneous tissue while the radial vessels, and medial and lateral antebrachial cutaneous nerves, are anastomosed to corresponding pelvic structures. This procedure requires an erection prothesis, has the potential for urological complications and results in forearm scarring, which may not be the preferred outcome in some transgender men. An alternative technique is the free sensate osteocutaneous fibula flap that provides coital ability without the need for additional erection prosthesis. In individuals not seeking erective capacity, a microphallus can be formed in individuals with clitoromegaly as a consequence of testosterone therapy via metoidioplasty.

2.4.4.4 Gender-affirming therapy outcomes

There is a substantial psychiatric co-morbidity in this population with a study in Ontario, Canada observing that 35.1% of its transgender cohort considered suicide in the past year, with 11.2% having attempted this (Bauer *et al.*, 2015).

Unfortunately, due to the inability to undertake randomised placebo controlled trials in this population there are significant limitations in our understanding of the efficacy of gender-affirming therapies to improve gender dysphoria and mental health outcomes in transgender populations. Existing data are typically cross-sectional or do not have sufficient power to draw strong conclusions (Johansson *et al.*, 2010; Heylens *et al.*, 2014; Ruppin & Pfäfflin, 2015). It has been demonstrated that transgender people report lower health-related quality of life scores than the general population (Nobili *et al.*, 2018).

However, larger studies with prolonged periods of follow up are now providing a better insight into the efficacy of this intervention. In a study of 359 individuals with two years follow up, gender dysphoria and mental health outcomes improved in those receiving GAHT therapy (Fisher *et al.*, 2016), suggesting a degree of efficacy of this therapy.

In a sample of 324 individuals who had undergone gender-affirming surgery in Sweden between 1973 and 2003, rates of mortality and psychiatric morbidity were higher compared to matched cisgender controls (Dhejne *et al.*, 2011). Therefore, improvements in gender dysphoria may not be sufficient to prevent higher rates of morbidity and mortality transgender populations receiving surgery.

A recent data linkage study examined the influence of gender-affirming surgery on mental health by utilising the Swedish Total Population Register (n=9,747,324), which was linked to the National Patient Register and the Prescribed Drug Register (Bränström & Pachankis, 2020). In this cohort, mental health outcomes of transgender individuals (n=2,679) were compared to the general population. Transgender people were six times more likely to have a mood and anxiety related health care appointment, and more than three times likely to have received antidepressant or anxiolytic medications. Importantly, they were more than six times at risk of hospitalisation following a suicide attempt.

Duration of exposure to GAHT was not associated with improvements in the likelihood of mental health treatment. However, duration following surgical

management resulted in reduced mental health treatment (adjusted OR 0.92; 95% CI 0.87, 0.98). This study has a number of limitations including that completed suicide attempts could not be assessed and that mental health utilisation is not a perfect proxy for mental health. However, it does provide some evidence to support improving access to gender-affirming treatments and highlights the burden of mental health in this population.

A recent systematic review assessing the impact of GAHT, including 20 studies and 15 prospective cohorts, suggests that it may be associated with improvements in quality of life scores and decreases in depression and anxiety symptoms (Baker *et al.*, 2021). However, due to significant methodological limitations in this systematic review the strength of evidence was low. Moreover, conclusions regarding the impact of GAHT upon suicide could not be ascertained. Importantly, no study demonstrated any harms for these outcomes. These data are in line with a meta-analysis published in 2010, which demonstrated that in the 28 eligible studies identified, comprising of 1,833 individuals with gender dysphoria who underwent GAHT, there were improvements in gender dysphoria, psychological function and quality of life (Murad *et al.*, 2010). Similarly, a meta-analysis published in 2018 by Nobili *et* al suggested that quality of life did improve following the commencement of GAHT. However, again the evidence assessed was of very low quality and had a moderate to high risk of bias (Nobili *et al.*, 2018).

2.4.5 Cardiovascular disease in transgender people

2.4.5.1 Ischaemic heart disease

The association between being transgender, the utilisation of GAHT and the development of cardiovascular disease has been investigated since the 1980s. In a retrospective analysis of 425 transgender people treated with hormone therapy with 4 years of follow up, no associations were identified between being transgender, MI or mortality (Asscheman et al., 1989). A decade later, a retrospective analysis was undertaken of transgender women (n=816) and transgender men (n=293) receiving GAHT with 7,734 and 2,418 patient years of

follow up, respectively (van Kesteren *et al.*, 1997). Compared to transgender men, an decrease in the standardised incidence ratio (SIR) of MI (SIR 0.5 (95% CI 0.24,0.91)) but not MI standardised mortality was observed in cisgender men. Within this analysis transgender men did not demonstrate an increase in MI or MI-related mortality compared with comparator groups.

More recent evidence suggests the mode of gender-affirming care may influence outcomes in this population. A cohort study of 966 transgender women demonstrated a 51% higher mortality than the general population (Asscheman *et al.*, 2011). Conversely, in the 365 transgender men in this analysis, there was no significantly increased mortality. Importantly, cardiovascular mortality was three times higher for transgender women using ethinylestradiol (HR 3.12; 95% CI 1.28, 7.63), indicating that the type of oestrogen used may modulate cardiovascular risk. Ethinylestradiol undergoes extensive first pass metabolism, and its metabolites are believed to be pro-thrombotic and may contribute to this risk (Ezuruike *et al.*, 2018).

Longer-term hormone therapy was assessed in a cross-sectional study of 214 transgender women and 138 transgender men who received hormonal therapy for mean of 7.4 years and were age- and sex-matched on a 1:3 ratio with cisgender individuals (Wierckx *et al.*, 2013). Within this sample the rate of MI in transgender men were not found to be significantly higher than cisgender populations. However, transgender women experienced a significantly increased risk of MI compared to cisgender women with rates of 18.7 per 1,000 individuals. However, rates were not significantly increased compared to cisgender men (12.5 per 1,000).

The impact of gender-affirming surgery was assessed in a population-based matched cohort study of 324 transgender people in Sweden between 1973 and 2003 (Dhejne *et al.*, 2011). This demonstrated that compared to cisgender individuals matched for natal sex, the overall mortality of transgender individuals who had undergone surgery was higher during the follow up period (adjusted HR 2.8; 95% CI 1.8, 4.3). The HR for cardiovascular death in this population compared

to cisgender individuals was 2.5 (95% 1.2, 5.3) following adjustment for psychiatric morbidity and immigrant status. The cause of this difference is uncertain but may represent the effect of relative periods of hypogonadism. However, in a cohort of 100 transgender people who had undergone gender-affirming surgical management and received an average of 10 years hormonal therapy, only two MI events were observed in transgender women and no cases in transgender men (Wierckx *et al.*, 2012).

Our understanding of the prevalence of cardiovascular conditions in transgender individuals has been enhanced by BRFSS analyses (Meyer *et al.*, 2017; Nokoff *et al.*, 2018; Alzahrani *et al.*, 2019). These data are collected via random sampling and are considered to be representative of the wider US population. However, the BRFSS demonstrates a number of limitations including its cross-sectional nature, which cannot be used to infer causality. Moreover, gender-affirming healthcare status is not confirmed in these analyses, and cardiovascular outcomes are subject to recall bias as a consequence of being self-reported (Connelly & Delles, 2021). However, their capacity to engage with transgender individuals at a population level and produce national prevalence estimates adds significant value to their utility.

Analyses from the BRFSS have demonstrated that the risk of MI in transgender individuals compared to cisgender individuals is increased (OR 1.82; 95% 1.22, 2.72) (Meyer *et al.*, 2017). Nokoff *et al* showed that in transgender women there was an increased risk of MI (OR 2.9; 95% CI 1.6,5.3) compared to cisgender women but not men (Nokoff *et al.*, 2018). Within this analysis, there was no increased risk of MI in transgender men. However, a subsequent BRFSS analysis demonstrated that transgender men had a greater than fourfold risk of MI compared to cisgender women after adjusting for cardiovascular risk factors (Alzahrani *et al.*, 2019). This association was not demonstrated in transgender women.

Population-based research using electronic health records can potentially overcome the limitations that are inherent in the BRFSS design. In a large retrospective Kaiser Health system analysis of 2,842 transgender women and 2,118

transgender men receiving GAHT, the risk of MI was almost twice as high in transgender women compared to cisgender women (HR 1.8; 95% CI 1.1, 2.9) but not cisgender men (HR 0.9; 95% CI 0.6, 1.5) (Getahun *et al.*, 2018). This suggests that the increased risk of MI in transgender women may occur as a consequence of residual risk associated with their natal sex. In transgender men, no differences were observed in MI risk when compared to cisgender women or men.

Taken together, the evidence obtained across these studies are mixed. Although earlier research suggested an increased risk of ischaemic heart disease, and in particular MI, in transgender women, this association becomes less clear with more recent and larger analyses. If there is no increased risk of MI in transgender women compared to cisgender men, then increased rates of MI compared to cisgender women may be mediated by natal sex rather than the introduction of gender affirming healthcare or particular risk factors associated with being transgender, such as minority stress (Caceres & Streed, 2021).

The higher risk evident in older studies may indeed be associated with the utilisation of older formulations of oestrogen therapy, such as ethinylestradiol or conjugated equine oestrogens that have been linked to increased cardiovascular risk in the cisgender population (Weill *et al.*, 2016). Of note, the doses of ethinylestradiol utilised is cisgender populations are significantly lower than is used in transgender women, and a dose response relationship between cardiovascular and thrombotic risk and this hormone has previously been reported (Weill *et al.*, 2016).

Most studies reviewed recruited transgender women below the age of 50 years and little is known of the impact of cross-sex oestrogen on long-term cardiovascular health. It is also intriguing that little evidence exists for the increased risk of MI in transgender men receiving testosterone. If oestrogen is supressed in these individuals, the mechanism by which they receive cardioprotection is unclear and merits further investigation.

As discussed in recent meta-analysis, there is a distinct lack of data regarding meaningful health outcomes, such as ischaemic heart disease, in the available literature (Maraka *et al.*, 2017). Consequently, the capacity to provide guidance on these health outcomes when studies have not been completed utilising appropriate control populations or study design is limited.

2.4.5.2 Hypertension

Hypertension remains the leading modifiable risk factor resulting in cardiovascular disease and sex steroids are believed to regulate blood pressure and mediate sex differences in this condition (Colafella & Denton, 2018). In the 1980s, Asscheman *et al* demonstrated that the crude incidence of hypertension in 202 transgender women was elevated, while the association was not evident in transgender men (Asscheman *et al.*, 1989). However, this diagnosis of hypertension, which was defined in this study as blood pressure greater than 160/95 mmHg, was not found to be increased in either transgender men or women when compared to cisgender people of their natal sex. Similarly, in an analysis of transgender individuals utilising the BRFSS, the prevalence of self-reported hypertension in 369 transgender women and 239 transgender men was not increased compared to cisgender people (Nokoff *et al.*, 2018).

These studies demonstrate significant limitations and do not address the potential effect of duration of exogenous sex hormone exposure and age of the recipient upon blood pressure and the development of hypertension (Gooren & T'Sjoen, 2018a). Further research is required using modern definitions of hypertension to assess whether the risk of this condition is altered with long-term GAHT exposure. The influence of GAHT upon the blood pressure of people who are transgender is the focus of the systematic review contained within Chapter 3 of this thesis.

2.4.5.3 Cerebrovascular disease

The risk of cerebrovascular disease, including ischaemic stroke and transient ischemic attacks (TIA) has been investigated in a number of studies. With respect

to TIA, in a cohort study of 303 transgender women the incidence of TIA was not significantly different from cisgender men in the four years of follow up (Asscheman *et al.*, 1989). However, in a cohort of 214 transgender women and 138 transgender men with over seven years of GAHT use, a higher prevalence of TIA and cerebrovascular disease (23.4 per 1,000) was observed in transgender women (Wierckx *et al.*, 2013). Compared to their natal sex (9.4 per 1,000), the rates of these events were significantly more common, however, were not statistically different from cisgender women (14.9 per 1,000). Importantly, TIA or cerebrovascular disease events were not observed in transgender men.

In an analysis by van Kesteren *et al* the SIR for cerebrovascular disease in transgender women was not increased (van Kesteren *et al.*, 1997). Similarly, stroke-related mortality was not found to be increased in a cohort of 966 transgender women and 375 transgender men who were followed up for a median for 18.5 years. Indeed within the cohort of transgender men, no cases of stroke were identified (Asscheman *et al.*, 2011).

In stroke analyses from the BRFSS, no difference in cerebrovascular disease was evident in transgender people compared to cisgender populations (Meyer *et al.*, 2017). Similarly, when assessing transgender men and women separately, compared to cisgender people, there was no increased risk (Nokoff *et al.*, 2018). However, in an analysis undertaken by Getahun *et al*, elevated rates of ischaemic stroke in transgender women compared to either cisgender men or women were observed (Getahun *et al.*, 2018). In a subcohort of 853 transgender women commencing GAHT during the study observation period, the occurrence of ischaemic stroke did not differ greatly compared to cisgender populations during the first 6 years of follow up. However, substantive increases in ischaemic stroke rates occurred following this timepoint when compared to either cisgender men (HR 9.9; 95% CI 3.0,33.1) or women (HR 4.1; 95% CI 1.5,11.4). These results suggest an interaction between the commencement of gender-affirming medical and either duration of use or age, and stroke risk. This temporal effect, in addition to this study's large sample size and power, may in part explain why increases in rates of ischaemic stroke, or cerebrovascular disease, have not previously been

reported. This requires further investigation and validation, however, if this risk holds true, stroke prevention in transgender women should become a health priority.

2.4.5.4 Venous thromboembolism

Gender-affirming hormonal therapy may also promote the development of thrombosis. Both oestrogen and testosterone have been associated with thrombosis formation and disease in cisgender populations (Abou-Ismail *et al.*, 2020; Walker *et al.*, 2020). In a retrospective assessment of 303 transgender women receiving hormonal therapy, the rates of venous thromboembolism were increased by 20-45-fold (Asscheman *et al.*, 1989). However, more recent analyses demonstrate a moderate increased association with venous thromboembolism. In an analysis by Seal *et al,* venous thromboembolism occurred in 1.2% of oral oestrogen using transgender women (Seal *et al.*, 2012). This appeared to be more commonly associated with conjugated equine oestrogens (4%) compared to ethinylestradiol (0.7%) or oestrogen valerate (0.6%).

However, higher rates of venous thromboembolism (5.1%) were observed in a case control-study of 214 transgender women with an average oestrogen exposure of 7.7 years, thereby suggesting that this risk may increase over time (Wierckx *et al.*, 2013). This is supported by Getahun's analysis, whereby the risk of venous thromboembolism was proportional to the time exposed to oestrogen therapy (Getahun *et al.*, 2018). In this study, the 8-year venous thrombosis risk difference was found to be 16.7 (95% CI 6.4, 27.5) per 1,000 persons compared to 4.1 (95% CI 1.6, 6.7) at 2 years in transgender women compared to cisgender men.

Of note, not all studies demonstrated an increased risk of thrombosis in relation to oestrogen exposure. In the evaluation of 162 transgender women, no cases of venous thromboembolism were demonstrated during an average follow up of 64 months (Ott *et al.*, 2010). Similarly, in a retrospective study of 676 transgender women with a combined oestrogen exposure of 1,286 years, only one individual

(0.15%) in the cohort developed venous thromboembolism with an incidence of 7.8 events per 100,000 person-years (Arnold *et al.*, 2016).

Curiously, despite associations existing with testosterone therapy and the development of venous thromboembolism in cisgender men (Walker *et al.*, 2020), there appears to be a paucity of data regarding a similar relationship in transgender men receiving this hormone.

2.4.5.5 Cardiometabolic risk factors

The utilisation of GAHT may result in altered cardiometabolic risk. A recent meta-analysis of 29 studies comprising 4,731 transgender participants, of which 68% were transgender women, has examined the effect of hormone therapy on lipid profiles in this population (Maraka *et al.*, 2017). In transgender women, no differences in LDL, HDL or total cholesterol were observed. Conversely, transgender men exposed to testosterone demonstrated increases in LDL (17.8 mg/dL; 95% CI 3.5, 32.1) but decreases in HDL (-8.5 mg/dL; 95% CI -13,-3.9). No differences were observed in total cholesterol in this population. Interestingly, increases in triglycerides were apparent in both transgender women (31.9 mg/DL; 95% CI 3.9, 59.9) and transgender men (21.4 mg/dL; 95% CI 0.14,42.6). These studies, however, demonstrated significant heterogenicity and poor quality evidence, as is broadly evident in this field of research.

Moreover, the presence of type 2 diabetes may unfavourably alter cardiovascular risk (Rawshani *et al.*, 2017). In cisgender populations, individuals with type 2 diabetes have up to three times the risk of experiencing cardiovascular events compared to those without diabetes (Morrish *et al.*, 2001). In a cohort study of 966 transgender women receiving cyproterone acetate and either oral or transdermal oestrogen, with a median follow up of 18.5 years, no significant difference in diabetes-associated SMR compared to cisgender populations was observed (SMR 0.85, 95% CI 0.41,1.32) (Asscheman *et al.*, 2011). In this observational study, no cases of type 2 diabetes were demonstrated in the 365 transgender men assessed. However, a greater prevalence of type 2 diabetes in

transgender women (42 per 1000 cases) and transgender men (36.2 per 1,000 cases) was demonstrated in a cross-sectional study undertaken in 214 transgender women and 138 transgender men (Wierckx *et al.*, 2013). Conversely, analyses from the BRFSS demonstrated no difference in the prevalence of self-reported type 2 diabetes in transgender versus cisgender populations (Meyer *et al.*, 2017; Nokoff *et al.*, 2018).

Assessments of insulin resistance, such as the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) index have also been assessed in transgender populations. After two years of hormonal therapy there was a significant increase in HOMA-IR in transgender women (3.6 vs 6.6) (Colizzi *et al.*, 2015). Over the same time period, there was not a significant increase in this measurement of insulin resistance in transgender men. Gava *et al* demonstrated no difference after one year in HOMA-IR in transgender women utilising either cyproterone acetate or leuprolide in combination with transdermal oestrogen patches (Gava *et al.*, 2016). Importantly, fasting blood glucose levels have been observed to be higher in transgender women using transdermal oestrogen, however, no association has been demonstrated with oral ethinylestradiol (Elbers *et al.*, 2003; Colizzi *et al.*, 2015). Consequently, the relationship between being transgender, the use of GAHT and the development of type 2 diabetes and dysglycaemia remains uncertain.

2.4.5.6 Transgender cardiovascular guidance

The WPATH (Coleman *et al.*, 2012a) and the US Endocrine Society (Hembree *et al.*, 2017) have developed guidelines on the management of people who are transgender. Due to the paucity of data available to guide clinical decision making, these guidelines rely heavily on clinical experience from those with expertise in the management of individuals who are transgender. It is evident that much of the guidance provided is extrapolated from recommendations that exist for the management of cisgender men and women who are undergoing treatment with testosterone or oestrogen therapy, respectively (Unger, 2016).

With respect to cardiovascular health, the 7th version of WPATH guidelines published in 2012 has limited reference to the management of cardiovascular risk in these populations (Coleman *et al.*, 2012a). This guidance suggests that feminising hormone therapy is likely to increase the risk of hypertension and type 2 diabetes. With respect to the former it is stated the incidence of overt hypertension in this population is unknown. Conversely, masculinising hormone therapy may possibly increase blood pressure, however, was not thought to increase the risk of overt hypertension. With respect to cardiovascular disease, oestrogen use was stated to increase the risk of cardiovascular events in patients over age 50 with underlying cardiovascular risk factors. Masculinising therapy is stated to increase the risk of cardiovascular disease in people with underlying risk factors but not healthy individuals.

WPATH state that general clinical guidelines may either over- or underestimate the cost-effectiveness of screening for cardiovascular risk in these individuals who are receiving hormone therapy. It is therefore recommended that clinicians review their national evidence-based guidelines and discuss screening with their patients after taking into account baseline risk. However, no formal recommendations or levels or evidence are provided in these guidelines regarding cardiovascular risk management.

WPATH released the Standards Of Care version 8 in December 2022 and provides additional guidance on this topic (Coleman *et al.*, 2022). These updated guidelines emphasise that information relating to traditional cardiovascular and cerebrovascular risk factors should be obtained from transgender individuals in order to provide regular cardiovascular risk assessment. Moreover, the assessment and management of cardiovascular risk factors should be tailored to that individual. However, these guidelines also acknowledge that there are currently insufficient data to adjust predictive risk equations, which are often reliant on sex, to the transgender population. Suggestions included either using natal sex, affirmed gender or a weighted average of these to outputs, after taking into account longitudinal exposure to GAHT. Nevertheless, there are currently insufficient data to support this approach.

The Endocrine Society guidelines strongly encourage tobacco cessation in transgender women to reduce the potential risk of thrombosis and cardiovascular complications (Hembree *et al.*, 2017). Moreover, these guidelines stipulate that there is an increased risk of coronary artery disease, cerebrovascular disease and hypertension in transgender men (Hembree *et al.*, 2017). These risks are also suggested to be present in transgender women, with the exception of hypertension. Consequently, transgender men, and not women, are suggested to have their blood pressure measured at regular intervals. However, these suggested interventions and putative risks are not supported by empirical studies or formal levels of evidence.

Consequently, evidence-based guidance on cardiovascular risk management is extremely limited in this population. No substantive cardiovascular risk guidance for transgender people have been published despite evidence of their potential cardiovascular vulnerability.

2.4.6 Section summary

This thesis section has outlined the current evidence relating to the effects of gender-affirming therapies, and in particular exogenous testosterone and oestrogen, on cardiovascular health outcomes in transgender people. A paradoxical relationship exists between our understanding of the potential cardioprotective effects of oestrogen and the cardiovascular outcomes observed in transgender women (Iorga *et al.*, 2017). These data suggest the existence of an increased risk of MI and ischaemic stroke in this population. Whether this is a consequence of hormone therapy or legacy effect of sex assigned at birth is unclear as not all studies demonstrate increased risk compared with cisgender men. Alternatively, androgen depletion in these individuals may also be considered a potential mechanism by which cardiovascular health is altered (Hu *et al.*, 2020). Importantly, these studies do not assess the influence of gender related mechanisms in these populations, and it is unclear whether any putative risk occurs as a consequence of sex hormone- or sociocultural mechanisms.

Conversely, studies relating to the cardiovascular risk in transgender men have been inconsistent. It remains unclear why the introduction of testosterone in transgender men may not elevate cardiovascular risk in this population, or whether bias in existing research precludes the detection of increased risk in this population.

However, numerous limitations exist in the research described in this section. In particular, most of the studies identified are retrospective. Furthermore, the hormonal formulations, mode of administration and doses are not standardised and hormone level targets are not presented (Connelly *et al.*, 2019). Moreover, the effect of health inequalities, mental health disorders, and adverse health behaviours such as smoking and substance abuse are often not included within analyses (Reisner *et al.*, 2016). Consequently, the study of cardiovascular health and disease in transgender people is required to implement better clinical care and evidence-based guidance. Enhancing our understanding of the influence of gender-affirming interventions and cardiovascular outcomes in transgender people could be fundamental in informing the management of cardiovascular risk in this population.

2.5 Chapter summary

In this chapter, the means by which sex and gender may influence the cardiovascular health of cisgender and transgender populations are discussed. This provides an overview of the complexity of sex and gender, and how variations in these traits may facilitate the development of cardiovascular disease (e.g. Section 2.2), thereby establishing a foundation of understanding for future chapters of this thesis.

In section 2.3 of this chapter, the different components of gender are discussed along with methods by which gender can be measured to assess the impact of this upon cardiovascular health. A particular focus of this section is the GENESIS-PRAXY study. In Chapter 4 of this thesis, the GENESIS-PRAXY gender stratification questionnaire is adapted and implemented in a UK population. Moreover, a simple

masculinity-femininity questionnaire is piloted as a potential alternative means of stratifying gender in cardiovascular research. This section also provides context for the potential role of miRNA in mediating sex and gender influences in cardiovascular disease. This is relevant to Chapter 5, where samples obtained from the original GENESIS-PRAXY study were obtained in order to perform a bioinformatic analysis of differentially expressed miRNA in sex and gender stratified individuals who have experienced ACS.

Section 2.4 of this chapter highlights the paucity of data relating to cardiovascular health outcomes in transgender individuals. Within this thesis, Chapter 3 presents a systemic review of the effect of GAHT on the blood pressure of transgender individuals with the aim of expanding our knowledge of this relationship. Moreover, Chapter 6 presents preliminary analysis from the Vascular Effects of Sex Steroids in Transgender Adults (VESSEL) study. This cohort study aimed to conduct vascular phenotyping procedures, including flow-mediated dilatation (FMD), peripheral artery tonometry (PAT), and pulse wave analysis (PWA) and velocity (PWV) in transgender individuals on long-term hormone therapy compared to cisgender individuals.

Fundamentally, these sections serve to elucidate the means by which sex and gender can modulate cardiovascular pathophysiology, in order to better understand the methodological approaches adopted within this thesis to investigate this relationship.

Chapter 3 Transgender Adults, Gender-Affirming Hormone Therapy & Blood Pressure: A Systematic Review

3.1 Chapter overview

In this chapter, the associations between blood pressure and masculinising (i.e. testosterone) and feminising (i.e. oestrogen) gender-affirming hormone therapies (GAHT) in transgender individuals are explored through a systematic review of the literature. The results of this chapter have been published in the *Journal of Hypertension*: Connelly PJ, Clark A, Touyz RM, Delles C. Transgender adults, gender-affirming hormone therapy and blood pressure: A systematic review. *J Hypertens* 2021;39:223–230.

3.2 Introduction

GAHT including testosterone, oestrogen, gonadotropin-releasing hormone (GnRH) analogues and anti-androgens, aim to align the secondary sex characteristics of transgender people with their gender identity (T'Sjoen *et al.*, 2019). Due to the lack of epidemiological and mechanistic data, there is ambiguity as to whether GAHT influences the cardiovascular health of people who are transgender (Streed *et al.*, 2017; Irwig, 2018; Connelly *et al.*, 2019).

Sex hormones play a pivotal role in the modulation of blood pressure (Dubey *et al.*, 2002). The blood pressure of cisgender men and women are broadly equivalent before puberty, however, following the rise of sex hormone secretion occurring during this phase, there are profound sex-dependent differences in blood pressure and the prevalence of hypertension (Jackson *et al.*, 2007).

The influence of oestrogen on female blood pressure can be observed during the menstrual cycle, where blood pressure inversely relates to circulating oestrogen levels (Caroccia *et al.*, 2016). Via oestrogen receptors (ERs), estradiol modulates vasorelaxation and vasoconstriction, and endothelial function via nitric oxide-dependant mechanisms (Iorga *et al.*, 2017). Moreover, oestrogen may modulate the RAAS where levels of renin, plasma renin activity and aldosterone are higher during phases of increased oestrogen secretion (Chidambaram *et al.*, 2002).

Conversely, testosterone increases renin levels and expression/activity of angiotensin converting enzyme and Angiotensin II receptor type 1, while downregulating Angiotensin II receptor type 2, thereby favouring a relatively vasoconstrictive phenotype (Te Riet *et al.*, 2015).

It would therefore be anticipated that trends in blood pressure observed in cisgender populations, where males demonstrate significantly higher blood pressure and rates of hypertension compared to pre-menopausal females, would be apparent in transgender populations (Staessen *et al.*, 1991; Virani *et al.*, 2021). Consequently, decreases in blood pressure would be expected following the introduction of oestrogen in transgender women, and increases in blood pressure of transgender men commencing testosterone.

However, this hypothesis is not supported by current data. Asscheman *et al*, observed an increase in the crude incidence of hypertension in transgender women but not transgender men (Asscheman *et al.*, 1989). Moreover, in data provided by the Behavioural Risk Factor Surveillance System (BRFSS), no differences between transgender men and women were observed when compared to cisgender populations with respect to self-reported hypertension (Nokoff *et al.*, 2018).

Therefore due to lacking or inconsistent data, international guidelines for the management of people who are transgender set forth by the Endocrine Society and World Professional Association for Transgender Health have been cautious in their recommendations regarding blood pressure control in this population (Coleman *et al.*, 2012b; Hembree *et al.*, 2017).

Prior to the publication of this systematic review, current recommendations did not suggest the routine monitoring of blood pressure in transgender populations (Coleman *et al.*, 2012b; Hembree *et al.*, 2017). However, no data from these guidelines provided the effect size of GAHT on blood pressure or whether specific blood pressure targets are recommended in this population. Additionally, there were no formal assessments of the quality of evidence (i.e., levels of evidence) provided for this recommendation.

Given the potential concerns regarding the cardiovascular health of transgender populations (Irwig, 2018) and the importance of hypertension as a modifiable risk factor (Yusuf *et al.*, 2020) it is imperative that the effect of GAHT on blood pressure is understood and if harmful acted upon to reduce cardiovascular risk. Consequently, a systematic review of the literature to assess the effect of GAHT on the blood pressure of people who are transgender was undertaken.

3.3 Hypothesis & aims

3.3.1 Hypothesis

The null hypothesis was that no difference in blood pressure would be observed following the introduction of hormonal therapy in transgender individuals.

3.3.2 Aims

- To systematically review the effect of GAHT on the blood pressure of transgender individuals.

- To assess the quality of evidence provided in the identified studies.

3.4 Methods

3.4.1 Eligibility criteria

In this systematic review, randomized trials and observational studies of transgender individuals who used GAHT were included irrespective of gender-affirming surgical status. Only studies with populations older than 16 years of age were included. Review articles, commentaries and letters not containing original data were excluded from this systematic review.

Studies were required to have defined populations of transgender individuals prescribed hormone therapy (i.e. transmasculine individuals prescribed testosterone and transfeminine individuals prescribed oestrogen, antiandrogens (cyproterone acetate, finasteride or spironolactone) or GnRH analogues). Studies were included with any dose, formulations or route of formulations of these treatments.

Individuals who are transgender and were receiving GAHT may be gender non-binary (i.e. their gender identity or expression does not conform to binary gender), and may not consider themselves as simply men or women (Meerwijk & Sevelius, 2017). Therefore for the purposes of this systematic review, transgender people prescribed oestrogen (with or without anti-androgens or GnRH analogues) were categorised as transfeminine, while transgender individuals receiving with testosterone were considered transmasculine.

3.4.2 Search strategy

The systematic review is reported according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Moher *et al.*, 2009). The protocol for this systematic review was registered on PROSPERO (CRD42020186202). English language articles published until January 13, 2020, were searched in the PubMed/MEDLINE, SCOPUS, and Cochrane Library databases for inclusion in the systematic review by Dr Paul Connelly.

The following search terms were utilised in this analysis: ‘blood pressure’ AND 'cross-sex' OR 'gender affirming' OR 'gender dysphoric' OR 'gender incongruent' OR 'person, transsexual' OR 'person, transgender' OR 'persons, transsexual' OR 'persons, transgender' OR 'trans feminization' OR 'trans man' OR 'trans masculinization' OR 'trans woman' OR 'transgender' OR 'transgender female' OR 'transgender male' OR 'transgender man' OR 'transgender person' OR 'transgender persons' OR 'transfeminine' OR 'transmasculine' OR 'transsexual' OR 'transsexual person' OR 'transsexual persons' OR 'trans woman'.

3.4.3 Data extraction

Two investigators (Dr Paul Connelly & Miss Anna Clark) conducted the literature search and data extraction independently. Following the removal of duplicate records, study titles and abstracts were screened to identify potentially eligible articles for inclusion in the full text review. Selected articles then underwent full text screening, confirmation of eligibility, and data extraction.

The data extracted from these articles contained the first author's name, publication year, country of where the study was undertaken, number of participants included in that study, duration of follow-up following the commencement of GAHT, the formulation, dose and route of administration of GAHT, and systolic and diastolic blood pressure before and after the introduction of hormone therapy. Along each aspect of this process, if any disagreement between the two reviewers emerged, consensus was obtained following the consultation of a third reviewer (Professor Christian Delles).

3.4.4 Quality assessment

The quality of the studies included were assessed by the 'National Institute of Health Quality Assessment Tool for Before–After (Pre–Post) Studies with No Control Group' (Table 3-1) (National Institutes of Health, 2014). This quality assessment tool consists of twelve components uses to assess the risk of selection, reporting and observer bias. The quality of included studies was again evaluated by two independent assessors (Dr Paul Connelly & Miss Anna Clark). Based on the results of this assessment, each included study was classified as either poor, fair or good quality.

3.4.5 Statistical analysis

Inter-assessor agreement was assessed by calculated Cohen's kappa (Cohen, 1960). This provides a score, which if greater 0.8 demonstrates excellent interrater agreement. If scores are demonstrated between 0.4 and 0.8, agreement is

considered to be fair and scores of less than 0.4 are considered to have poor agreement. All analyses were performed using R version 4.0.2.

Quality Assessment Tool for Before-After (Pre-Post) Studies with No Control Group
1. Was the study question or objective clearly stated?
2. Were eligibility/selection criteria for the study population prespecified and clearly described?
3. Were the participants in the study representative of those who would be eligible for the test/service/intervention in the general or clinical population of interest?
4. Were all eligible participants that met the prespecified entry criteria enrolled?
5. Was the sample size sufficiently large to provide confidence in the findings?
6. Was the test/service/intervention clearly described and delivered consistently across the study population?
7. Were the outcome measures prespecified, clearly defined, valid, reliable, and assessed consistently across all study participants?
8. Were the people assessing the outcomes blinded to the participants' exposures/interventions?
9. Was the loss to follow-up after baseline 20% or less? Were those lost to follow-up accounted for in the analysis?
10. Did the statistical methods examine changes in outcome measures from before to after the intervention? Were statistical tests done that provided p values for the pre-to-post changes?
11. Were outcome measures of interest taken multiple times before the intervention and multiple times after the intervention (i.e., did they use an interrupted time-series design)?
12. If the intervention was conducted at a group level (e.g., a whole hospital, a community, etc.) did the statistical analysis take into account the use of individual-level data to determine effects at the group level?

Table 3-1. Quality assessment tool questions for before- after studies.

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3.5 Results

3.5.1 Studies included

Following the search, 600 non-duplicated potentially relevant research articles were identified (Figure 3-1). No additional records were identified from other databases. In total, the for eligibility of 84 full text articles were reviewed, however, 83.3% (n=70) of these texts were excluded. Thirty-two articles were not original investigations, 33 did not report relevant outcome data, 4 contained adolescent populations (Hannema *et al.*, 2017; Jarin *et al.*, 2017; Olson-Kennedy *et al.*, 2018; Stoffers *et al.*, 2019) and 1 did not provide blood pressure data prior to commencing hormonal therapy (Angus *et al.*, 2019). Consequently, after applying these exclusions criteria 14 studies were included in systematic review. All of the included studies included were found to be pre-post observational studies that were uncontrolled.

3.5.2 Study characteristics

A total of 1,309 participants were included within these 14 research articles. Approximately 50% of these were transmasculine individuals utilising testosterone therapy or transfeminine individuals utilising oestrogen therapy. These studies took place between 1989 and 2019 and follow-up for these individuals ranged between 4 months and 5 years. The majority of these studies (78.5%) were undertaken in Europe, while the rest were undertaken in North America.

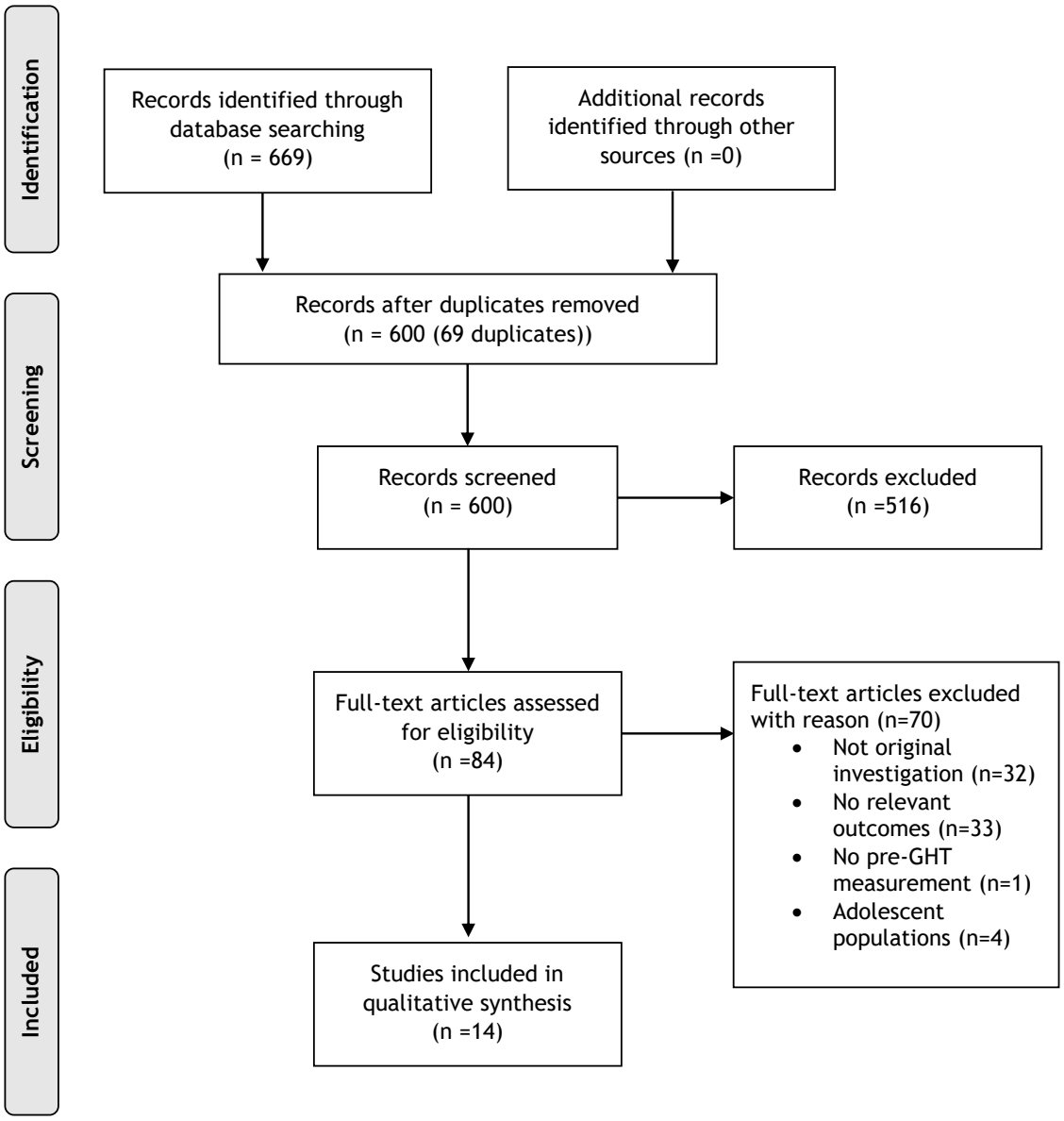

Figure 3-1. PRISMA flow diagram for study selection.
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3.5.3 Blood pressure measurement

With regards to the standardised measurement of blood pressure, only a minority of studies (n=4) defined the position (i.e. standing versus sitting) of blood pressure measurement (Elbers *et al.*, 2003; Colizzi *et al.*, 2015; van Velzen *et al.*, 2019). Three studies recorded collecting multiple blood pressure measurements (Elbers

et al., 2003; Giltay *et al.*, 2004; Colizzi *et al.*, 2015). Lastly, only one study defined the blood pressure device (BP-8800; Colin Corporation, Hayashi, Japan) (Elbers *et al.*, 2003), which was a non-validated blood pressure measurement device.

3.5.4 Transfeminine individuals

A total of 10 studies included data on the blood pressure of transfeminine individuals receiving oestrogen therapy (Table 3-2). Sixty hundred and sixty-six participants were included in these studies, which were undertaken between 1989 and 2019. The mean sample size of these studies was 66 individuals and the largest study included a total of 242 individuals (van Velzen *et al.*, 2019).These participants had a mean follow up of 16.4 (SD 7.9) months, which ranged between 6 and 31.7 months.

The studies presented varied outcomes related to blood pressure. Following the introduction of oestrogen therapy, three studies demonstrated an increase in systolic blood pressure between 7.2 and 17.8 mm Hg. In a further three studies, a decrease in systolic blood pressure between 3.4 and 10 mmHg were noted. Diastolic blood pressure was found to be elevate between 3.2 and 5.7 mmHg in two studies and unchanged in the remaining studies.

A variety of dosages, formulations and route of administration of oestrogen therapy were utilised in participants included in the studies identified in this systematic review:

- Oral estradiol valerate (n=6)
- Estradiol patches (n=6)
- Estradiol gel (n=2)
- Oral ethynyl estradiol (n=2)
- Oral conjugated oestrogen (n=2)
- Sublingual micronized 17Β estradiol (n=1)
- Intramuscular (IM) estradiol valerate(n=2)

- Unspecified oral oestrogen (n=1)

Four studies utilised age-dependent formulations, where transdermal oestrogen patches were provided in place of oral oestrogen formulations for those over the age of 40-45 years, which is typical of clinical practice (Wierckx *et al.*, 2014b; Quirós *et al.*, 2015; Auer *et al.*, 2018; van Velzen *et al.*, 2019). In addition to oestrogen, a number of studies utilised adjunct therapies. These included cyproterone acetate (n=7), spironolactone (n=3) and flutamide (n=1). Progestogens were utilised in 2 studies while no study was identified that included the use of GnRH analogues.

3.5.5 Transmasculine individuals

In this analysis, thirteen studies conducted between 2003 and 2019 were identified that included systolic and diastolic blood pressure measurements in transmasculine individuals (Table 3-3). The mean sample size was 50 individuals, and ranged between 11 and 97 people. Three studies consisting of less than 20 individuals were included in this analysis (Elbers *et al.*, 2003; Fernandez & Tannock, 2016; Vita *et al.*, 2018). Participants were followed up for a mean 18.9 (SD 14.2) months and this ranged between 4 months and 5 years.

A variety of testosterone formulations were also utilised in participants included in the studies identified in this systematic review. These included:

- IM testosterone undecanoate (n=8)
- IM testosterone esters (n=7)
- Transdermal testosterone gel (n=2) or patches (n=1)
- Oral testosterone undecanoate(n=1)
- Subcutaneous testosterone cypionate (n=1)

There was significant variation in the impact of testosterone on blood pressure among these studies. Among the included studies, a majority (n=9) did not show a statistically significant alteration in systolic blood pressure. Conversely, three

studies observed a significant increase in systolic blood pressure, with the reported range falling between 4.3 and 13.3 mmHg (Mueller *et al.*, 2007, 2010; Colizzi *et al.*, 2015). A single study described a decrease systolic blood pressure of 4.6 mmHg (Giltay *et al.*, 2004). Lastly, one study observed a statically significant increase in diastolic blood pressure of 3.7 mmHg (95% CI 0.24, 7.2) (Colizzi *et al.*, 2015), whereas the majority of studies did not report a change in this measure.

3.5.6 Evidence quality

An assessment of the study quality was undertaken using the Quality Assessment Tool for Before–After Studies with no control group (Table 3-1)(National Institutes of Health, 2014). Inter-rater agreement was calculated as 92.8% with an expected agreement of 69.4%. Cohen's kappa was 0.77 (SE 0.22) indicating fair agreement between assessors. The majority of the evaluated studies exhibited poor to fair quality (Table 3-4). Only two publications were found to possess a good quality rating (Colizzi *et al.*, 2015; van Velzen *et al.*, 2019). Since all included studies followed an uncontrolled quasi-experimental before-after design, their evaluative strength was inherently limited. As a result no study fully satisfied all the quality criteria evaluated in this analysis. Most studies did not provide power calculations or showed evidence of being underpowered. Consequently, these could not be relied upon to demonstrate statistically significant changes in blood pressure. Moreover, the absence of blinding, the limited use of repeated measurements, and the broad heterogeneity among the study cohorts further diminished the quality of studies included. Lastly, the administered interventions encompassed various doses, formulations, and combinations of therapies, which contributed to the overall heterogeneity observed across the studies.

Author, Year	Country	Follow up (months)	Sample size, n	Mean age (years) (SD)	Intervention	Mean difference in BP before & after GAHT (mmHg) [95% CI]	
						SBP	DBP
Prior *et al.*, 1989	Canada	12	23	30.7 (6.2)	0.625 to 2.5 mg conjugated oestrogen, followed by 10 mg medroxy-progesterone/day during weeks 3 and 4 or continuously if gonadotrophins increased, in addition 100– 200 mg spironolactone/day	-7.3 [-15.3, 0.7]	-2.6 [-9.3, 4.1]
Elbers *et al.*, 2003	The Netherlands	12	20	26 (6)	100mg oral ethinyl estradiol and 100 mg cyproterone acetate/day	+7.2 [0.2, 14.6]	+5.7 [0.0, 11.4]
Wierckx *et al.*, 2014	Belgium, The Netherlands	12	53	30.3 (14.1)e	<45 Years (n=40) 100mg/24 h transdermal 17ß-estradiol patch. If intolerant of this, 2 mg transdermal 17ß estradiol gel twice daily or 4 mg estradiol valerate per day	-5.4 [-10.8, 0.0]	-0.1 [-4.0, 3.8]
Colizzi *et al.*, 2015	Spain	24	79	30.2 (9.6)	2.12 +/- 0.57 mg transdermal estradiol gel/day and 100 mg cyproterone acetate/day	+17.8 [14.4, 21.2]	+3.2 [0.3, 6.1]
Quirós *et al.*, 2015	Spain	24	150	32.4 (10.1)	Oral oestrogen (conjugated equine oestrogens or estradiol valerate) and either cyproterone acetate or flutamide. >40 Years were recommended transdermal oestrogens (dose/frequency/formulation unspecified)	+6.4 [3.5, 9.3]	+3.7 [1.4, 5.9]

Deutsch *et al.*, 2015	USA	6	16	29 (9.4)	2 mg sublingual micronized 17b estradiol twice daily (n=14) or 20 mg IM estradiol valerate/2 weeks (n=1) or 100mg estradiol via transdermal patch (n=1) in addition to 50–100 mg spironolactone/day (n=15)	-10 [-17.0, -2.9]	-11 [-19.7, -2.3]
Fernandez & Tannock, 2016	USA	18	33	31 (10)	1.71 mg oral oestrogen (type unspecified)/day (50%), transdermal oestrogen (dose/type/ frequency unspecified) (14%) or IM oestrogen (36%) (dose/type/frequency unspecified), and 100 mg spironolactone/day	-6.0 [-13.9, 1.9]	-5.0 [-10.6, 0.6)
Vita *et al.*, 2018	Italy	31.7	21	25.2 (7)	2–6 mg oral estradiol valerate/day and 50–100 mg cyproterone acetate/day (if not undergoing sex reassignment surgery). Patients (n =4) receiving ethylestradiol were switched to estradiol valerate. Progesterone was also utilized (n=3) (type/route/ frequency unspecified)	-6.1 [-11.0, -1.2]	-4.0 [-9.1, 1.1]
Auer *et al.*, 2018	Germany	12	24	34.8 (1.4)	2 mg oral estradiol valerate twice daily or 100mg transdermal 17b estradiol patch/day (if >45 years) and 50 mg cyproterone acetate/day	-6.7 [-13.9, 0.6]	-1.7 [-6.1, 2.7]

van Velzen *et al.*, 2019	Belgium, The Netherlands	12	242	32.3 (12.6)	2 mg oral estradiol valerate twice daily (n=144) or 100mg transdermal estradiol patch/day (if age >45 years, n=98), and 50 mg cyproterone acetate/day	-3.4 [-5.9, -0.9]	-1.8 [-4.1, 0.5]

Table 3-2. Blood pressure in oestrogen exposed transfeminine individuals.

BP: blood pressure; DBP: diastolic blood pressure; GAHT: gender-affirming hormone therapy; SBP: systolic blood pressure.

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Author, Year	Country	Follow-up (months)	Sample size, n	Mean age (years) (SD)	Intervention	Mean difference in BP before & after GAHT (mmHg) [95% CI]	
						SBP	DBP
Elbers *et al.*, 2003	The Netherlands	12	17	23 (5)	250 mg IM testosterone esters/2 weeks	+1.0 [−5.1, 7.1]	−0.2 [−4.6, 4.2]
Giltay *et al.*, 2004	The Netherlands	4	81	36.7 (10)	250 mg IM testosterone esters/2 weeks (n = 61) or oral testosterone undecanoate 160–240 mg/day (n = 20)	−4.6 [−8.3, −0.9]	−2.1 [−4.4, 0.2]
Mueller *et al.*, 2007	Germany	12	35	29.6 (8.9)	1 g IM testosterone undecanoate/12 weeks	+4.3 [−1.5, 10.1]	+2.9 [−0.3, 6.1]
Mueller *et al.*, 2010	Germany	24	45	30.4 (9.1)	1 g IM testosterone undecanoate/12 weeks	+5.2 [0.5, 9.9]	+2.8 [−0.0, 5.6]
Wierckx *et al.*, 2014	Belgium, The Netherlands	12	53	24.5 (7.0)a	1 g IM testosterone undecanoate at baseline, then 6 weeks, then/12 weeks thereafter. If intolerant of testosterone undecanoate, 250 mg IM testosterone esters/2 weeks	+4.1 [−0.5, 8.7]	+2.3 [−1.5, 6.1]
Colizzi *et al.*, 2015	Spain	24	43	28.8 (5.6)	250 mg IM testosterone ester/21.16 ± 3.17 days	+13.3 [9.6, 13.3]	+3.7 [0.24, 7.2]
Quirós *et al.*, 2015	Spain	24	97	28.6 (8.6)	50 mg transdermal testosterone gel/day or 1 g IM testosterone undecanoate 1 g/12 weeks	+2.2 [−0.9, 5.3]	+1.5 [−1.1, 4.1]

Deutsch *et al.*, 2015	USA	6	34	27 (6.9)	50–70 mg subcutaneous testosterone cypionate/week (n = 31), 5 g gel/day (n = 2) or 4 mg transdermal patch/day (n = 1)	+3.0 [−3.7, 9.7]	−2 [−6.0, 2.0]
Fernandez & Tannock, 2016	USA	18	19	27 (7)	~150 mg IM testosterone/2 weeks (type unspecified)	−2.0 [−12.6, 8.6]	−1 [−7.9, 5.9]
Vita *et al.*, 2018	Italy	25.5	11	25.1 (3.7)	IM testosterone enanthate (n = 10) or undecanoate (n = 1) (dose/frequency unspecified)	+6.2 [0.5, 11.8]	+3.1 [−1.9, 8.1]

Table 3-3. Blood pressure in testosterone exposed transmasculine individuals.

BP: blood pressure; DBP: diastolic blood pressure; GAHT: gender-affirming hormone therapy; SBP: systolic blood pressure. Reproduced with permissions (Connelly *et al.*, 2021c).

Author, year	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12	Quality Rating
Prior *et al*, 1989	Y	Y	Y	Y	NR	N	N	NR	Y	Y	N	NA	Fair
Elbers *et al*, 2003	Y	Y	Y	Y	NR	Y	Y	NR	Y	Y	N	NA	Fair
Giltay *et al*, 2004	Y	Y	Y	Y	N	N	Y	N	Y	Y	N	NA	Fair
Mueller *et al*, 2007	Y	Y	Y	Y	NR	Y	N	N	Y	Y	N	NA	Fair
Mueller *et al*, 2010	Y	Y	Y	Y	NR	Y	N	N	Y	Y	N	NA	Fair
Wierckx *et al*, 2014	Y	Y	Y	Y	NR	N	N	N	Y	Y	N	NA	Fair
Colizzi *et al*, 2015	Y	Y	Y	Y	Y	Y	Y	N	Y	Y	N	NA	Good
Quiros *et al*, 2015	Y	Y	Y	Y	Y	N	N	N	N	Y	N	NA	Fair
Deutsch *et al*, 2015	Y	Y	Y	Y	NR	N	Y	N	Y	Y	N	NA	Fair
Fernandez *et al*, 2016	Y	Y	Y	Y	NR	N	N	N	N	Y	N	NA	Poor
Vita *et al*, 2018	Y	Y	Y	Y	NR	N	Y	N	Y	Y	N	NA	Fair
Auer *et al*, 2018	Y	Y	Y	Y	NR	N	N	N	Y	Y	N	NA	Fair
Gava *et al*, 2018	Y	Y	Y	Y	NR	N	N	N	Y	Y	N	NA	Fair
van Velzen *et al*, 2019	Y	Y	Y	Y	Y	N	Y	N	N	Y	N	NA	Good

Table 3-4. Quality assessment of studies assessing the effect of GAHT on blood pressure in transgender individuals.

Reproduced with permissions (Connelly *et al.*, 2021c).

3.6 Discussion

This systematic review appraises the evidence relating to the effect of commencing GAHT on the blood pressure of people who are transgender. A total of 14 studies were identified encompassing 1,309 transgender individuals receiving GAHT, who demonstrated blood pressure measurements before and after the initiation of these therapies.

The evidence-base for the effect of hormonal therapy on blood pressure of transgender individuals is extremely limited. Many methodological limitations were demonstrated in the included studies. Consequently, the majority of studies rated as poor to fair in the quality assessment undertaken. These weaknesses included the use of multiple hormonal formulations, doses and combinations within a single cohort. As these interventions lacked of standardisation, the results obtained may have been confounded and biased as blood pressure effects may be dose and formula dependent (Kyriacou & Lewis, 2016). These limitations may explain in part the significant variation observed in the effect these therapies upon blood pressure.

Moreover, these studies included often short-term follow up and lacked repeated measures. In a longitudinal analysis of testosterone therapy in hypogonadal cisgender men, the effect of lowering blood pressure occurred and was maintained over a period of several years, and therefore clinically significant changes in blood pressure may not have been observed in the limited follow up periods (Saad *et al.*, 2020). In addition, the majority of these studies were inadequately powered to detect clinically significant changes in blood pressure as a consequence of their limited sample size (Krzywinski & Altman, 2013).

With respect to the measurement of blood pressure, only one study reported the blood pressure apparatus used (Elbers *et al.*, 2003). In this instance, the blood pressure device (Colin, BP-8800) has been deemed not to be validated for clinical

research as a consequence of demonstrating inconsistent aberration patterns (Naschitz *et al.*, 2000). Moreover, these studies did not provide detail on blood pressure measurement protocols. Failure to standardise this technique or the use of non-validated methods of blood pressure measurement may have substantive deleterious effects on reproducibility and utility of these results. In recent guidance on blood pressure assessment in clinical based research, multiple blood pressure readings are recommended from a validated and calibrated blood pressure device from which the mean should be derived and utilised in that analysis (Muntner *et al.*, 2019).

Importantly, the studies included in this systematic review were all uncontrolled before-after studies, and as a consequence demonstrate inherently flawed evaluative methodologies (Grimshaw, 2000). Consequently, a meta-analysis was not viable as pre-post effects in this context would be influenced by participant characteristics (i.e. intra-individual variation) and cannot be distinguished from the intervention (i.e. GAHT) (Cuijpers *et al.*, 2017). Furthermore, the lack of standardisation in the methodologies and outcome end points of these studies impedes direct comparisons and meta-analysis. Lastly, as pre-post blood pressure effects were likely to provide biased outcomes, and not prove reliable with regard to treatment effects, it was concluded that this analytical tool should not be employed.

A significant limitation of this systematic review is that it did not incorporate evidence of the development or prevalence of hypertension in this population. In an observational study conducted by Asscheman *et al* an increase in the crude incidence of hypertension was observed in transgender women (Asscheman *et al.*, 1989). This increase was not observed in transgender men. However, when comparing the standardised incidence ratio of hypertension, which was defined as >160/95 mmHg in this analysis, there was no difference between cisgender and transgender populations. Given that this blood pressure cut off is well above modern definitions of hypertension, these rates may have been under reported

(Mancia *et al.*, 2023). However, in a recent BRFSS survey, self-reported hypertension was not observed to be higher in transgender populations (Nokoff *et al.*, 2018). As many individuals with hypertension are unaware that they may have this condition, this analysis may also have underestimated the prevalence of hypertension in this population (Ning *et al.*, 2016).

Within this systematic review, the majority of studies investigating the effects of testosterone therapy on transmasculine individuals revealed no significant alterations in either systolic or diastolic blood pressures. The vascular influences of testosterone have been demonstrated in a number of models. Testosterone can elicit coronary artery and aortic vasodilation (Chou *et al.*, 1996; Ding & Stallone, 2001; English *et al.*, 2002). These effects appear to be rapid, non-genomic, and independent of the conversion of testosterone to oestrogen, as demonstrated by the use of aromatase inhibitors and ER antagonists (Yue *et al.*, 1995; Tep-areenan *et al.*, 2002). This may also be AR independent, given that the antagonist flutamide does not inhibit the vasodilatory effect of testosterone (Cairrão *et al.*, 2008). Consequently, this sex steroid likely interacts with a number of membrane bound receptors and ion channels (e.g. GPRC6A, OXER1, TRPM8 and ZIP9) to bring about alterations in vasoreactivity (Lorigo *et al.*, 2020).

Testosterone has also been shown to promote vasoconstrictory responses. This sex steroid increases thromboxane A2 receptor density in aortic smooth muscle cells and enhances vasoconstrictor responses in female animal models (Matsuda *et al.*, 1995; Chinnathambi *et al.*, 2014). In addition, ovariectomised female spontaneously hypertensive rats treated with testosterone demonstrate equivalent blood pressure levels to males, an effect attributable to upregulation of the RAAS (Reckelhoff *et al.*, 2000; Lucas-Herald *et al.*, 2017; Morselli *et al.*, 2017b; Colafella & Denton, 2018). Androgens have also been shown to downregulate the expression of angiotensin II type-2 receptors, which promote vasodilatation, in females (Mishra *et al.*, 2016). Moreover, increases in endothelin-1, a potent vasoconstrictor, have been observed in transmasculine cohorts

receiving testosterone therapy (Polderman *et al.*, 1993). Consequently, the influences of this sex hormone upon vascular function and the development of hypertension may be sex-specific and warrants further mechanistic investigation (Santos *et al.*, 2023).

In transfeminine individuals using oestrogen therapies, both increases and decreases in systolic blood pressure were demonstrated in the studies reviewed. Oestrogen has been found to enhance vasodilation at a cellular level through various mechanisms that are both dependent and independent of the endothelium. It has been demonstrated that that activation of ER subtype, ERα, and binding to the nuclear oestrogen response element of renin expressing juxtaglomerular cells is necessary for basal renin expression, which suggests that oestrogen may be involved in RAAS regulation (Lu *et al.*, 2016). In the endothelium, oestrogen promotes the expression and activation of endothelial nitric oxide synthase (eNOS), leading to an increase in the availability of nitric oxide, which facilitates vasodilation. Moreover, oestrogen stimulates rises in endothelium-derived hyperpolarizing factor and prostacyclin, while reducing the levels of endothelin-1 (Akishita *et al.*, 1998; Chambliss *et al.*, 2000; Liu *et al.*, 2001; Sumi & Ignarro, 2003; Sobrino *et al.*, 2010). Furthermore, oestrogen influences calcium flux in vascular smooth muscle cells, thereby promoting vasorelaxation independently of the endothelium (Han *et al.*, 1995).

Consequently, the putative relationship between oestrogen and increasing blood pressure in transfeminine individuals and the lack of response noted in transmasculine individuals suggests significant limitations in our understanding of the influence of sex and gender on blood pressure regulation. The development of hypertension, and the means by which sex hormones modulate blood pressure, is complex and involves numerous systems (Colafella & Denton, 2018). Mechanistic research addressing the consequences of sex-chromosome and -steroid interactions in blood pressure regulation is therefore merited.

To conclude, due to the limitations evident in the studies assessed in this systematic review, which objectively demonstrated limited quality, it is not possible to provide clinical recommendations of the effects of GAHT on the blood pressure in transgender individuals. Stronger evidence is warranted to clarify whether oestrogen or testosterone modulates the blood pressure of transgender individual and whether these alterations lead to increased cardiovascular risk, and what interventions can be undertaken to ameliorate this. In particular, as the majority of the studies included in this analysis were undertaken in younger individuals, research focusing on the interaction between blood pressure, hormonal therapies and advancing age will be required to evaluate whether blood pressure lowering interventions are required in those utilising long-term hormonal therapies (Gooren & T'Sjoen, 2018b).

3.7 Future perspectives

The prevalence of the transgender population continues to increase with as many as 390 adults per 100,000 of the US population identifying as transgender (Bradford *et al.*, 2013; Meerwijk & Sevelius, 2017). As a consequence, the undertaking of good quality research is required to ensure better care and evidence-based guidance in this increasing population. Therefore, recommendations aimed at improving the quality of research in this field have been developed and are set out in Table 3-5.

Randomized controlled trials (RCT) are the gold standard in clinical research and facilitate the reduction of bias and allow the examination of causal inference of an intervention. However, RCTs may not be acceptable or ethical, for instance the use of placebo therapies, in transgender populations and are associated with significant costs and sample size considerations (T'Sjoen *et al.*, 2019). A concerted research effort is therefore required to improve upon research methodologies utilised in the future studies.

Quasi-experimental uncontrolled before-after studies have intrinsically weak evaluative designs and are therefore at high risk of bias (Grimshaw, 2000). These could be improved through the introduction of cisgender controls, and preferably would include cisgender men and women to allow comparison with sex assigned at birth and affirmed gender. Another potential means of reducing bias would be the inclusion of an interrupted time series or self-controlled case series design (Hudson *et al.*, 2019). This would permit the effect of the intervention to be estimated after accounting for the underlying trend of blood pressure dynamics in transgender individuals (Grimshaw, 2000). Multiple study visits with repeated blood pressure measurements should be undertaken at baseline and during intervention follow up. Ideally, the utilisation of ambulatory blood pressure monitoring should be encouraged, as this provides a stronger prediction of target end-organ damage and cardiovascular events (Conen & Bamberg, 2008).

Since the publication of this systematic review (Connelly *et al.*, 2021c), Banks *et al* have longitudinally assessed the blood pressures of 470 transgender individuals following the commencement of GAHT (Banks *et al.*, 2021). In that analysis the blood pressures of this cohort were assessed at baseline and at follow-up appointments over a period of 57 months. After four months of commencing GAHT, prolonged increases were observed in systolic blood pressures (2.6 mmHg) in transgender men receiving testosterone. Conversely, decreases in systolic blood pressure of 4 mmHg were observed in transgender women, while no significant change in diastolic blood pressure was demonstrated in either transgender men or women.

To date, this is the most comprehensive blood pressure analysis in transgender individuals. As a consequence, the most recent iterations of the World Professional Association for Transgender Health (WPATH) Standards Of Care guidance, of which this systematic review is cited, now suggest the monitoring of blood pressure before and after the introduction of testosterone therapy, particularly within the first 2 to 4 months after commencing treatment (Coleman *et al.*, 2022). However,

there are no current recommendations for blood pressure measurement in the longer-term, or specific guidance relating to transgender women utilising oestrogen therapy.

Research Question	Recommendations for future research
Does gender-affirming hormone therapy alter the blood pressure of transgender men and/or women?	Interrupted time series or controlled cohort study measuring blood pressure before and after the introduction of gender-affirming hormone therapy using validated office or ideally ambulatory blood pressure recordings.
Are alterations in blood pressure associated with increased cardiovascular risk in transgender men and women?	Prospective longitudinal observational cohort study of transgender people for cardiovascular disease in relation to components of blood pressure.
Do blood pressure-lowering interventions reduce cardiovascular risk in transgender men and women?	Randomized controlled study of transgender individuals with SBP > 130 mmHg to SBP targets of <120 mmHg (intensive treatment) or less than 140 mmHg (standard treatment) with a primary composite outcome of myocardial infarction, other acute coronary syndromes, stroke, heart failure or death from cardiovascular causes.

Table 3-5. Recommendations for blood pressure research in transgender people.
SBP: systolic blood pressure; DBP: diastolic blood pressure. Reproduced with permissions (Connelly *et al.*, 2021c).

Importantly, the blood pressure response to GAHT may be idiosyncratic. Within the analysis conducted by Banks *et al,* an increase in blood pressure of greater than 5 mmHg was observed in a quarter of transgender women receiving oestrogen therapy (Banks *et al.*, 2021). Conversely, similar numbers of transgender men experienced a decrease in blood pressure. As the response to GAHT is not uniform,

routine blood pressure monitoring in transgender individuals may therefore be merited. Moreover, it is imperative that the mechanisms by which these divergent responses occur are elucidated to identify individuals at higher risk and improve our understanding of hormonal regulation of blood pressure and the development of hypertension.

Chapter 4 Gender Stratification in Cardiovascular Research

4.1 Chapter overview

In this chapter, a gender stratification questionnaire adapted from the Gender and Sex Determinants of Cardiovascular Disease: From Bench to Beyond-Premature Acute Coronary Syndrome (GENESIS-PRAXY) study is assessed in a UK cohort to generate gender scores via principal component analysis for future use in cardiovascular research. These scores are compared to a simple masculinity-femininity score.

4.2 Introduction

Sex and gender variables are often conflated in clinical research (Clayton & Tannenbaum, 2016). As demonstrated in Chapter 2, the impact by which sex (i.e. sex hormones, chromosomal complement and sex-specific factors) and gender (sociocultural behaviours and attitudes) interact to modulate the development cardiovascular disease are being increasingly recognised (Azizi *et al.*, 2022; Visniauskas *et al.*, 2022). However, despite advances in our understanding of the relationship between these concepts, research has been limited by the lack of adequate quantitative tools to assesses the effects of gender upon cardiovascular outcomes (Nielsen *et al.*, 2021).

The GENESIS-PRAXY Study was a prospective observational cohort study conducted between 2009 and 2013, which provided significant progress in the field of sex and gender research in relation to cardiovascular disease (Pelletier *et al.*, 2016). Associations between sex, gender and recurrent acute coronary syndrome (ACS) and major adverse cardiac events (i.e., ACS, cardiac mortality, revascularisation) were investigated over a 12-month follow-up period following the index ACS episode. A GENESIS-PRAXY Gender Index (GGI) was constructed to assess the combined influence of gender factors derived from a number of gender-dimensions (i.e. identity, relations, roles and institutional) on cardiovascular risk

factors and outcomes, while distinguishing such variables from biological sex (Connelly *et al.*, 2021a).

The GGI uses an approach consistent with gender diagnosticity, which was pioneered by Lipa and Connelly in the 1990s. Gender diagnosticity uses Bayesian probability (Figure 4-1), whereby a diagnostic ratio is derived as the probability estimate that a trait is possessed by an individual within a group divided by that person's probability estimate this trait is possessed more generally (van de Schoot *et al.*, 2021). Simply put, Bayes Theorem can determine the probability of a specific trait in a group of individuals with specific characteristic after taking into account how common that trait is and the likelihood of such characteristics in those with and without this trait. As described by Lipa and Connelly, 'If an individual difference is defined by the behaviour of two indexing groups, A and B, then the discriminant function computed from a given set of diagnostic indicators will yield a Bayesian probability that describes how "A-like" or "B-like" an individual is' (Lippa & Connelly, 1990).

$$P(A|B) = \frac{P(B|A)P(A)}{P(B)}$$

Figure 4-1. Bayes' Theorem.
P(A|B) is a conditional probability (i.e., posterior probability: the probability of *A* given *B*). *P(B|A)* is also a conditional probability (i.e., likelihood: the probability of *B* given A). *P(A) and P(B)* are the probabilities of observing *A* (i.e., prior probability) and *B* (i.e., marginal possibility) respectively.

Consequently, gender diagnosticity proposes that an individual may be predicted to be a man or woman from several gender-associated factors (e.g. an individual's occupational preference or carer status). Gender diagnostic probability in a particular population of men and women are computed following gender-related variable discriminant analysis, whereby Bayes' theorem is applied to individuals' discriminant function scores to assess the probability that an individual belongs to

a particular gender social group (i.e. is either a man or woman). Specifically, the discriminant analysis produces diagnostic probabilities identifying how man- or woman-like an individual is based upon gender-predictor variables (Lippa & Connelly, 1990).

The gender diagnosticity method demonstrates several advantages over pre-existing gender scales, such as the BRSI (Bem, 1974). Scales of masculinity and femininity typically characterise gender according to stereotypes, whereby this process identifies objective behavioural traits within a given population that differs between men and women. This approach also therefore identifies meaningful, and potentially clinically relevant, differences in gender, rather than providing only a classification. This procedure also facilitates the incorporation of a variety or gender dimensions, as gender diagnostic indicators, and highlights that this dimensionality may differ between masculine and feminine social groups. Moreover, this facilitates the measure of gender-related factors, which may demonstrate variance in time and sociocultural norms, and therefore is considered to have greater predictive utility in comparison to fixed scales of gender stereotypes (Lippa & Hershberger, 1999).

Using this methodology, GENESIS-PRAXY constructed a gender index (i.e. GGI) comprising several gender-related variables, which were derived from four constructs of gender: identity, roles, relations and institutionalised gender (Pelletier *et al.*, 2015). A composite measure of these gender factors was computed including: 1) household primary earner; 2) personal income; 3) number of hours per week spent doing housework; 4) level of stress at home; 4) BSRI masculinity score; and 5) BSRI femininity score. These components permitted to the calculation of a propensity score (i.e., the conditional probability of being a woman versus a man) based on these gender-factors. The GGI ranged from 0 to 100, with the higher scores relating to feminine characteristics derived from this model.

However, several limitations are evident within the implementation of the GENESIS-PRAXY gender questionnaire that must be addressed. This was restricted to predominantly male, older individuals, residing in North America who had experienced ACS, which may act as a source of selection bias. This questionnaire has not been cross-validated in non-patient or broader-patient populations (Nielsen *et al.*, 2021). Furthermore, in addition to gender characteristics being conditional to this cohort, these may alter in time in accordance to sociocultural perceptions and expectations of gender. Therefore, the application of gender scores, derived from GENESIS-PRAXY questionnaire components, may not be directly applicable to other cohorts. Consequently, the main objective of this chapter is to adapt and implement this questionnaire and analysis methods in the UK for future use in cardiovascular research. Moreover, this chapter aims to compare the GGI directly with a simple scale of perceived and self-reported masculinity and femininity as an alternative means of gender data collection.

4.3 Hypothesis & aims

4.3.1 Hypothesis

- The GENESIS-PRAXY gender-stratification questionnaire can be applied to a UK cohort and will demonstrate a continuum of gender scores in males and females.

4.3.2 Aims

- Adapt and implement the GENESIS-PRAXY questionnaire in a UK cohort.

- Generate gender scores for this population using principal component analysis (PCA).

- Compare the GENESIS-PRAXY questionnaire to a simple masculinity-femininity score.

4.4 Methods

4.4.1 Questionnaire composition & modification

The GENESIS-PRAXY questionnaire was modified for use in a UK cohort (Appendix i). The original questionnaire comprises elements targeting four main aspects of gender including: 1) gender relations, 2) gender identity, 3) gender roles, and 4) institutionalised gender. The gender variables measured include: primary earner status; employment status; hours of work per week; child care responsibility; child disciplining responsibility; hours of housework per week; and housework responsibility status; work, home and total stress experienced; confidence in stress management abilities; marital status; personal income; and level of education (Pelletier *et al.*, 2015).

In addition, pre-existing measures of gender-related variables were included within the original GENESIS-PRAXY questionnaire such as: gender-related personality traits assessed via the BSRI (Bem, 1974); social support–related variables derived from Enhancing Recovery in Coronary Heart Disease Patients Social Support Instrument (The ENRICHD investigators, 2000); perceived community and national social standing from the MacArthur Perceived Social Standing Scale (Adler *et al.*, 2000); and job value and job quality deficits-related variables modified from the Canadian Policy Research Network-Ekos Changing Employment Relationships Survey Questionnaire (Pelletier *et al.*, 2015).

In accordance with guidelines produced by Beaton *et al* adaptation of this questionnaire was required for a UK cohort (Beaton *et al.*, 2000). Such guidance is the most widely practiced of its type as demonstrated in a recent systematic review of cross-cultural adaptation and psychometric validation of research

instruments (Arafat *et al.*, 2016). Following review of the original questionnaire, the research team (i.e., Dr Paul Connelly, Professor Christian Delles, Miss Anna Clark) examined and modified the questionnaire to ensure semantic, idiomatic, experimental, and conceptual equivalence. These adaptions were reviewed by the lead researcher of GENESIS-PRAXY, Professor Louise Pilote, to ensure this modified questionnaire adequately ensured content consistency and face validity of the source questionnaire. The involvement of students of the School of Cardiovascular and Metabolic Health, University of Glasgow (i.e. the intended recipient of this questionnaire) was sought prior to the implementation of this questionnaire to ensure question clarity and comprehension.

Modifications included the replacement of Canadian income divisions with Scottish income tax thresholds, inclusion of private health insurance and clarification of paid maternity or parental leave beyond statutory entitlements, and direct reference to Scotland with respect to scales of subjective social status. Additional demographic questions addressed gender identity (i.e. classification of sex assigned and birth and gender identity), ethnicity, age, stage, school and programme of University study, and living situation (e.g. living with parents or in private accommodation). Lastly, Likert scales of how masculine and feminine a person feels and how these individuals believe they are perceived were included. Participants were invited to rate 'on a scale of 1 to 7 how masculine do you feel?', with 1 representing not being masculine at all and 7 being extremely masculine. Then participants rated 'on a scale of 1 to 7 how masculine do you think other people perceive you?'. In a similar fashion, participants were asked to rate how feminine they felt and how they are perceived.

Prior to completing this anonymised questionnaire, it was explained to participants that they would answer questions relating to gender-based characteristics. However, no examples of stereotypical masculine and feminine behaviour, or gender-based characteristics, were provided prior to commencing this questionnaire.

4.4.2 Participant recruitment

This final Modified GENESIS-PRAXY Questionnaire (MGPQ) was constructed in English using the Online Surveys tool and distributed to students of the college of Medical, Veterinary and Life Sciences, University of Glasgow via anonymised email distribution lists (Appendix i). This was constructed by and administered by Dr Paul Connelly. Being a registered student of this institution was considered the only inclusion criteria of this questionnaire. With the permission of the head of the college of Medical, Veterinary and Life Sciences, university postmasters released an email to all students within this college. This email outlined the purpose of this study and provided a URL to the online questionnaire (Appendix ii). This questionnaire was sent out on the 21st January 2020. A further email was sent out on the 4th February 2020. This questionnaire was open for participation for a one month period.

4.4.3 Ethical considerations & confidentiality

Ethical approval was granted by the college of Medical, Veterinary and Life Sciences Ethics Committee at the University of Glasgow (200190067). Participants provided electronic written informed consent to participate (Appendix iii). Permission from the Head of the College of Medical, Veterinary and Life Sciences, Professor Dame Anna Dominiczak, was granted to provide access to generic student email lists, which were used for distribution of this electronic questionnaire. This was hosted by 'Online Surveys', which provides an online questionnaire tool, services and policies that are General Data Protection Regulation (GDPR) compliant. Completion of this questionnaire was completely anonymised. Prior to consenting and completing the self-administered questionnaire, participants were provided with a patient information sheet (Appendix iii) and privacy notice explaining what data was collected, why the data is required, the legal process for processing data, how long the data will be stored and the participants rights with respect to stored data (Appendix iii).

4.4.4 Gender score construction

In order to calculate gender scores, a similar statistical approach was utilised to the original GENESIS-PRAXY questionnaire analysis (Pelletier *et al.*, 2015). From participant responses, BSRI masculine and feminine scores were determined (Auster, 2016). The BSRI masculinity score was calculated as the mean of the self-rated Likert scales of 10 personality characteristics on the masculinity scale (i.e., defence of beliefs, leadership abilities, independence, willingness to take risks, assertion, dominance, strong personality, willingness to take a stand, forcefulness, and aggression). The femininity score was calculated as the mean of the self-ratings of the 10 personality characteristics on the femininity scale (i.e., affectionate, eager to soothe hurt feelings, sympathetic, warm, sensitive, tender, understanding, loves children, compassionate, and gentle). The remaining neutral characteristics were discarded.

Prior to PCA, correlational analysis was utilised to assess collinearity between gender-related variables (Kraemer, 2006). A correlation matrix was produced with correlation coefficients. For each variable pair that demonstrated correlation coefficients equal or greater than 0.8 (i.e., a very high correlation), one of the two variables was excluded at random via computation.

PCA is a multivariate statistical method used to reduce the dimensionality of a complex dataset while retaining a high degree of variability and statistical information (Greenacre *et al.*, 2022). Consequently, this increases the interpretability of the dataset while minimising the loss of data. PCA is a widely used statistical procedure in the analysis of questionnaires comprising a large number of items as a means of data reduction. This procedures aims to identify a smaller number of uncorrelated variables called principal components that accounts for the majority of the variability in the data.

PCA is achieved through the maximisation of variance through generation of new uncorrelated variables (i.e. principal components) that are linear functions of variables within the source dataset (Jollife & Cadima, 2016). These principal components demonstrate geometric properties that facilitate the interpretation of the essential features of multidimensional and complex datasets. They are ordered by the degree of variance they account for whereby the first component represents the linear combination that accounts for the largest possible variation. Succeeding components represents the linear combination that accounts for the largest variation not captured by the previous component with the restriction that they must be orthogonal to the previous component. The number of principal components cannot exceed the number of original variables (Subbuthai *et al.*, 2012).

For this analysis the principal axis method of PCA was used to extract components. This was achieved by identifying new uncorrelated variables (i.e., principal components) that are linear combinations of those evident within a given dataset that maximise variance. The number of components generated cannot exceed the number of original variables.

Assumptions of sampling and data adequacy were undertaken prior to analysis to ensure appropriateness of analysis via PCA (Shrestha, 2021). Sampling adequacy was assessed via the Kaiser Meyer Olkin test among proposed variables. This contrasts the magnitudes of the correlation coefficients to the magnitudes of the partial correlation coefficients. In other words, this assesses sampling adequacy for each variable in the model and for the overall model. A minimum Kaiser-Meyer-Olkin index of sampling adequacy of 0.6 was considered sufficient, whereas values less than 0.6 indicate the sampling is not adequate. Bartlett's test of sphericity was also utilised. This tests the null hypothesis that correlation matrix is an identity matrix (i.e. orthogonal). In an identity matrix variables are unrelated and not ideal for PCA. Rejection of this null hypothesis (i.e. demonstrations that the correlation matrix is not an identity matrix) is recommended for PCA.

Component eigenvalues are representative of the proportion of total variance accounted for by that component, whereby values of greater or equal to 1.0 are judged to be significant. Scree plots were produced, which represent a simple graphical line plot of the eigenvalues of each successive component. The number of components to be retained was determined using Kaiser's criterion (eigenvalue ≥ 1) and points of inflection (i.e. Cattell's scree test), evident on the plot following visual inspection (Dinno, 2009). Parallel analysis was also undertaken to aid in the selection of components utilising Monte-Carlo simulation of eigenvalues obtained by random data generation (Franklin *et al.*, 1995).

Following the extraction of components, varimax rotation, a means of orthogonal rotation, was utilised to maximise the variance of the squared loadings (Abdi & Williams, 2010). This produces a small number of large loadings and a large number of small to zero loadings for retained components, thereby simplifying interpretation. Loadings within this context represent the size and direction of an item's relationship with a given principal component. An item was considered to have loaded onto a particular component when loadings were equal to or exceeded 0.4 for a specific component, and remained less than 0.4 for other components, taking into account the directionality of that loading (Pelletier *et al.*, 2015).

Items loaded on retained components were then assessed via logistic regression for their association with female sex. Stepwise regression was employed, whereby a series of regressions were undertaken and items not significantly associated with sex were removed based on respective P-values (Pelletier *et al.*, 2015). Logistic regression coefficients from the final model were then used to calculate a gender score (i.e., the propensity score of being female multiplied by 100). This score (i.e. the GGI) reflects an estimate of the conditional probability of being female versus male while taking account gender variables retained in the PCA (Pelletier *et al.*, 2015). This GGI was utilised to categorise participants in accordance with masculine and feminine characteristics captured by the questionnaire, and

consists of a score between 0 and 100, whereby higher scores represent reflect higher level feminine characteristics within this sample. Conversely, lower scores are associated with more masculine traits, while intermediate score represent masculine and feminine characteristics.

In a similar fashion, Likert scales assessing self-assigned masculinity and femininity were included within a logistic regression model, whereby female sex was treated as the dependent variable. Logistic regression coefficients from this model were then used to calculate a masculinity-femininity score (i.e., the propensity score of being female multiplied by 100), which was compared to the GGI.

4.4.5 Statistical analysis

All statistical analyses were completed using R version 4.2.2 (R Core Team, Vienna, Austria), with P-values of <0.05 considered to be statistically significant. Data were expressed as means (standard deviation) unless otherwise stated. Missing data was handled via multiple imputation by chained equations. The C-statistic was utilised as a measure of goodness of fit for binary outcomes in the logistic regression model. Gender score distributions were assessed via a two-sample Kolmogorov-Smirnov test. Receiver operating characteristic (ROC) curves were utilised to compare composite gender (I.e. GGI) and masculinity-femininity scores. DeLong's test was utilised to assess differences between ROC curve areas under the curve (AUC). Parametric and non-parametric analyses were undertaken where appropriate.

4.5 Results

4.5.1 Participant demographics

The questionnaire was received by a total of 7,664 email addresses of students of the College of Medical, Veterinary and Life Sciences, University of Glasgow. 460

participants (6.0%) completed this between January and March 2020. For the primary cisgender analysis, 17 participants (3.7%) were excluded. These individuals identified as transgender women (n=2), transgender men (n=4) or gender non-conforming (n=11). Additionally, one member of an external college who completed this analysis was excluded.

	Male	Female
n (%)	104 (23.3%)	342 (76.7%)
Caucasian, n (%)	87 (83.6%)	300 (87.7%)
Age (years), median (IQR)	23 (21 - 26)	22 (20 - 26)
Undergraduate student, n (%)	62 (59.2%)	216 (63.2%)
Life Science Degree Programme, n (%)	33 (31.7%)	119 (34.8%)
Rented accommodation, n (%)	69 (66.3%)	247 (72.2%)

Table 4-1. Demographics of questionnaire respondents.

Therefore, in total 446 individuals were included within this analysis of which 23.3% were cisgender men and 76.7% were cisgender women (Table 4-1). The majority of students were Caucasian (86.8%). The median age of respondents was 22 (IQR 20, 26). Most respondents were undergraduate students (62.8%), and were members of life science (34.1%), medical (26.0%) and graduate (26.0%)) degree programmes. Rented accommodation was the most common home circumstance of those who completed the questionnaire (70.9%), while 15.7% resided in their parental home.

4.5.2 Correlation Analysis

A correlation matrix was produced from all questions in the MGPQ. Question 6 (For the children or other people living with you, to what level are you directly

responsible for caring for them?) demonstrated high correlation (correlation coefficient= 0.94) with question 7 (For the children or other people living with you, to what level are you directly responsible for disciplining them?). Following random deselection of question 7, the correlation matrix was repeated with no evidence of significant correlation between any questions (Figure 4-2).

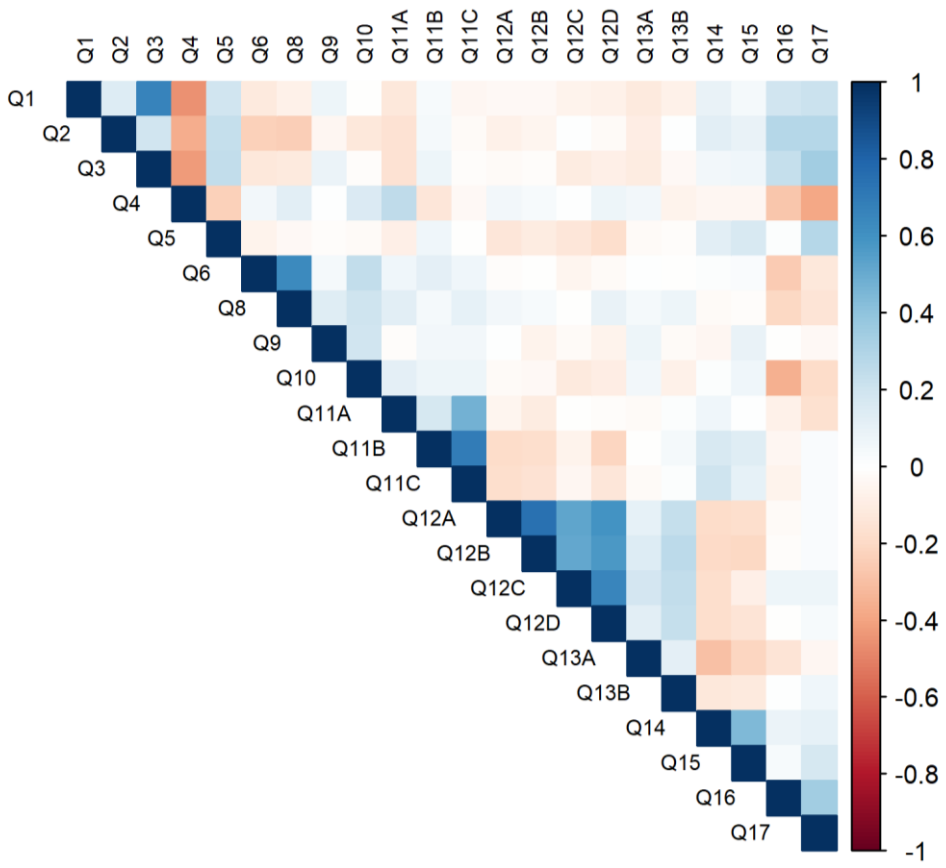

Figure 4-2. Correlogram of MGPQ responses.

This correlogram demonstrates the relationship between values of questionnaire variables and their corresponding correlation coefficient (r) values.

4.5.3 Principal component analysis

The Kaiser-Meyer-Olkin measure of Sampling Adequacy (KMO=0.69) and Bartlett's test of sphericity ($X^2(231)=2908.3$, $P<0.001$) demonstrated both sample size and data were satisfactory for conducting PCA (Shrestha, 2021). A scree plot was

utilised to determine the number of principal components to retain in the PCA (Figure 4-3). A total of 7 components demonstrating eigenvalues of greater than 1 (i.e. Kaiser's criterion) were identified, which accounted for a cumulative variance of 63% (Dinno, 2009). Visual inspection of the scree plot demonstrated multiple inflection points (i.e., Cattell's scree test), and suggested the inclusion of between 2 to 6 components.

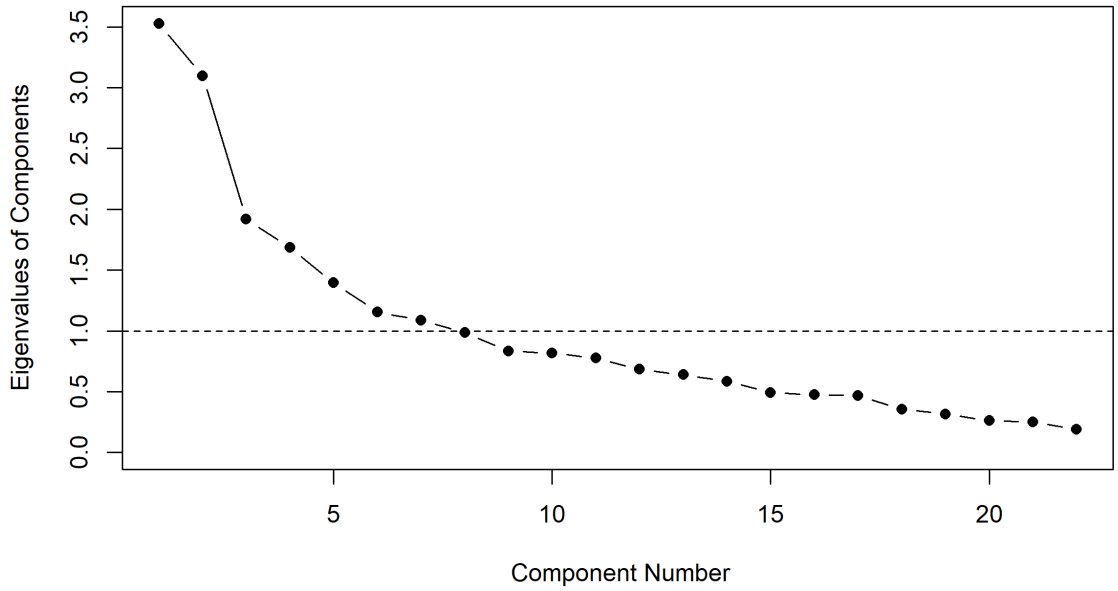

Figure 4-3. Scree plot representing principal component eigenvalues.

Consequently, parallel analysis was employed as a superior means of selecting principal components in this analysis (Franklin *et al.*, 1995). A Monte-Carlo simulation of eigenvalues obtained by randomly generated sets of data (n = 1000) of the same size (number of variables and observations) was utilised (Figure 4-4). This identified five components with eigen values greater than those produced in a simulated analysis to be extracted, which accounted for 53% of the cumulative variance of the PCA.

Principal components underwent varimax rotation, which minimises the number of variables with high loadings across multiple components (Kaiser, 1974). Following rotation, eigenvalues were of the 5 rotated principal components were

3.53, 3.10, 1.92, 1.68, and 1.39. Thereafter, loadings for each rotated component were then assessed. An item was considered to have loaded onto a particular component when loadings were equal to or exceeded 0.4 for a specific component, and remained less than 0.4 for other components (Pelletier *et al.*, 2015). Using these criteria, a total of 21 gender-related variables were found to load on the six retained rotated components (Table 4-2). These variables were then utilised within the subsequent logistic regression analyses. There were no instances where a questionnaire item was found to be loaded on more than one component.

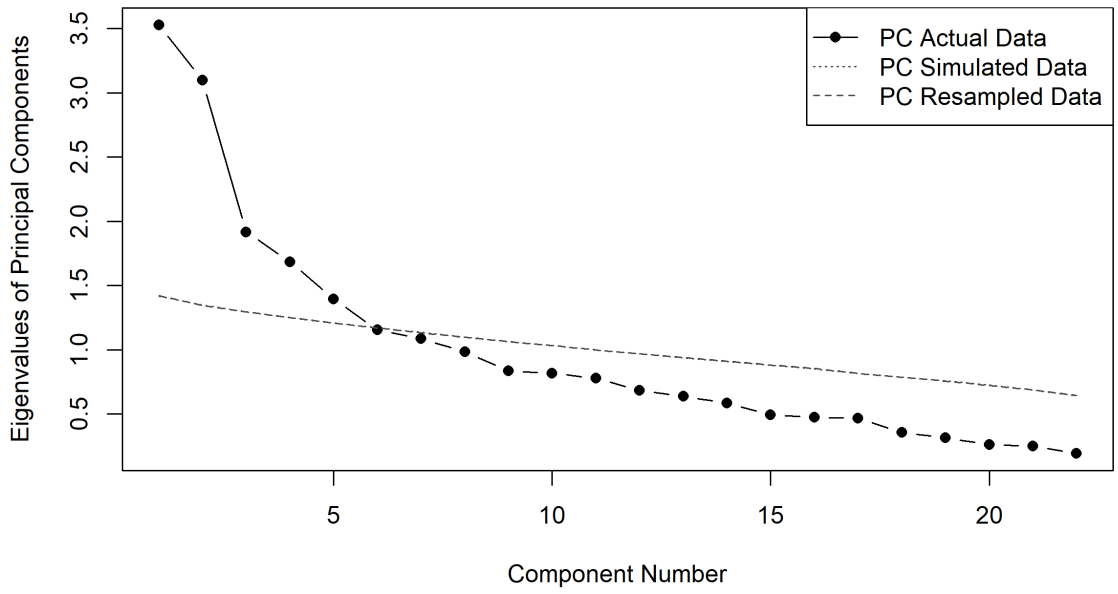

Figure 4-4. Parallel analysis of principal components.

This figure demonstrates eigenvalues from simulated data and corrected eigenvalues obtained from resampling the original data. PC: Principal components.

	RC1	RC2	RC3	RC4	RC5
Q1 – Work status	-0.06	0.75*	-0.08	0.06	-0.03
Q2 – Education level	-0.01	0.41*	0.00	-0.40	0.16
Q3 – Current Job	-0.05	0.80*	-0.04	0.02	-0.03
Q4 – Hours worked	-0.02	-0.75*	-0.05	0.13	0.02
Q5 – Work benefits	-0.15	0.45*	-0.01	0.00	0.15
Q6 – Children care responsibility	0.05	-0.08	0.05	0.76*	0.13
Q8 – Children sickness responsibility	0.14	-0.07	0.07	0.76*	0.08
Q10 – Housework responsibility	-0.12	-0.05	0.04	0.60*	-0.03
Q11A – Stress at work	-0.01	-0.31	0.59*	0.06	0.03
Q11B – Stress at home	-0.11	0.17	0.81*	0.09	0.04
Q11C – Overall stress	-0.06	0.01	0.91*	0.06	0.08
Q12A – Someone available to listen to you	0.82*	-0.03	-0.16	0.05	-0.08

Q12B - Someone available to give you advice	0.81*	-0.01	-0.16	0.03	-0.12
Q12C - Someone available to give you affection	0.79*	-0.05	0.04	-0.11	-0.06
Q12D - Someone available to trust and confide	0.83*	-0.11	-0.11	-0.03	-0.04
Q13A -BSRI masculinity score	0.12	-0.05	0.07	0.09	-0.60*
Q13B – BSRI femininity score	0.43*	0.05	0.19	-0.04	-0.18
Q14 – Social standing in your community	-0.14	0.06	0.16	-0.03	0.76*
Q15 – Social standing nationally	-0.11	0.10	0.07	0.06	0.73*
Q16 – Primary earner status	0.07	0.33	-0.02	-0.51*	0.15
Q17 - Personal income	0.15	0.56*	0.03	-0.26	0.22

Table 4-2. Rotated component loadings of questionnaire items.

*refers to items significantly loaded on given components (cut-off ≥0.4). RC: rotated components; BSRI: Bem sex role inventory.

4.5.4 Logistic regression model

The 21 gender related variables identified via PCA were then assessed via logistic regression analysis to determine which variables were associated with female sex. Five key questionnaire components within the logistic regression model were

identified (Table 4-3). These included ‘Is there someone available to give you good advice about a problem?’, the BSRI masculinity and femininity scores, and ‘Where would you place yourself on this ladder?’ in reference to perceived social standing within the participant’s community and personal income. The C-statistic of this model was 0.71, thereby suggesting that these variables were performing better than chance to predict biological sex (i.e. C-statistic=0.5) (Pelletier *et al.*, 2015). This model permitted the calculation of probability of an individual being female, thereby producing a composite gender score (i.e. GGI) of between 0 and 100, with a higher score representing an increased probability of being female.

	Coefficient	Standard Error	P-value
Someone available to give good advice	0.37	0.12	0.002
BSRI masculinity score	-0.40	0.14	0.005
BSRI femininity score	0.30	0.13	0.021
Where you feel you stand in your community	-0.20	0.07	0.007
Personal income	0.31	0.07	<0.001

Table 4-3. Gender score logistic regression model

4.5.5 Distribution of gender scores

Scaled density plots for GGI scores in males and females are depicted in Figure 4-5. This is accompanied by gender score distribution demonstrated in the GENESIS-PRAXY study (Pelletier *et al.*, 2016). In this student cohort, gender scores derived from the MGPQ demonstrated two distinct albeit overlapping distributions ascertained via the Kolmogorov-Smirnov test (D=0.32, P<0.001). Median gender scores were 82 (IQR 74.0, 89.0) and 71 (IQR 54.8, 83.0) in women and men, respectively.

Participant demographics were then stratified according to gender score tertile (Table 4-4). Tertile 1 ranged between 12 and 75; tertile 2 ranged between 76 and 85; and tertile 3 ranged between 86 and 96. Higher scores were found to be associated with younger age, being an undergraduate, and rented accommodation (P<0.01).Importantly, although the percentage of participants scoring significantly on depression or anxiety HADS questionnaire item (Anxiety: 59.7%; depression: 21.1%), there was no association with gender score tertile in this population (p>0.05).

Demographics	Tertile 1	Tertile 2	Tertile 3	P-value
n	149	149	148	-
Gender score, median (IQR)	64 (54, 70)	81 (78, 83)	90 (87.75, 92)	<0.001
Age (years), median (IQR)	25 (22, 30)	22 (21, 25)	22 (20, 24)	<0.001
Males, n (%)	60 (40.3%)	25 (16.8%)	19 (12.8%)	<0.001
Caucasian, n (%)	125 (83.9%)	136 (91.3%)	126 (85.1%)	0.14
Undergraduate, n (%)	65 (43.6%)	99 (66.4%)	114 (77%)	<0.001
Rented accommodation, n (%)	100 (67.1%)	106 (71.1%)	110 (74.3%)	<0.01
Depression (HADS ≥ 8)	38 (25.5%)	26 (17.5%)	30 (20.3%)	0.21
Anxiety (HADS ≥ 8)	92 (61.7%)	85 (57.1%)	94 (63.5%)	0.29

Table 4-4. Participant demographics stratified by gender score tertiles.

IQR: interquartile range, HADS: Hospital Anxiety & Depression Score

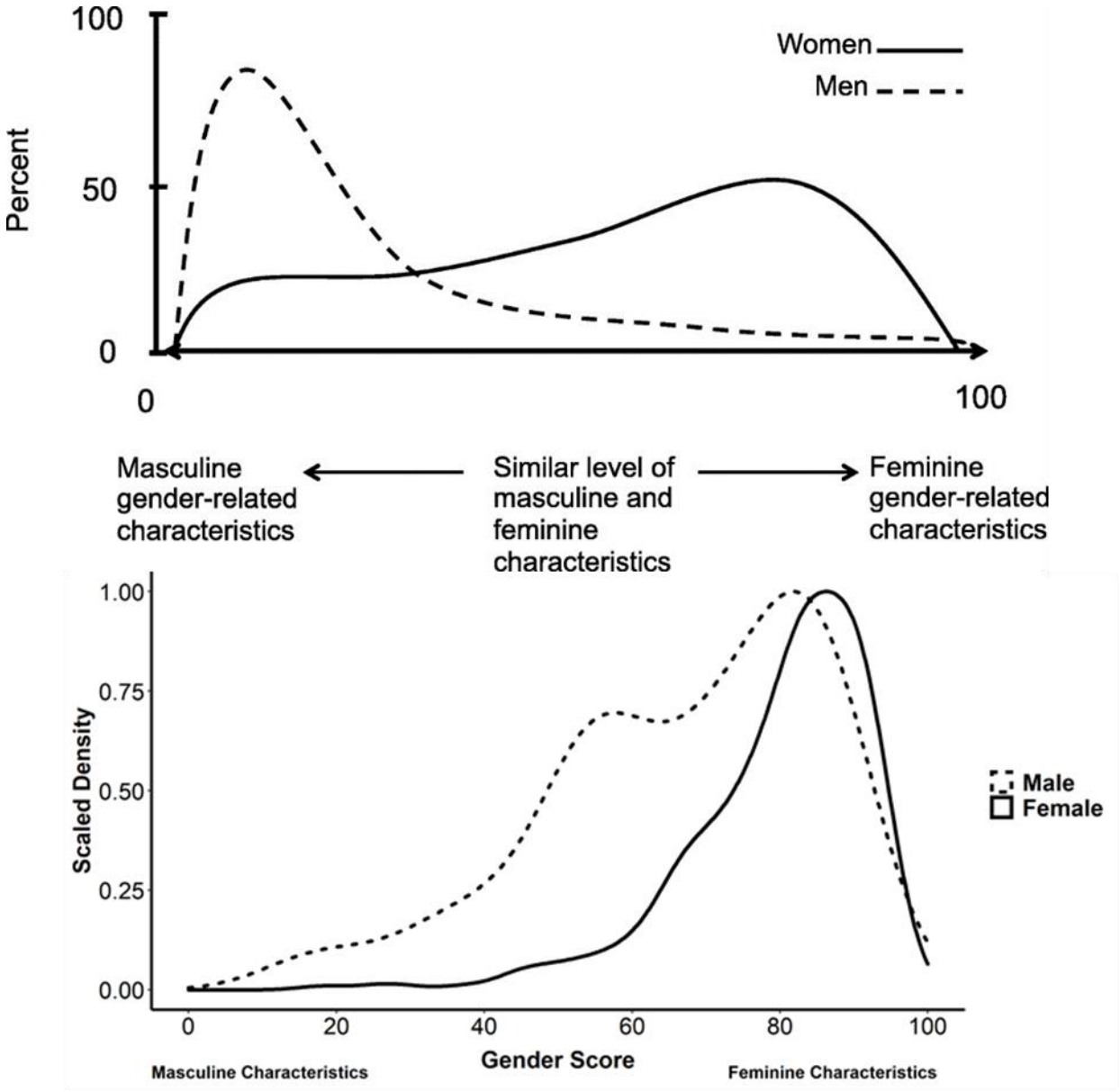

Figure 4-5. Gender Score distributions.

This figure demonstrates the gender score distributions from the GENSIS-PRAXY study (top) and results obtained from this analysis (bottom). Reproduced with permission (Pelletier *et al.*, 2015).

4.5.6 Masculinity-femininity scores

Likert scales assessing self-assigned masculinity and femininity were also assessed in this population. The results of these scales were assessed in a logistic regression

model with female sex being the dependent variable (Table 4-5). The C-statistic for this model was determined to be 0.97.

Propensity scores for female sex were then calculated. Median scores were 98 (IQR 95, 99) and 9 (IQR 3, 43) in females and males respectively. Scaled density plots for the distribution of self-assigned masculinity-femininity scores are depicted in Figure 4-6. These distributions were distinct (D=0.83, P<0.001), and demonstrate a continuum of responses in both males and females.

	Coefficient	Standard Error	P-value
Femininity	1.01	0.17	<0.001
Masculinity	-1.14	0.18	<0.001

Table 4-5. Self-assigned masculinity and femininity score logistic regression model.

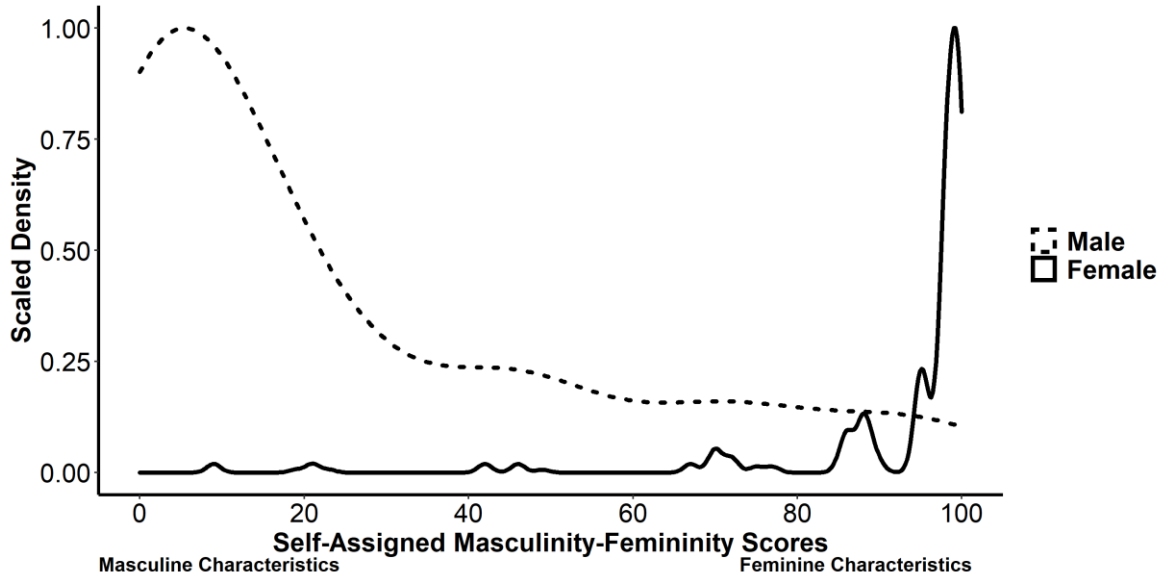

Figure 4-6. Composite masculinity-femininity scores.

This figure demonstrates Scaled density plot of Composite masculinity-femininity scores in males and females.

4.5.7 Gender scores comparison

Comparing the GENESIS-PRAXY composite gender score with our composite masculinity-femininity score within this study population, the self-assigned score had a greater sensitivity. ROC curves were generated in Figure 4-7, the AUC of the Gender Score was 71.4 (95% CI 0.66, 0.77) compared to 0.97 (95% CI 0.95, 0.98) in the perceived masculinity-femininity score (P<0.001). Although Spearman's correlation demonstrated no relationship between Gender Scores and the Masculinity-Femininity Score in males (rho=0.16, P=0.09), a correlation was evident in females (rho=0.15, P=0.006), which may reflect different sample sizes between these groups.

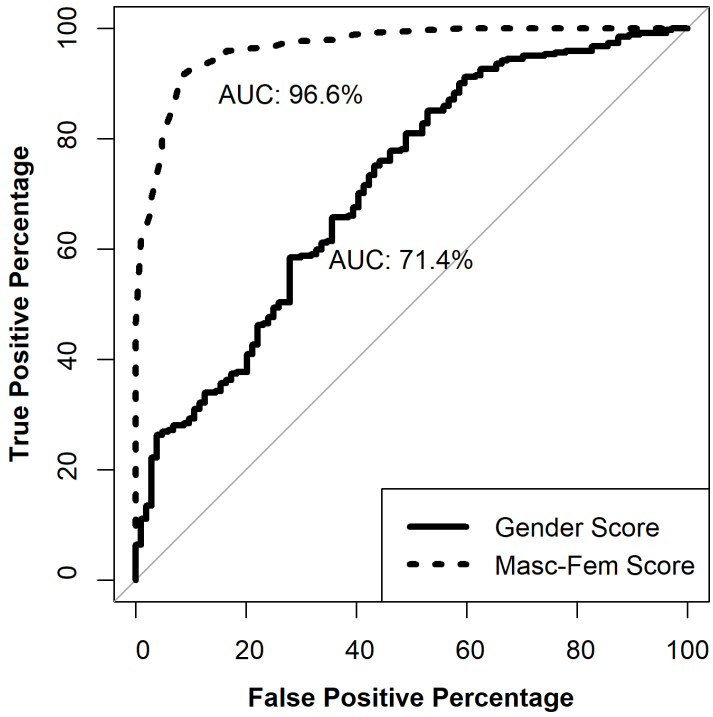

Figure 4-7. ROC curves for composite gender and masculinity-femininity scores.

4.5.8 Self-assigned versus perceived gender

Self-assigned masculinity and femininity were assessed in addition to how that that person believed others to perceive these traits (Figure 4-8). There were no significant differences demonstrated between self-assigned and perceived femininity in either males and/or females ($P>0.05$). However, both sexes demonstrated conflict regarding their internal sense of masculinity, and how masculine they felt they were perceived by others ($P<0.001$). This discrepancy between self-assigned and perceived masculinity was evident when the study population was stratified by sex also.

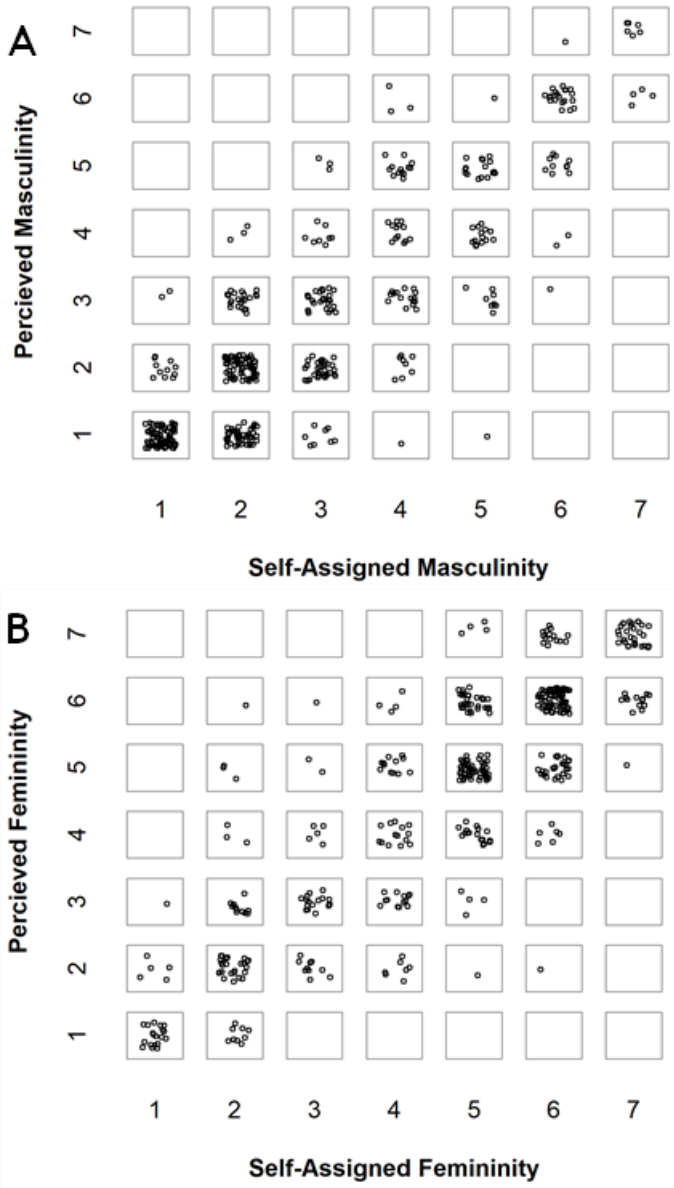

Figure 4-8. Self-assigned and perceived masculinity (A) and femininity (B) Likert scales. Participants were asked: 1) On a scale of 1 to 7 how masculine do you feel?; 2) On a scale of 1 to 7 how masculine do you think other people perceive you?; 3) On a scale of 1 to 7 how feminine do you feel?; 4) On a scale of 1 to 7 how feminine do you think other people perceive you?. In these scales, 1 represents not being masculine (or feminine) at all, and 7 represents being extremely masculine (or feminine).

4.6 Discussion

Clinical research typically dichotomously stratifies individuals by sex (i.e. either male or female). However, the omission of the gender spectrum may impede analysis (Connelly *et al.*, 2021a). Gender is an integral psychosocial determinant of health and wellbeing. An individual's health such as smoking, alcohol and physical exercise, are greatly influenced by gender (O'Neil *et al.*, 2018). Moreover, it can also impact upon mental wellbeing, socio-economic opportunity and health equity (Haider *et al.*, 2020). Cardiovascular disease exemplifies this relationship whereby lower socioeconomic status, high levels of psychosocial stressors, and lower quality of life at the time of the ACS is more evident in women than men (Bucholz *et al.*, 2017). Consequently, the measurement and incorporation of gender into clinical research is imperative to correctly stratify study participants and incorporate this central and pervasive trait into analysis.

In this chapter the GENESIS-PRAXY questionnaire was successfully adapted and applied to a healthy UK population in order to validate its utility and analysis methods for future use in cardiovascular research. Distinct but overlapping gender scores distributions in 446 cisgender men and women were observed. These gender scores were derived from MGPQ items relating to: whether someone was available to provide good advice; where you felt you stood in your community, personal income; and BSRI masculinity and femininity scores. In addition, a simple self-assigned gender scale was utilised and found to be a more sensitive predictor of female sex than the GGI. Importantly, in both models a continuum of gender existed across sexes.

Several differences exist between these findings and the results of the original GENESIS-PRAXY study (Figure 4-5), which demonstrated more distinct distributions between men and women (Pelletier *et al.*, 2015). A major factor that may have contributed to this are components of the GGI model derived from the logistic regression analysis. In GENESIS-PRAXY the composite gender score (i.e. GGI) was

obtained from responses to household primary earner status, personal income, the number of hours per week dedicated to housework, the level of stress at home, and BSRI masculinity and femininity scores. Although some features of the composite gender score in this analysis were consistent with the GENESIS-PRAXY (i.e. personal income, BSRI masculinity and femininity scores) components such as advice availability and perceived community standing were found to be significantly associated with female sex in this analysis, therefore providing a different model by which the GGI is calculated.

Moreover, these differences may be a consequence of the populations sampled. The GENESIS-PRAXY cohort was derived 273 women (30%) and 636 men (70%) aged 18 to 55 years who experienced ACS and with significant cardiovascular risk factors (Pelletier *et al.*, 2016). Adverse health behaviours leading to poorer cardiovascular health are intrinsically linked to societal inequalities, which may manifest within the context of gender (Short & Mollborn, 2015). Consequently, this may have contributed to sampling bias within GENESIS-PRAXY and explain some differences observed in this analysis. This study population in this chapter was also significantly younger with a median age of 22 years compared to 43 years in the GENESIS-PRAXY cohort. Younger individuals may not have adopted traditional gendered characteristics, or the items within the questionnaire may not reflect modern gender stereotypes. Interestingly, the lowest gender score tertile group was older than the other groups (Table 4-4). In a recent cross-temporal meta-analysis of U.S. college students BSRI evaluations, femininity scores have decreased significantly across the last three decades, which may indicate cultural changes in expected feminine gender norms (Donnelly & Twenge, 2017). Additionally, given that the participants in this study were students, they may not be a representative sample, whereby non-students may elicit differing gender roles and relations at equivalent ages, and contributed to participant bias (Ebert *et al.*, 2014).

Additionally, this questionnaire yielded a low response rate, with only 6% of recipients completing it. This may have introduced bias, whereby the individuals choosing to respond to this questionnaire may not be representative of the broader population. Similarly, self-selection bias could have influenced participation, with individuals completing the questionnaire due to specific interests or opinions on the topic. Consequently, this may partially account for the overrepresentation of transgender individuals in the respondents, and impacts upon the external validity of these findings. It is therefore possible that the differences observed in gender score distributions evident in this chapter compared to the original GENSIS-PRAXY analysis may have arose as a consequence of these biases.

Furthermore, GENESIS-PRAXY collected data between 2009-2013, whereas this study was completed almost a decade later. It is therefore possible that gender expectations have evolved during this time period thereby rendering traditional gender norms and expectations obsolete and resulting in a more homogenous gender spectrum (Ellemers, 2018). Although multiple indicators of gender inequality have improved dramatically since the 1970s, in recent decades this has slowed or indeed halted, and therefore this is unlikely to account for differences observed within this analysis over a relatively short period of time (England *et al.*, 2020).

Importantly, these populations were also sampled in different geographical locations and cultures. In GENSIS-PRAXY most recruiting centres (n=24) were in Canada, while the United Sates and Switzerland provided a single recruiting centre each. Consideration should be given to the possibility that the disparities between the outcomes of this questionnaire and GENESIS-PRAXY may reflect distinct cultural differences between Scottish and North American populations. Gender roles, expectations, and behaviours may differ between groups as a result of geographic locations and related societal norms (Schmitt et al., 2017). However, this could also indicate a failure of this questionnaire to adequately capture gender within this population.

It is therefore unsurprising that the results varied between these analyses given the demographic, temporal and sociocultural differences that existed between populations. This should not be a barrier to the implementation of gender scores, however, Bayesian principles and the relative contribution of gender within a given cohort should be considered. Consequently, measurement of gender via questionnaires may need to be tailored to the age, location and culture of participants, and some questions may need to be altered over time to reflect societal changes norms (Donnelly & Twenge, 2017). Importantly, the GENESIS-PRAXY methodology has recently been applied to data extracted from the Austrian Health Interview Survey and Canadian Community Health Survey, which demonstrates significant overlap of gender scores between males and females at a population level, which may suggest that the overlapping but distinct gender score distributions may be appropriate (Azizi *et al.*, 2021).

A major strength of this analysis is the distribution of male and female participants. Within the original GENESIS-PRAXY analysis ~70% of participants were men, however, the inverse it true in this analysis. Given that the outcome variable in the construction of the composite gender score was female sex, a broader distribution of females within this study may have contributed to the altered distribution of GGI scores observed.

Interestingly, in this analysis higher (feminine) scores were found to be associated with younger age, being an undergraduate, and rented accommodation ($P<0.01$). Although, no association was found between GGI scores and either anxiety or depression, it is important to note that 59.7% and 21.1% individuals scored highly on HADS questionnaire anxiety and depression scores, respectively. This may have impacted the distribution of scores within this population. Importantly, this questionnaire signposted individuals to the University of Glasgow Counselling & Psychological services webpage if they were feeling low, stressed or not coping.

In comparison, in the GENESIS-PRAXY study higher GGI scores were associated with anxiety, and may be a pathway by which social roles or personal characteristics traditionally ascribed to woman elevate recurrent ACS risk (Pelletier *et al.*, 2015, 2016). These results have important ramifications for the general wellbeing of students, who are at substantial risk of these conditions (Ibrahim *et al.*, 2013).

This analysis also aimed to compare the GENESIS-PRAXY questionnaire to a simple masculinity-femininity score via the inclusion of Likert scales assessing self-assigned masculinity and femininity. Following the construction of a composite masculinity-femininity score, the median scores in females and males were 98 (IQR 95, 99) and 9 (IQR 3, 43), respectively. Overall a spectrum of overlapping scores were observed in either sex, however, the distribution was much more disparate than observed in the GENESIS-PRAXY study (Pelletier *et al.*, 2015). Importantly, there were no differences between self-assigned and perceived feminine gender, suggesting that an individual's perception and expression of feminine traits expression was congruent. However, this relationship did not hold true for masculine traits. The variance between self-assigned and perceived masculinity, suggests an apparent difficulty in masculine gender expression in men and women, which should be taken into account when constructing measurements of gender. As the self-assigned composite score demonstrated greater sensitivity compared to the GGI in this cohort, its inclusion may demonstrate utility in future cardiovascular research.

There are a number of limitations in this model of gender stratification, which are shared with the original GENESIS-PRAXY investigation. Firstly, sex was used as a dependent variable with the logistic regression analysis to predict feminine traits (Pelletier *et al.*, 2015). Although sex is an imperfect proxy for gender, this limitation was at least in part offset through the recognition that individuals demonstrated congruence between the sex they were assigned at birth and their gender identity. Importantly, there is a need to undertake analyses such as this

using more representative samples within the general population and in individuals with a variety of cardiovascular diseases.

These measures of gender should be applied and validated in sufficiently powered analyses of transgender individuals, which would have not been possible in an analysis of this size. Future studies should be conducted to identify the validity of the MGPQ in both transgender and mixed populations of sufficient power to ensure validity. Given that 3.7% of respondents were self-reported as transgender, the inclusion of this population in gender-related research is imperative to the understanding of the broader role of gender in health and disease (Rytz *et al.*, 2023).

A further limitation of stratifying gender via composite gender scores it that is restricts gender to a one-dimensional spectrum. As such individuals are considered to have masculine, neutral or feminine gender. This does not fully reflect the complex gender-related behavioural traits whereby an individual may exhibit contrasting gender identities, roles and behaviours in different facets of their life. Recently researchers have developed the Stanford Gender-Related Variables for Health Research tool for gender assessment. This embraces a multi-dimensional approach whereby specific gender-related behaviours and attitudes are examined to understand their contribution to health and disease processes. However, this method has yet to be applied in the context of cardiovascular disease. Importantly, the impact of race on these quantitative tools to measure gender are required, as the majority of studies in this field have been conducted in broadly Caucasian populations (Nielsen *et al.*, 2021).

To conclude, this chapter has adapted and implemented a gender score questionnaire in a UK sample and piloted a simple masculinity-femininity question as a potential means of measuring gender in cardiovascular research. The utilisation of the latter requires assessment in the context of clinical research to identify whether the health implications of gender are captured by this method.

In both gender score models, evidence of masculine and feminine genders were observed regardless of sex. As a consequence, it is recommended that in addition to sex variables, a gender stratification method such as the GGI is incorporated into cardiovascular research programmes to better stratify the influences of these discrete concepts.

Chapter 5 Sex & Gender Dependent Differential MicroRNA Expression in Acute Coronary Syndrome

5.1 Chapter overview

In the previous chapter, a gender score questionnaire was adapted and implemented in a UK sample as a means of measuring gender in cardiovascular research in order to provide distinct information of the pathophysiological consequences of gender in addition to biological sex. In this chapter, a bioinformatic analysis of sex and gender stratified differentially expressed microRNA (miRNAs), obtained via next generation sequencing (NGS), in human plasma of individuals who have experienced acute coronary syndrome (ACS) is undertaken. Regulatory network analysis was then used predict sex and gender-dependent miRNA-gene interactions in this condition. This research provides novel insight into our understanding of the potential underlying pathophysiological processes associated with sex and gender in ACS.

5.2 Introduction

Despite advances in medical therapies, cardiovascular disease continues to be a leading global cause of death (Roth *et al.*, 2018). In Europe mortality relating to cardiovascular disease accounts for 49% of deaths in women and 40% of deaths in men (Townsend *et al.*, 2016). In recent decades there has been an observed reduction in the mortality associated with cardiovascular disease (Shah *et al.*, 2021). However, this trend is more readily apparent in men compared to women (Gupta *et al.*, 2014; Haider *et al.*, 2020).

A substantial burden of the morbidity and mortality in cardiovascular disease is a consequence of the development of ACS. ACS encompasses several conditions that promote myocardial ischaemia or injury. These include: 1) ST elevation myocardial infarction (STEMI), resulting from complete coronary thrombosis resulting in myocardial necrosis; 2) non-ST elevation myocardial infarction (NSTEMI), where there is partial coronary artery thrombosis and myocardial

necrosis; and 3) unstable angina, which results from a partially occluded coronary artery in the absence of myocardial necrosis (Pagidipati & Peterson, 2016). Concerningly, the incidence of hospitalisation relating to ACS and case fatality rates in young women have increased, despite decreasing in men (Arora *et al.*, 2019; Vaccarino, 2019).

Sex differences in coronary artery anatomy and physiology may account for some of the disparities in outcomes observed between males and females (Haider *et al.*, 2020). Females experience increased coronary blood flow, smaller epicardial coronary artery diameter and higher endothelial shear stress when compared to males, which may alter coronary endothelial function and modulate atherosclerosis pathophysiology and response to treatment (Hiteshi *et al.*, 2014; Patel *et al.*, 2016). In a prospective study of 697 patients (24% female) with ACS, females experienced less extensive coronary artery disease via angiography or radiofrequency intravascular ultrasound (Lansky *et al.*, 2012). Moreover, comparatively these lesions were associated with less plaque rupture, necrotic core and calcium. These features appear to be modified by age, whereby females demonstrate increasing plaque rupture and vulnerability later in life (Seegers *et al.*, 2022). Consequently, the underlying pathophysiology of ACS is sex-dependent, whereby plaque rupture occurs more predominantly in males, while plaque erosion and thrombosis occurs more frequently in females (Haider *et al.*, 2020).

In addition to obstructive coronary artery disease, 6-8% of myocardial infarction (MI) occur in non-obstructive coronary arteries (MINOCA) (Thygesen *et al.*, 2018). The underlying pathophysiology of this condition is diverse and includes plaque rupture or erosion with thrombosis, coronary artery dissection, epicardial coronary vasospasm, or coronary embolisation (Talebi *et al.*, 2021). This condition is associated with unfavourable outcomes and in comparison to obstructive disease patients are predominant younger and female (Pasupathy *et al.*, 2015; Parwani *et al.*, 2023). In an analysis of the Variation In Recovery: Role of Gender on Outcomes in Young Acute Myocardial Infarction Patients (VIRGO) study, females with MI were

five times more likely to have MINOCA compared to males (Safdar *et al.*, 2018). The sex-mediated mechanisms responsible for this condition remain unclear, but again demonstrate the stark differences evident between male and female coronary artery disease pathophysiology.

The mechanisms responsible for these differences are multifactorial and may represent a combination of both sex chromosome and hormone actions and interactions. For example, specific Y chromosome haplogroups are associated with increased risk of ischaemic heart disease, potentially via modulating the immune response and vascular inflammation (Charchar *et al.*, 2012; Eales *et al.*, 2019). Moreover, it is evident that oestrogen facilitates a number of anti-atherosclerotic mechanisms including the promotion of endothelial cell growth and repair, and the inhibition of vascular smooth muscle proliferation (Connelly *et al.*, 2019; Seegers *et al.*, 2022). Activation of the oestrogen receptor ERα in particular has been shown to promote endothelium-dependent vasodilatation via eNOS, endothelial proliferation and migration, and promotes carotid artery re-endothelialisation (Chambliss *et al.*, 2010; Kypreos *et al.*, 2014). Conversely, oestrogen has also been demonstrated to promote calcification in progressive atherosclerotic lesions via vascular smooth muscle differentiation to osteoblast-like cells (McRobb *et al.*, 2017). This demonstrates a potential mechanism by which oestrogen may in fact promote atherosclerosis in those with established atherosclerotic disease, and highlights that perturbation of oestrogen signalling may facilitate the development of ischaemic heart disease and ACS across the lifespan.

In addition to sex-mediated effects, components of gender are likely to contribute to differences observed in ACS outcomes between men and women. For instance, in a recent meta-analysis it has been demonstrated that there is less use of aspirin, statin and angiotensin-converting enzyme inhibitor primary care prescriptions in women compared to men (Zhao *et al.*, 2020). Multiple gender-mediated factors may facilitate this effect including lower patient awareness of the importance of

cardiovascular disease management in women, inadequate public health and healthcare provider engagement, and a failure of physicians to recognise the prevalence and importance of intensive disease management in this population. However, even provided with the context of pre-existing significant cardiovascular disease, such as admission to hospital with ACS, women have been shown to receive less acute and secondary prevention treatments compared to men (Hao *et al.*, 2019). These disparities and failures of cardiovascular disease management in women are not driven by sex-dependent biology but mediated via psychosocial gender-related factors.

With respect to specific components of gender, job insecurity and permanent work stress as aspects of gender roles, increase the odds of MI two-fold in men, but not women (Rosengren *et al.*, 2004; Netterstrøm *et al.*, 2010). Similarly, in the VIRGO study, women with acute myocardial infarction (MI) were found to have higher levels of socioeconomic deprivation and psychosocial stressors, and lower quality of life (Bucholz *et al.*, 2017). Moreover, in a study of 302,885 people aged 40-60, men who were married had a lower risk of MI compared to single or cohabiting men, which was not demonstrated in women, thereby demonstrating the differential impact of gender relations (Kilpi *et al.*, 2015).

Consequently, emerging evidence demonstrates the importance of gender, in addition to sex, in understanding discrepancies in ACS risk and outcomes in men and women. However, despite these advances, research of gender-related factors in cardiovascular disease has been hindered by our inability to appropriately measure gender and its constituent components (Clayton & Tannenbaum, 2016).

In the Gender and Sex Determinants of Cardiovascular Disease: From Bench to Beyond-Premature Acute Coronary Syndrome (GENESIS-PRAXY) study, recurrent ACS within 12 months occurred in 3% of males and females. Similarly, major adverse cardiovascular events (MACE) and all-cause mortality occurred in 8% and <1% in both males and females. In a sex adjusted cox proportional hazard

regression model, both recurrent ACS (HR 0.93, 95%CI 0.45, 1.92) and MACE (HR 0.71, 95%CI 0.41, 1.23) were not found to be statistically significant, suggesting that within this cohort of individuals with established ACS biological sex was not a determinant of future adverse cardiovascular outcomes.

In contrast, feminine characteristics resulting in a higher GENESIS-PRAXY Gender Index (GGI), as described in Chapter 4, was associated with an elevated risk of hypertension (OR 1.85 (95% CI 1.04, 3.29), diabetes (OR 2.07, 95% CI 1.00, 2.39), and recurrent ACS (OR 4.50, 95% CI 1.05, 19.27), which importantly was independent of sex (Pelletier *et al.*, 2016). Indeed, the rate of recurrent ACS was 5% in those with characteristics ascribed to women, when compared to either the gender 'neutral' (2%) or masculine groups (2%). Similarly, when the GGI methodology was applied within in an analysis of Canadian Community Health Survey (n = 63,522; 55% female), traits attributed to women were associated with adverse cardiovascular health and outcomes (Azizi *et al.*, 2021). Yet again, this relationship was independent of biological sex and baseline cardiovascular risk factors.

Given that differences in outcomes of the GENESIS-PRAXY groups can be observed in relation to gender the physiological consequences of gender stratification must be considered. Social epigenomics explores the mechanisms by which social experiences modulate epigenetic gene modification and regulation. It is hypothesised that this can be achieved though interaction between social and environmental stimuli promoting DNA methylation, post-translational histone modification, and regulation of non-coding RNAs, such as miRNA (Mancilla *et al.*, 2020).

Differential expression of multiple miRNA, including miR-21, miR-208a/b, miR-133a/b, miR-30 family, miR-19, and miR-20, have been shown to be associated with the development of ACS (Kaur *et al.*, 2020). miRNA may promote translational repression and/or mRNA degradation, and potentially gene upregulation, which

may modulate the development of ACS via multiple signalling mechanisms (Vasudevan, 2012). Moreover, sex-dependent miRNA expression has previously been demonstrating utilising the Cancer Genome Atlas database (Guo *et al.*, 2017), which enhances its candidacy as a putative biological mediator of gender traits.

However, our understanding of sex and gender differential miRNA expression in ACS is extremely limited. In the Nord-Trøndelag Health Study (HUNT) prospective nested case-control study circulating miRNA were investigated as putative predictive biomarkers of fatal acute MI (Bye *et al.*, 2016). Circulating miR-424-5p was found to be associated with a higher risk of acute MI exclusively males and miR-26a-5p in females. However, in a recent systematic review of miRNA dysregulation in ACS and stable coronary artery disease, only ~30% reported on the number of males and females assessed (Kaur *et al.*, 2020). Moreover, sex differences were not identified in the majority of these studies following adjustment for this in *post hoc* analyses.

Gender, or gender-related factors, have yet to be the subject of miRNA analysis in ACS. External exposures such as air pollution, smoking, physical activity, alcohol consumption, and even noise exposure have been shown to alter miRNA expression (Vrijens *et al.*, 2015; Panico *et al.*, 2021). Furthermore, in a murine model of social isolation following stroke, there was dysregulation of cerebral miR-297a-3p and miR-200c-3p, which are regulators of cell proliferation and impaired post-stroke recovery (Holmes *et al.*, 2020). Social isolation promotes the development for stroke and MI, which has been demonstrated to be a more potent risk factor in women compared to men, thereby highlighting the potential importance of miRNA as mediators of gender in the vasculature (Hakulinen *et al.*, 2018). Consequently, the opportunity to assess miRNA differential expression in a well gender-stratified cohort may facilitate a better understanding of the pathophysiological pathways contributing to differences observed in ACS between

men and women, that have not yet been fully elucidated in binary sex-based analyses.

This chapter will therefore explore the biological underpinnings of sex and gender-mediated effects on ACS via assessing differential miRNA expression, and associated gene networks, in a subgroup of the original GENESIS-PRAXY participants. Individuals at the most extreme ends of the gender spectrum were contrasted with respect to differential miRNA expression. To achieve this goal miRNA was extracted from this cohort and NGS was undertaken. A bioinformatics analysis of differentially miRNA then identified the up- or downregulation of miRNA in individuals stratified by both sex and gender. Network gene analysis was then employed to explore putative gene relationships, which may act as drivers of sex and gender-driven gene regulation in the context of ACS.

5.3 Hypothesis & aims

5.3.1 Hypothesis

- Plasma miRNA will be differentially expressed according to sex and gender phenotypes, stratified using the GENESIS-PRAXY gender-stratification questionnaire, in individuals with ACS.

5.3.2 Aims

- To extract miRNA from peripheral blood samples in a subgroup of feminine males/females and masculine males/females from the GENESIS-PRAXY cohort.

- Identify differentially expressed circulating miRNA in individuals who have experienced ACS, stratified by sex and gender.

- To undertake a bioinformatics analysis of differentially expressed miRNA and associated genes to investigate potential relationships between sex, gender and ACS.

- To perform regulatory network analysis to predict miRNA-gene interactions in relation to sex and gender-dependent differentially expressed miRNA in ACS.

5.4 Methods

5.4.1 Study population

GENESIS-PRAXY was a prospective study of ACS patients aged 18–55 admitted to 24 centres (24 in Canada, 1 in the United States of America, and 1 in Switzerland) between 2009 and 2013 (Pelletier *et al.*, 2016). Participants were eligible for inclusion if they were presenting with ACS, aged between 18-55 years, fluent in English and/or French, and able to provide informed consent. ACS was defined as having symptoms considered consistent with acute cardiac ischaemia within 24 hours of hospital presentation and at least one of the following:

- Electrocardiographic changes: transient ST-segment elevations of ≥ 1 mm, ST-segment depressions of ≥ 1 mm, new T wave inversions of ≥ 1 mm, pseudo-normalization of previously inverted T waves, new Q waves (10/3 the height of the R wave or ≥ 0.04 seconds), new R wave > S wave in lead V1 (posterior MI), new left bundle branch block (changes should be seen in ≥ 2 contiguous leads)

- Increase in cardiac enzymes: creatine kinase-MB (CK-MB) > 2 times the upper limit of the hospital's normal range or if no CK-MB available, then

total creatine phosphokinase > 2 times the upper limit of the hospital's normal range, positive troponin I, or positive troponin T.

This sub-study comprised of 36 individuals who were included within the GENESIS-PRAXY study, stratified by gender score with available plasma samples for analysis. All data included in this study was obtained via self-administered questionnaire and blood samples obtained during the first 48 hours of admission.

5.4.2 Gender stratification

The self-administered questionnaire permitted the calculation of a composite gender score (Pelletier *et al.*, 2016). This was achieved via combining composite measures of the gender-related characteristics (e.g. social roles). Retained component variables form a principal component analysis of these factors, were utilised in a logistic regression, where biological sex was included at the dependent variable to derive a gender-related score. Coefficient estimates were used to calculate a propensity score (gender score), which represents the probability for each participant being female. Higher scores represent characteristics ascribed to femininity (gender score 70-100), intermediate scores (gender score 31-69) are gender neutral, and lower scores denote masculine characteristics (gender score 0-30). This questionnaire was completed within 24 hours of admission due to ACS.

Males and females with the 9 highest and lowest gender scores, representing the most masculine and feminine participants were included in this substudy. If more than 9 individuals fell into these groups due to identical scoring, 9 individuals were chosen due to sample availability or at random. Four groups comprising of 9 masculine males, 9 masculine females, 9 feminine females and 9 masculine females were created.

5.4.3 Plasma sample preparation

Following informed consent, patients provided a blood sample within 48 hours of hospital admission with ACS. Peripheral blood from ACS patients was drawn into BD vacutainers (sodium citrate) and centrifuged at 3000 rpm for 10 min at room temperature (~20 °C). The upper plasma fraction was carefully transferred into fresh tubes, centrifuged again at 3000 rpm for 10 min at room temperature, then distributed into aliquots and stored locally at −80 °C until being transported in dry ice to the McGill University Health Centre in Montreal, Canada. Plasma samples showed no evidence of gross haemolysis as evaluated by visual inspection and Nanodrop absorbance measurement at 414 nm (haemoglobin). Blood samples were transferred on dry ice to the BHF Cardiovascular Research Centre, Glasgow, UK where they were thawed and aliquoted for miRNA extraction.

5.4.4 miRNA extraction

The miRNA was extracted from plasma samples using the miRNeasy Serum/Plasma kit (Qiagen, Manchester, UK) following the manufacturers protocol. This was undertaken by Dr Paul Connelly with assistance from Dr Sheon Samji. Plasma samples (200 µL) were thawed and lysed with 5 volumes (200 µL) of QIAzol Lysis Reagent. The lysate solution was incubated at room temperature (15–25°C) for 5 minutes. 200 µL of chloroform was added to the lysate sample and mixed for 15s and then incubated at room temperature for 3 minutes. Samples were then centrifuged at 12,000 x g at 4°C for 15 minutes.

The upper aqueous phase, containing RNA, was removed and 900 µL 100% ethanol was added mix thoroughly. Up to 700 µL of the sample was then transferred to a RNeasy MinElute spin column in a 2 ml Eppendorf. This was centrifuged at 8000 x g for 15 s at room temperature. The flow through was discarded and the step repeated. 700 µL Buffer RWT was added to the RNeasy MinElute spin column and centrifuged for 15s at 8000 x g and the flow through was discarded. 500 µL Buffer

RPE was then pipped onto the RNeasy MinElute spin column and centrifuged at 8000 x g for 15s and the flow-through was discarded. 500 µL 80% ethanol was then added onto the RNeasy MinElute spin column and centrifuge for 2 min at 8000 x g to wash the spin column membrane.

The RNeasy MinElute spin column was then transferred to a new Eppendorf and centrifuged at full speed for 5 minutes to dry the membrane. The RNeasy MinElute spin column was then transferred to a 1.5 mL collection tube and 14 µL RNase-free water was added to the centre of the spin column membrane and centrifuged at full speed for 1 minute to elute the RNA. The 12 µL eluate of miRNA was then obtained and frozen at -80°C for future analysis.

5.4.5 miRNA NGS library preparation & sequencing

MiRNA next generation sequencing (NGS) library preparation and sequencing were performed by Glasgow Polyomics. MiRNA quality analysis was performed using the Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). MiRNA sequencing libraries were prepared from the extracted miRNA using the Qiagen QIASeq miRNA library prep kit. To summarise, specifically designed 3' adapters were sequentially ligated to mature miRNAs. These ligated miRNAs were then reverse-transcribed to generate cDNA using a reverse-transcription primer with a unique molecular index (UMI) tag. The cDNA library was then amplified via polymerase chain reaction (PCR) (21 cycles) and subsequently purified prior to quality control assessment. Libraries were sequenced on the Illumina NextSeq 500 platform in 75 base, single end mode to an average of at least 15 million reads per sample. Raw data was de-multiplexed and FASTQ data files for each sample were generated.

5.4.6 miRNA NGS processing & analysis

MiRNA NGS output was provided in FASTQ format, a text-based format for storing sequencing data and quality scores. These FASTQ files contain nucleotide

sequencing data with associated quality scores. Prior to differential miRNA analysis, these data require processing as depicted in Figure 5-1.

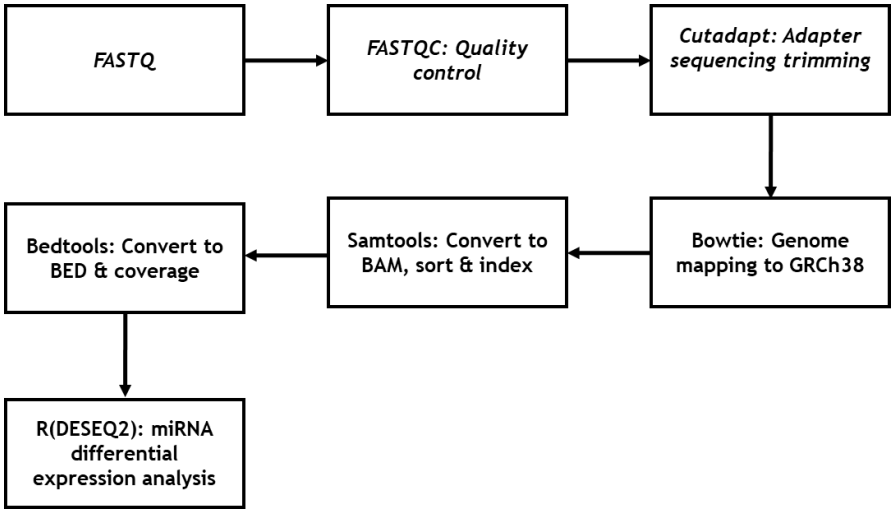

Figure 5-1. miRNA sequencing and processing pipeline.

These FASTQ files underwent initial quality control checks via FastQC (v0.11.9). This programme summarises and reports a variety of aspects of quality control including per base sequence quality across all bases at each position in the FastQ file, per sequence quality scores, per base sequence content, per sequence guanine-cytosine content, sequence duplication levels, overrepresented sequences and adapter content. These sample FastQC reports may be compiled into a singular report using MultiQC (v1.11) report encompassing all samples within the analysis (Ewels *et al.*, 2016).

NGS technologies introduce sequence tags, which are required for sample library preparation. These are ligated to target sequences and may overlap with sequenced regions. Consequently, these should be removed prior to downstream analysis. Adapter sequences were trimmed using the programme Cutadapt (v3.4) (Martin, 2011). For each FASTQ file, the adapter sequence for the QIAseq miRNA NGS 3' Adapter (AACTGTAGGCACCATCAAT) were trimmed and the reads were filtered to those with a Phred quality score (i.e. the measure of quality of the

identification of bases generated by automated sequencing) greater than 25, thereby retaining reads with base call accuracy of 99%. Moreover, processed reads shorter than 17 bases were discarded. These trimmed reads then underwent further quality control analysis using FastQC and MultiQC. FastQ Screen(v0.14.0) was also employed to quantifying the proportion of reads that map to a panel of reference human genome (Wingett & Andrews, 2018).

These trimmed reads were then aligned using the short read-aligner, Bowtie (v1.3.0) (Langmead *et al.*, 2009). Bowtie aligns reads with the aid of an index of a reference genome. The Bowtie index is a similar to that of the FM index (Ferragina & Manzini, 2000) in that both utilise the Burrows-Wheeler Transform as a means to increasing speed and efficiency. Firstly, the Bowtie build function was used to generate a collection of index files, which allow Bowtie to align reads to that reference genome. In this instance these index files were constructed from the Genome Reference Consortium Human Build 38 (HG38). Once created, Bowtie may align 35-base pair reads at a rate of over 25 million reads per CPU-hour. Bowtie aligned these reads against the human reference database and only mature miRNA sequence in miRBase were counted (Griffiths-Jones *et al.*, 2006).

The output of this alignment process was in Sequence Alignment Map (SAM) format as Bowtie does not write BAM files directly. Using SAMtools (v1.12), SAM files were converted to Binary Alignment Map (BAM) format, sorted and indexed to allow swift extraction of alignments which overlap particular genomic regions (Danecek *et al.*, 2021).

Bedtools (v2.30.0) was then used to convert BAM files to browser extensible data (BED) files using the bamtobed tool(Quinlan & Hall, 2010). The coverage tool was then applied to compute the depth and breadth of coverage of features in the sequence alignments of the BED files on the human genome coordinates of miRNAs. This was achieved using the hsa.gff3 file extracted from miRbase

(http://www.mirbase.org/ftp.shtml). Files were then imported to R (v4.2.2), where mature miRNA counts and sample data were merged for analysis.

5.4.7 Differential expression analysis

The programme DESeq2 was then used for differentially expression analysis on the compiled read data. Deseq2 may be used to identify differentially expressed miRNA and gene-wise dispersion via negative binomial generalized linear models (Love *et al.*, 2014). Prior to analysis reads were filtered to exclude miRNA where there less than two samples with less than 10 normalised read counts. Normalisation is achieved through estimates of size factors using the median ratio method (Anders & Huber, 2010). To overcome the high level of variance in logarithmic fold change estimates evident in miRNA with low read counts, logarithmic fold change shrinkage toward zero was employed. This empirical Bayesian procedure involves the generation of logarithmic fold change maximum-likelihood estimates via a generalised linear model, then fits a zero-centred normal distribution to the observed distribution of these estimates across all miRNA (Love *et al.*, 2014). Shrunken logarithmic fold changes are then utilised in the Wald test when assessing differential expression between groups (Wald, 1943). The Benjamini-Hochberg false discovery rate (FDR) of 5% was used for the adjustment of the resulting p-value. Consequently, miRNA were considered significantly differentially expressed if the fold change was >1.5 with an adjusted P-value (adj.P) of <0.05.

5.4.8 miRNA regulatory network analysis

miRNA regulatory network analysis was undertaken utilising the web-based tool, miRNet (v2.0) (Chang *et al.*, 2020). This integrated platform permits the creation and visual exploration of miRNA regulatory networks, and therefore the exploration of miRNA-gene interactions (Chang & Xia, 2023). The miRNet analytic process comprises of 3 components: 1) data input; 2) network creation; and 3)

network visual analytics (Chang *et al.*, 2020). Differentially expressed miRNAs were utilised as input for gene predictions and interactions were identified via miRTarBase v8.0 and TarBase v8.0 using mirBase accession IDs. Genes listed in these curated databases of miRNA–target interactions were selected for network construction (Papadopoulos *et al.*, 2009; Huang *et al.*, 2022). Minimum network connections was used as a filter for miRNA-gene interactions and enriched pathways, thereby excluding target nodes with less than 2 connections from the network (i.e. a degree filter of 2). Pathway enrichment was undertaken utilising Kyoto Encyclopaedia of Genes and Genomes (KEGG), Reactome, Gene ontology (GO) Biological Processes (GO-BP), GO Molecular Function (GO-MF), GO Cellular Component (GO-CC), and DisGeNET databases (Kanehisa & Goto, 2000; Young *et al.*, 2010; Jassal *et al.*, 2020; Piñero *et al.*, 2020). This incorporated all network genes and hypergeometrical testing was applied with an adjusted P-value <0.05 considered significant. Pathways demonstrating biological significance (i.e. related to cardiovascular physiology) and demonstrating a FDR of 5% were selected.

5.4.9 Ethics

In Quebec, a multicentre ethics review allowed for the McGill University Heath Centre to act as the central review board and coordinate ethics approval for all centres. All other centres received ethics approval from their respective hospital ethics review boards. Ethical approval was granted by the college of Medical, Veterinary and Life Sciences Ethics Committee at the University of Glasgow (200200018) following the implementation of a material transfer agreement.

5.4.10 Statistical analysis

All statistical analyses relating to this study were completed R version 4.0.2 (R Core Team, Vienna, Austria), with P-values of <0.05 considered to be statistically significant. The miRNA sequencing processing pipeline was undertaking on Ubuntu

20.04 LTS Windows Subsystem for Linux 2 (WSL2). Packages utilised in this miRNA NGS processing were obtained from the life science software distribution platform, Bioconda (Grüning *et al.*, 2018).

Data underwent pre-processing filtering to exclude poorly detectable miRNA, where the sum of counts per million mapped reads (CPM) for each miRNA for all samples were greater than 10. Statistical analysis of miRNA count data was undertaken using DESEQ2 (v1.30.1), a bioconductor package downloaded to R (v4.0.2) for differential expression analysis of RNA sequencing experiments (Love *et al.*, 2014). Poorly detectable miRNAs (i.e. those where the sum of the counter per million mapped reads were < 10) were filtered prior to the analysis. P-values and Benjamini-Hochberg FDR-correct P-values for differentially altered miRNAs were calculated in DESEQ2. Differentially expressed miRNAs were defined by FDR adj.P-values < 0.05. In the functional analysis hypergeometrical testing was employed, whereby adjusted P-values <0.05 were considered significant. Differences between groups for continuous data were assessed using a Mann-Whitney, Wilcoxon signed-rank or Kruskal–Wallis test as data were not normally distributed. Data are presented as median (interquartile range (IQR)) unless otherwise specified.

5.5 Results

5.5.1 miRNA study participant characteristics

miRNA was analysed in 36 individuals who had experienced ACS within 48 hours. These individuals were stratified according to gender scoring and had plasma samples available (Table 5-1). The median age of participants was 50 years (IQR 47.7, 53). There were no statistical differences in ages between groups (P>0.05) between groups. 94.4% of individuals were of Caucasian ethnicity. Median gender scores in males were 0.4 (IQR 0.1, 0.4) in the masculine (i.e. masculine males) and 83.8 (IQR 82.3, 85.9) in the feminine group (i.e. feminine males; P=0.004). Median gender scores in females were 4.4 (IQR 2.39, 6.3) in the masculine group (i.e. masculine females) and 98.6 (IQR 97.9, 98.8) in the feminine group (feminine females; P=0.004).

	Feminine Females	Feminine Males	Masculine Females	Masculine Males	P-value
n	9	9	9	9	-
Sex	Female	Male	Female	Male	-
Gender	Feminine	Feminine	Masculine	Masculine	-
Gender Score, median (IQR)	98.6 (97.9, 98.8)	83.8 (82.3, 85.9)	4.4 (2.9, 6.3)	0.4 (0.1, 0.4)	<0.001
Age, median (IQR)	50 (42, 51)	49 (48, 53)	53 (50, 53)	49 (48, 54)	0.59
Caucasian, n (%)	9 (100%)	9 (100%)	7 (77.8%)	9 (100%)	0.74
Smoker, n (%)	7 (77.8%)	8 (88.9%)	7 (77.8%)	6 (66.7%)	0.94

Table 5-1. Participants demographics

5.5.2 Sequencing quality control

FASTQ files underwent initial quality control assessments via FastQC. This was undertaken both before and after pre-processing steps such as filtering, quality and adapter trimming. FastQC assesses the quality of raw reads by providing per-base and per-sequencing evaluations of sample read quality. This quality control process, along with adapter trimming, was iteratively performed to ensure the extraction of low quality reads and adaptor sequences, which prevents their interference in future read mapping processes (Huang *et al.*, 2021).

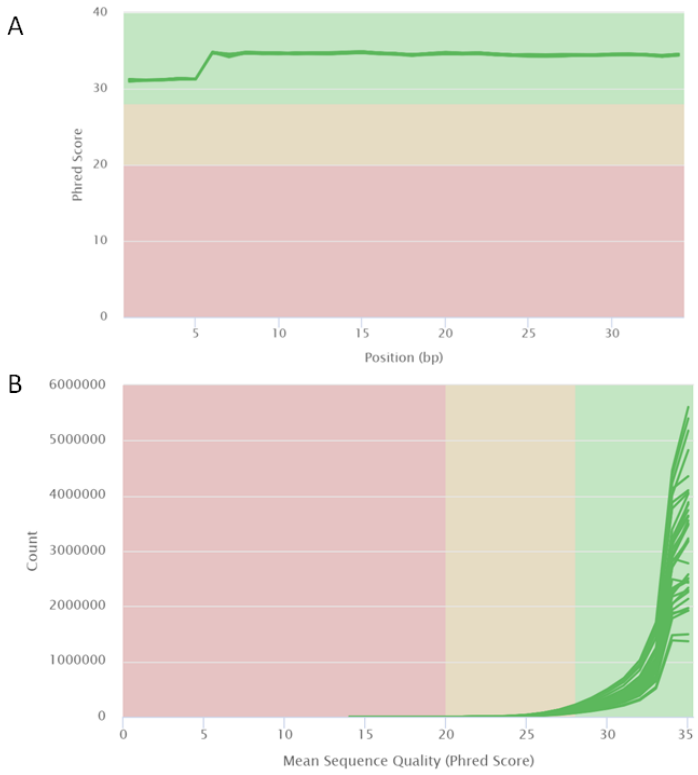

Figure 5-2. Post-sequencing quality control of FASTQ files using FastQC.
All samples were assessed for A) mean per-base and B) per-sequence quality via the Phred score.

Following the utilisation of FASTQC, MultiQC was used to visualise and inspect quality control outputs across multiple samples. Figure 5-2 demonstrates the sequence quality histogram representing the mean quality value across each base

position in the read. The Phred quality score indicates the measure of a base quality sequencing. These are calculated by comparing read signals to the probability of accurate base-reading and relates to base-calling error probabilities. This relationship is logarithmic ($Q = -10 \log_{10} P$), whereby scores of 30 or above demonstrate a probability of a base being called incorrectly of 0.001, and a base call accuracy of 99.9% (Zhang *et al.*, 2017). This quality control process was employed both before and after adapter sequencing trimming.

5.5.3 Differential expression of plasma miRNA

A total of 2,883 mature miRNAs were detected at any level across within the 36 samples. miRNA expression profiles were compared according to biological sex and gender using the following comparisons: male vs female; masculine males vs feminine males; and feminine females vs masculine females. Differential expression was confirmed using 1.5 fold change and adjusted P-values of less than 0.05 as cut-offs.

Dispersion, or variance, is representative of the relationship between the mean and variance (i.e. dispersion) of the expression of miRNA (Love *et al.*, 2014). To overcome the high level of variance, or dispersion, in logarithmic fold change estimates evident in miRNA with low read counts, logarithmic fold change shrinkage toward zero was employed in the differential expression analysis. This empirical Bayesian procedure involves the generation of logarithmic fold change maximum-likelihood estimates via a generalised linear model, then fits a zero-centred normal distribution to the observed distribution of these estimates across all miRNA (Love *et al.*, 2014). The dispersion plot in Figure 5-3 demonstrates dispersion estimates shrunk from the gene-wise estimates. Shrinkage of log fold change estimates in this context minimises the number of miRNA with high levels of dispersion, thereby reducing the impact of variance upon the differential analysis.

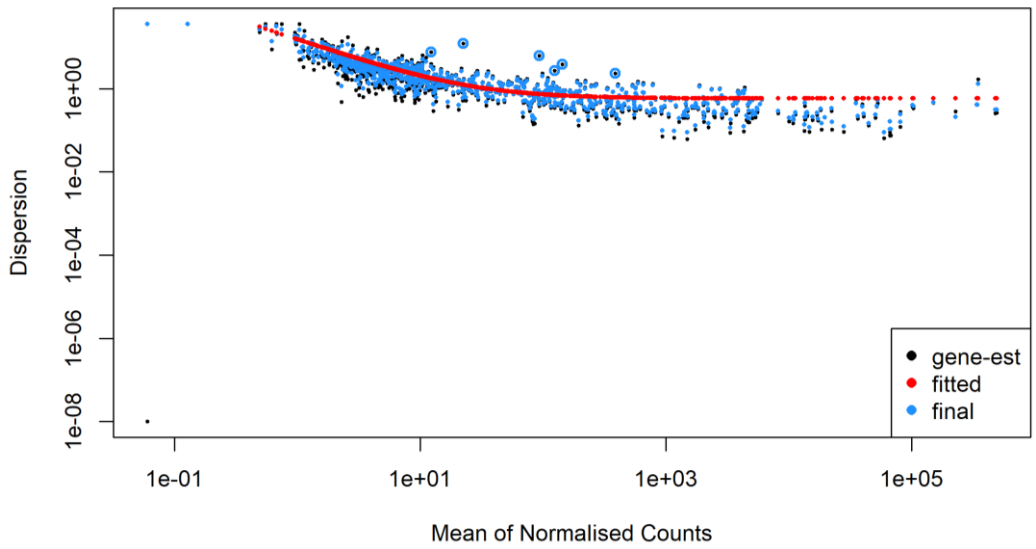

Figure 5-3. Dispersion plot.

This figure demonstrates dispersion (variance) estimates shrunk towards gene-wise estimates (black). Final shrunken estimates utilised in the analysis are depicted in blue. Black points circled in blue are detected as dispersion outliers. The overall trend of dispersion mean dependence is observed in red.

miRNA	miRNA Accession Number	Base Mean	Log2 Fold Change	LFCSE	P-value	Adjusted P-value
miR-664a-5p	MIMAT0005948	919.53	-0.61	0.24	0.00046	0.022
miR-3613-5p	MIMAT0017990	822.66	0.59	0.31	0.00125	0.049
miR-382-5p	MIMAT0000737	4194.09	-0.65	0.35	0.00050	0.022
miR-134-5p	MIMAT0000447	1871.95	-0.63	0.37	0.00046	0.022
miR-10b-5p	MIMAT0000254	2174.08	0.76	0.28	0.00009	0.016
miR-885-5p	MIMAT0004947	108.68	0.62	0.40	0.00021	0.018
miR-206	MIMAT0000462	145.77	-0.75	0.32	0.00013	0.016
miR-32-5p	MIMAT0000090	552.49	0.73	0.22	0.00003	0.009

Table 5-2. Sex-dependent differentially expressed plasma miRNA in ACS.

Base mean represents the average of normalised count values divided by size factors.

Biological sex was first assessed using this method and detected eight differentially expressed miRNA in females compared with males who had experienced ACS in the preceding 48 hours (Table 5-2). Of these differentially expressed miRNA, four were upregulated (miR-3613-5p, miR-10b-5p, miR-885-5p and miR-32-5p) and four downregulated (miR-664a-5p, miR-382-5p, miR-134-5p and miR-206) in this analysis (Figure 5-4). No miRNAs were up- or downregulated more than two-fold in this analysis.

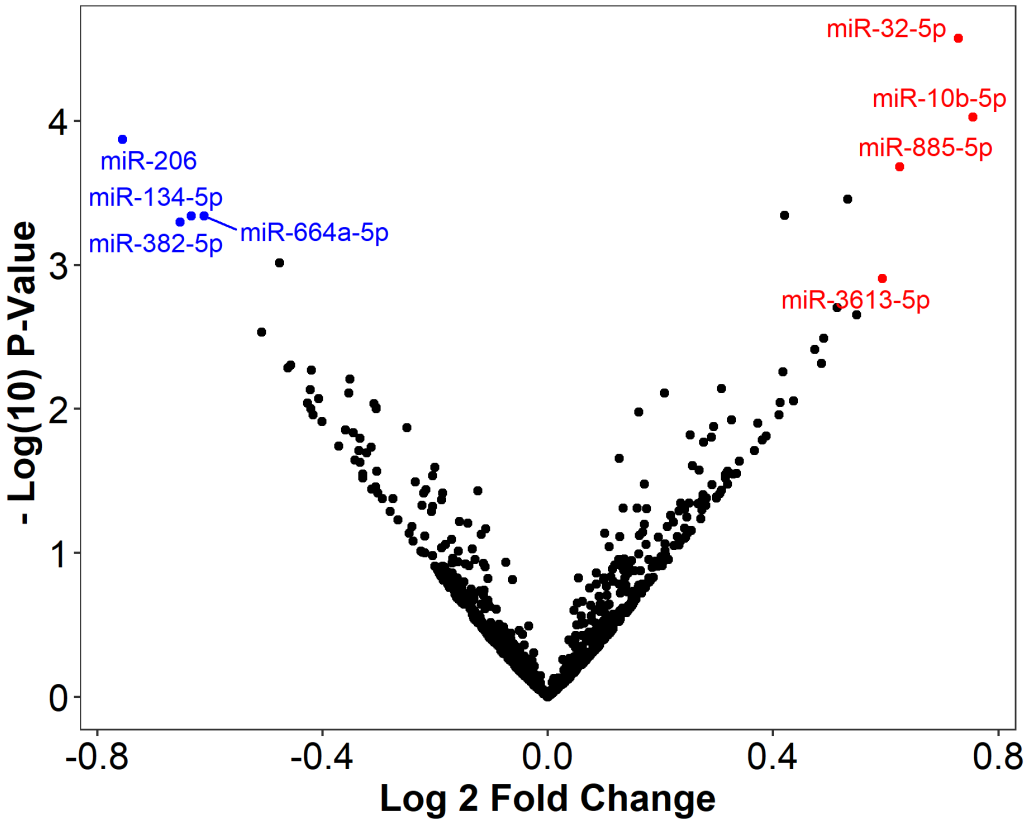

Figure 5-4. Volcano plot of sex-dependent differentially expressed miRNA in ACS. Differentially expressed miRNA in females compared to males who have experience ACS in the preceding 48 hour are depicted. The log fold change of miRNAs is plotted against the -$\log_{10}$ P-value. Upregulated (red), downregulated (blue) and non-differentially expressed (black) miRNA are depicted.

This was followed by a gender group analysis. Firstly, differential expression was assessed between feminine versus masculine males (Table 5-3). This demonstrated

upregulation of miR-4467 (log2 fold change=5.2, adj.P= 0.002) and downregulation of miR-3605-5p (log2 fold change=-1.54, adj.P =0.036). A 37 fold change was evident in miR-4467 (Figure 5-5). Further gender analyses were undertaken in feminine versus masculine females, however no significantly differentially expressed miRNA were identified (i.e. fold change was less or equal to 1.5 and/or an adjusted p-value of greater than 0.05).

miRNA	miRNA Accession Number	Base Mean	Log2 Fold Change	LFCSE	P-value	Adjusted P-value
miR-3605-5p	MIMAT0017981	70.69	-1.54	1.26	0.00008	0.036
miR-4467	MIMAT0018994	7.22	5.23	1.61	0.000002	0.002

Table 5-3. Gender-dependent differentially expressed miRNA in males with ACS.
Base mean represents the average of normalised count values divided by size factors. LFCSE represents the standard error of the log2FoldChange estimate.

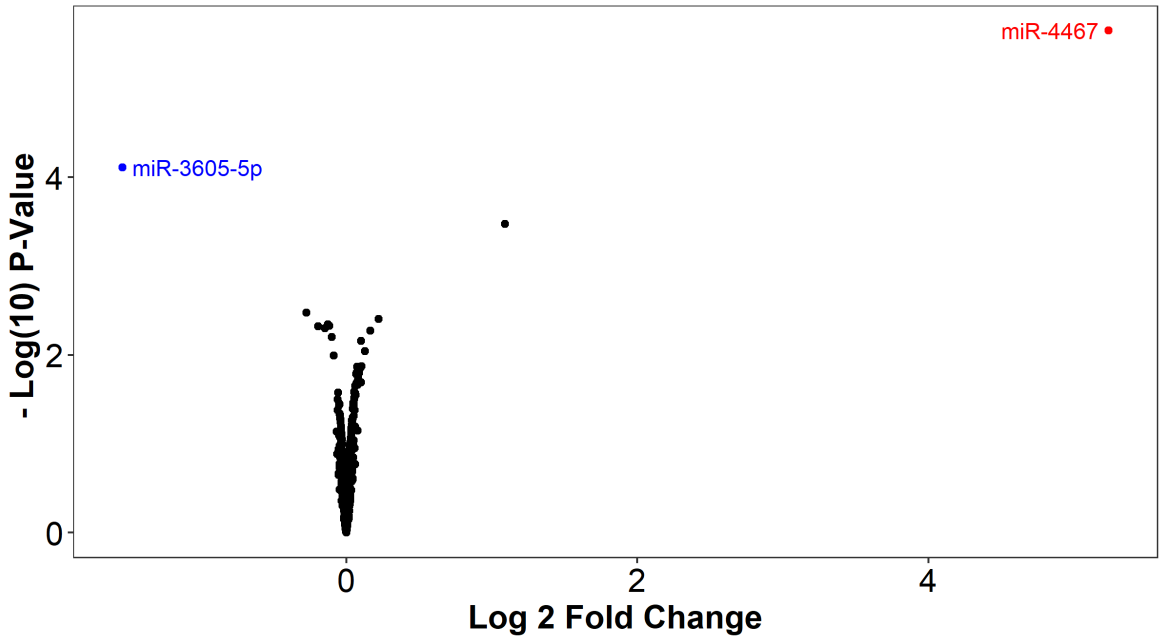

Figure 5-5. Volcano plot of gender-dependent differentially expressed miRNA.
Differentially expressed miRNA in feminine versus masculine males who have experience ACS in the preceding 48 hour are depicted. The log fold change of miRNAs is plotted against the -$\log_{10}$ P-value. Upregulated (red), downregulated (blue) and non-differentially expressed (black) miRNA are depicted.

5.5.4 miRNA regulatory network analysis

miRNA-gene interactions were explored using the miRNet (v2.0) platform. miRNA accession codes were utilised to identify *H. sapiens* gene predicted interactions in identified via miRTarBase v8.0 and TarBase v8.0 databases. Minimum network connections was used as a filter for miRNA-gene interactions, which excluded target nodes with less than two connections from the network (i.e. a degree filter of 2).

Differentially expressed miRNA between males and females demonstrated a total of 3,953 gene interactions and 4,783 edges (i.e. interactions between elements of a network) across the eight identified miRNA (Dragomir *et al.*, 2018). Following the adjustment of the degree filter to two this was limited to 114 gene

interactions and 361 edges. The resulting miRNA-gene interaction network can be demonstrated in Figure 5-6.

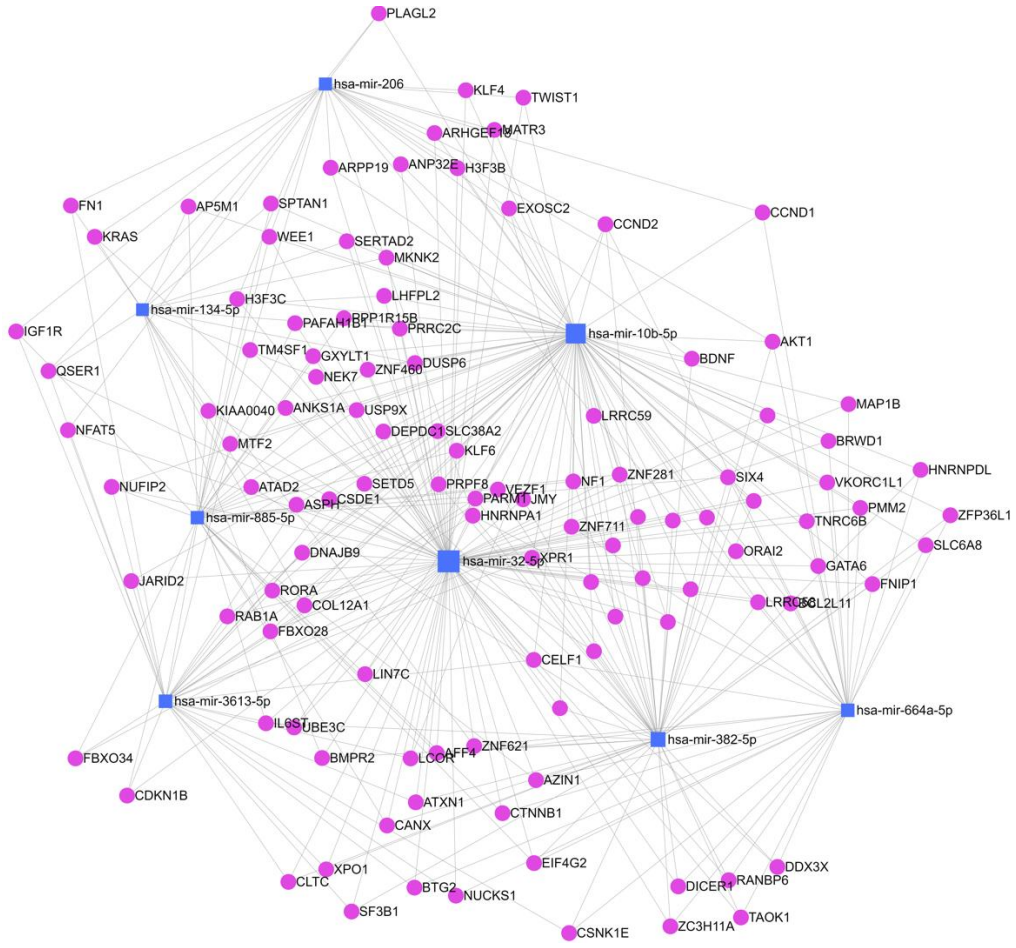

Figure 5-6. Sex-dependent miRNA-target gene interaction networks.
Blue squares represent miRNA, while circles represent gene interactions.

Functional analysis was performed on eight mature miRNAs identified in the analysis of biological sex. Pathway enrichment was undertaken utilising KEGG, Reactome, GO-BP, GO-MF, GO-CC, and DisGeNET databases (Kanehisa & Goto, 2000; Young *et al.*, 2010; Jassal *et al.*, 2020; Piñero *et al.*, 2020). This incorporated all network genes and hypergeometrical testing, whereby adjusted P-values <0.05 were considered significant. Importantly, pathway analysis included consideration of biological significance. Data on pathway enrichment is provided alongside the associated rich factor, which represents ratio of

differentially expressed gene counts in a pathway to all genes annotated in that pathway. Higher rich factor values denote a greater degree of pathway enrichment.

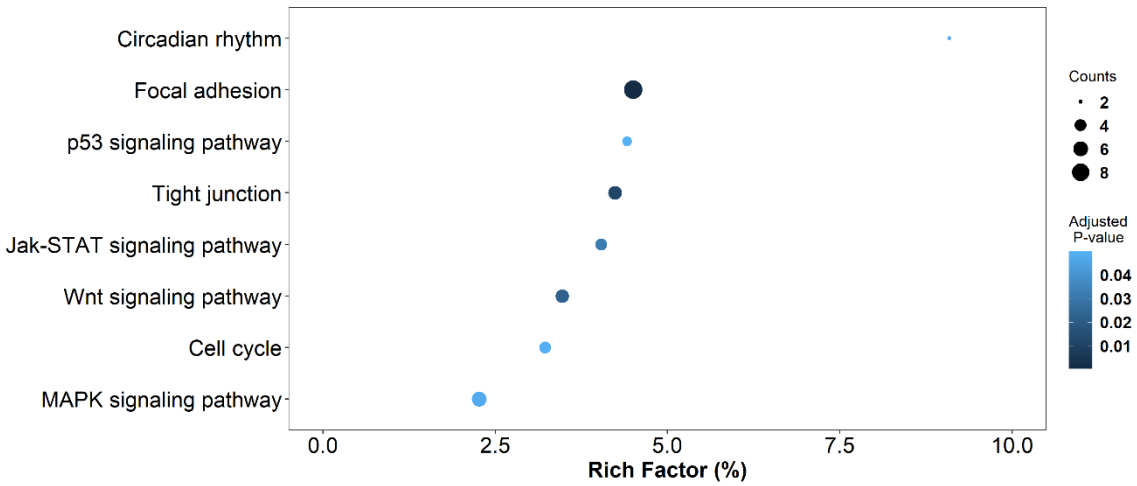

Figure 5-7. Sex-dependent KEGG pathway enrichment analysis.
Rich factor represents the ratio of differentially expressed genes annotated in a pathway to the number of all genes annotated within this pathway.

In total 297 pathways across these databases (DisGeNET 33.7%, GO-BP 33.7%, Reactome 24.9%, KEGG 7.4%, GO-MF 0.3%) were significantly enriched. Enriched KEGG pathways (Figure 5-7) included focal adhesion (adj.P=0.0003), tight junctions (adj.P=0.01), Wnt signalling (adj.P=0.022), Jak-STAT signalling (adj.P =0.035), circadian rhythm (adj.P=0.047), MAPK signalling (adj.P =0.047), Cell cycle (adj.P=0.049), and p53 signalling (adj.P=0.049). Genes identified from this network include: *CCND2, AKT1, WEE1, BMPR2, DST, MKNK2, H3F3B, IL6ST, PTEN, NF1, RORA, KRAS, CCND1, BDNF, IGF1R, NFAT5, CTNNB1, CDKN1B, FN1, COL5A1, DUSP6, COL1A2, SPTAN1*, and *CSNK1E* (Figure 5-8).

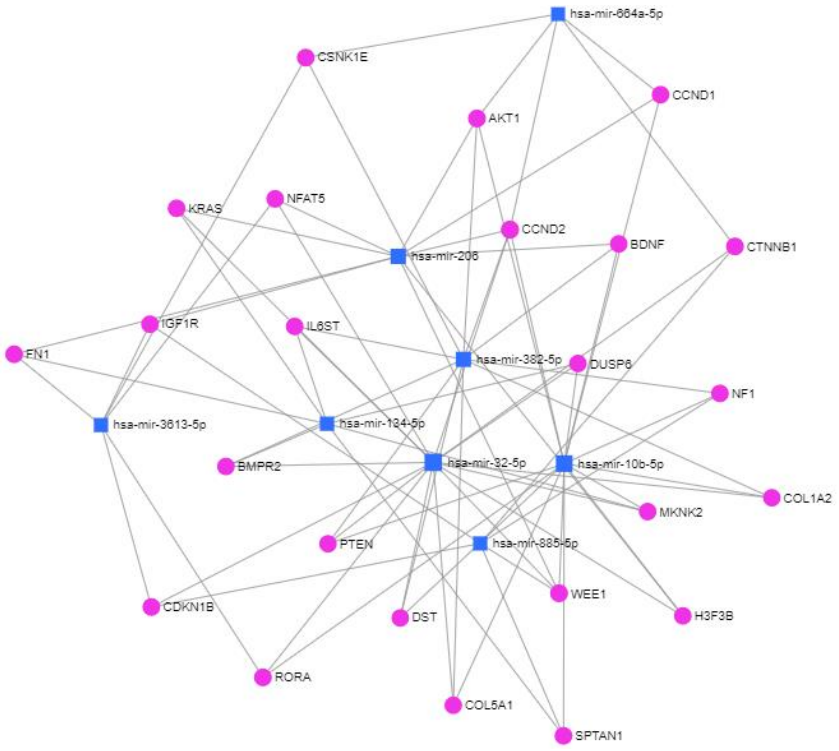

Figure 5-8. Sex-dependent KEGG enrichment analysis

This figure demonstrates miRNA (blue squares) and gene (purple) networks identified in the KEGG enrichment analysis.

Enriched Reactomes pathway (Figure 5-9) included negative regulation of the PI3K/AKT network, syndecan interactions, signalling by NGF, Collagen formation, signalling by PDGF, NGF signalling via TRKA from the plasma membrane, Assembly of collagen fibrils and other multimeric structures, Cell Cycle (Mitotic), Cell Cycle, and signalling by ERBB4 (adj.P<0.02). Genes involves in this network included: *AKT1, BMPR2, DST, H3F3B, PTEN, BCL2L11, TNRC6B, KRAS, CCND1, WEE1, ARPP19, XPO1, ARHGEF18, COL12A1, FN1, COL5A1, RAB1A, DUSP6, TAOK1, COL1A2, CSNK1E, PAFAH1B1*, and *CDKN1B*.

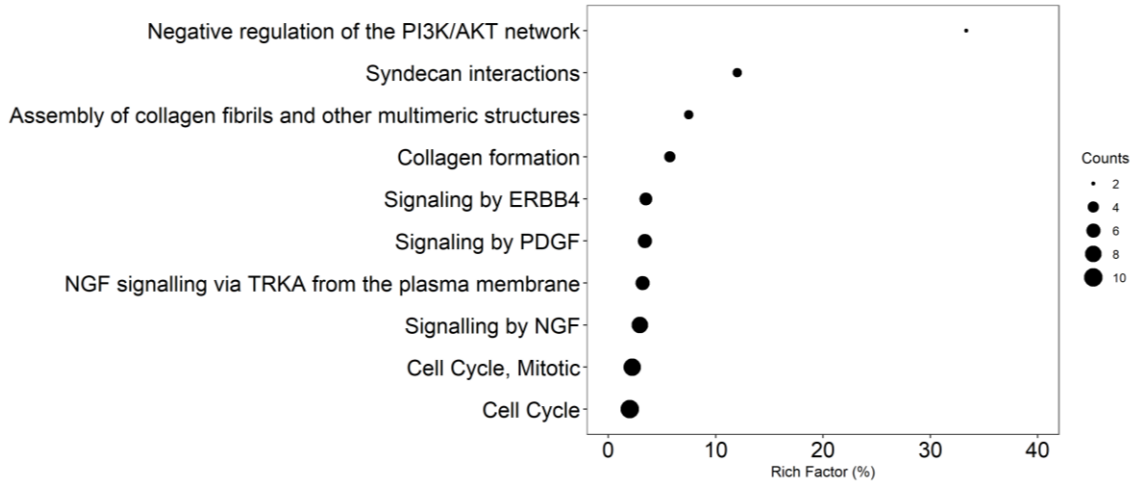

Figure 5-9. Sex-dependent Reactome pathway enrichment analysis.

GO-BP enriched pathways (Figure 5-10) included regulation of anatomical structure morphogenesis, regulation of growth, negative regulation of signal transduction, regulation of cell cycle, vasculature development, regulation of cellular component organization, cell development, phosphatidylinositol-mediated signalling, growth, and positive regulation of cell cycle (adj.P<0.001). Genes relating to this pathway included: *CCND2, FNIP1, AKT1, WEE1, EIF4G2, SIX4, JMY, CELF1, BMPR2, DST, DDX3X, GATA6, MKNK2, H3F3B, IL6ST, MAP1B, PAFAH1B1, PTEN, ATXN1, TWIST1, BTG2, KLF4, SERTAD2, BCL2L11, NF1, EXOSC2, NEK7, KRAS, CCND1, BDNF, IGF1R, ADM, ZFP36L1, CLTC, XPO1, VEZF1, DICER1, CTNNB1, JARID2, ANKS1A, AFF4, ARHGEF18, CDKN1B, FN1, COL5A1, DUSP6, TAOK1, COL1A2, BRWD1, SPTAN1*, and *CSNK1E*.

GO-MF enrichment analysis only demonstrated one significantly enriched pathway, whereby these differentially expressed miRNA were associated with single-stranded RNA binding (rich factor= 9.5%; adj.P= 0.019). Genes relating to this network included: *DDX3X, ATXN1, HNRNPA1,* and *HNRNPDL*.

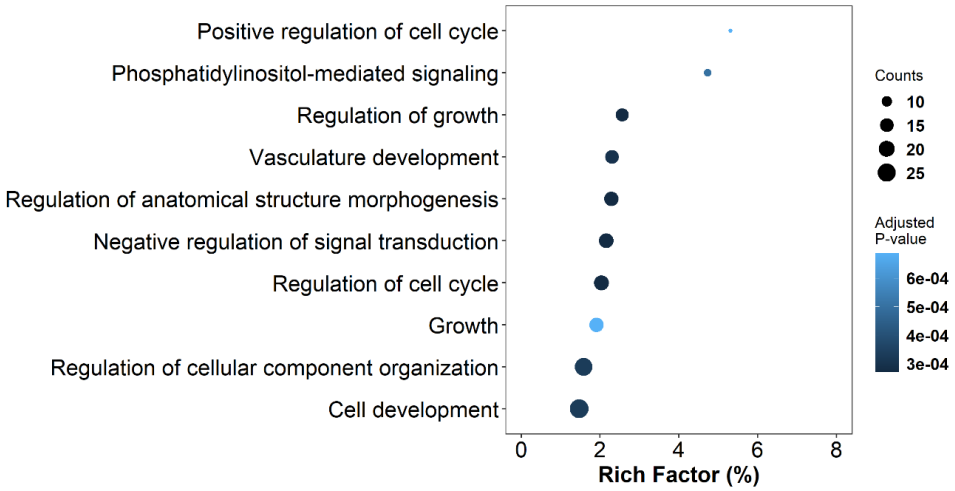

Figure 5-10. Sex-dependent GO-BP pathway enrichment analysis.

Lastly, enrichment analysis demonstrated associations with DisGeNET cardiovascular abnormalities, myocardial ischemia, cardiovascular diseases, hyperandrogenism, heart failure (right-sided), right ventricular failure, intimal fibrosis, and arterial intimal fibrosis pathways (adj.P<0.01). Genes involved in this network included: *DST*, *BMPR2*, *IL6ST*, *PAFAH1B1*, *KLF4*, *NF1*, *BDNF*, *CANX*, *DUSP6*, and *CCND1* (Figure 5-11).

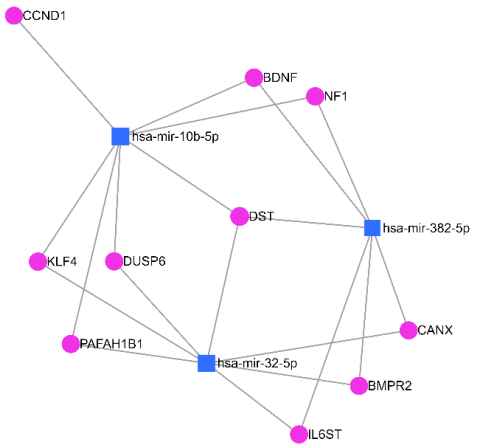

Figure 5-11. Sex-dependent DisGeNET enrichment miRNA networks

This figure demonstrates miRNA (blue squares) and gene (purple) networks identified via DisGeNET enrichment analysis.

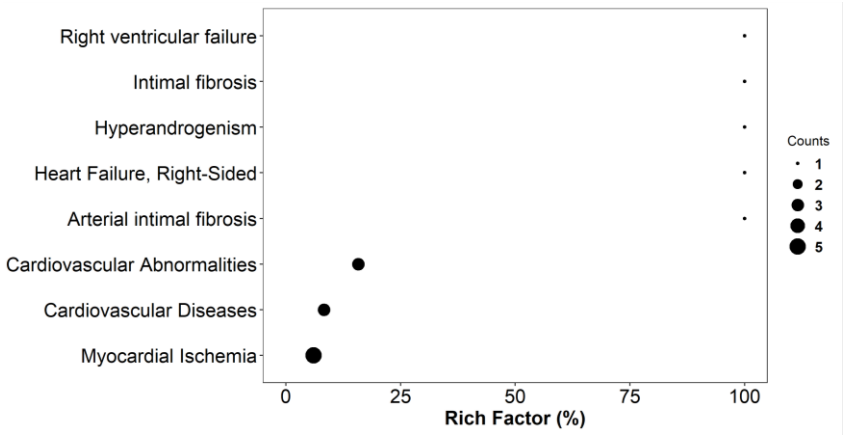

Figure 5-12. Sex-dependent DisGeNET pathway enrichment analysis

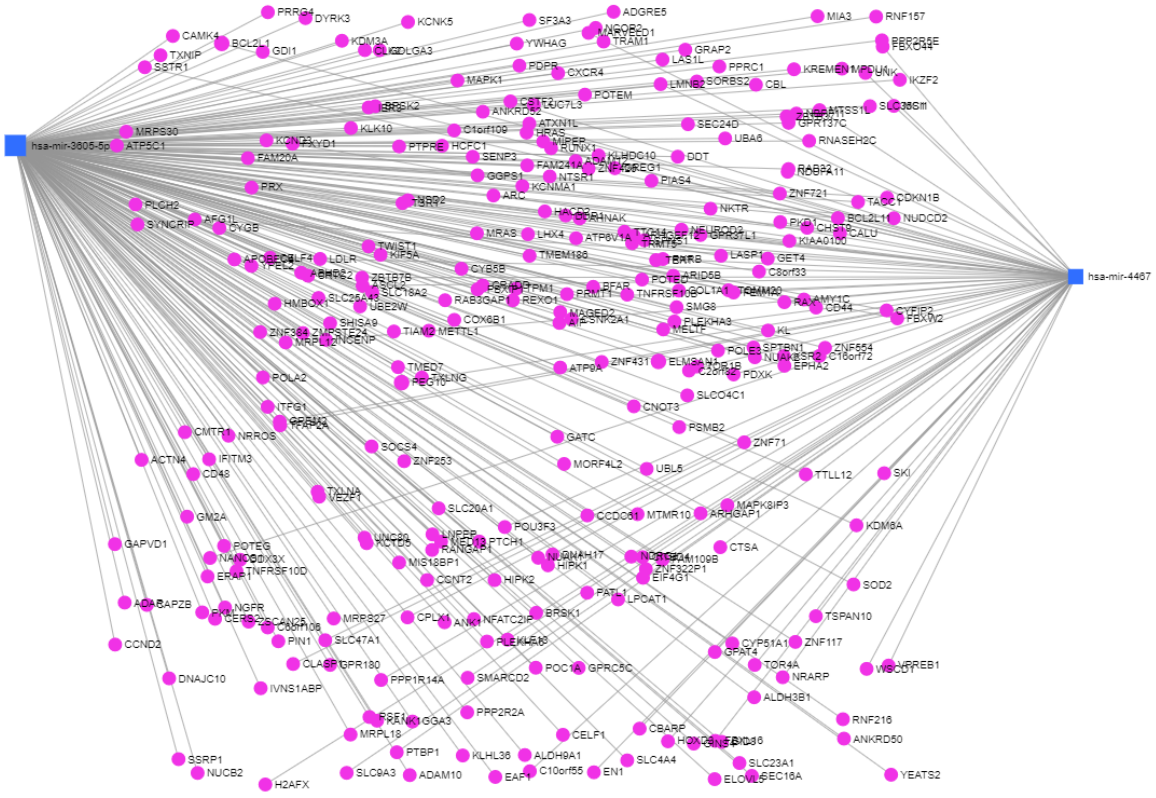

Figure 5-13. Gender-dependent miRNA-target gene interaction networks in males. Differentially expressed miRNA in feminine versus masculine males following ACS. Blue squares represent miRNA, while purple circles represent gene interactions.

Differentially expressed miRNA between feminine and masculine males demonstrated a total of 295 gene interactions and 298 edges across the 2 identified miRNA (Figure 5-13). Due to the relatively low number of gene interactions, no degree filter was added during this analysis. Typically, this is only required in the context of large (i.e. >2,500 nodes) networks (Chang & Xia, 2023).

Pathway enrichment was again undertaken utilising KEGG, Reactome, GO-BP, GO-MF, GO-CC, and DisGeNET databases (Kanehisa & Goto, 2000; Young *et al.*, 2010; Jassal *et al.*, 2020; Piñero *et al.*, 2020). Of these databases, there was no evidence of pathway enrichment in KEGG or Reactome databases. In total 106 pathways across the remaining databases (DisGeNET 94.3%, GO-BP 1.9%, GO-MF 1.9%, GO-CC 1.9%) were significantly enriched.

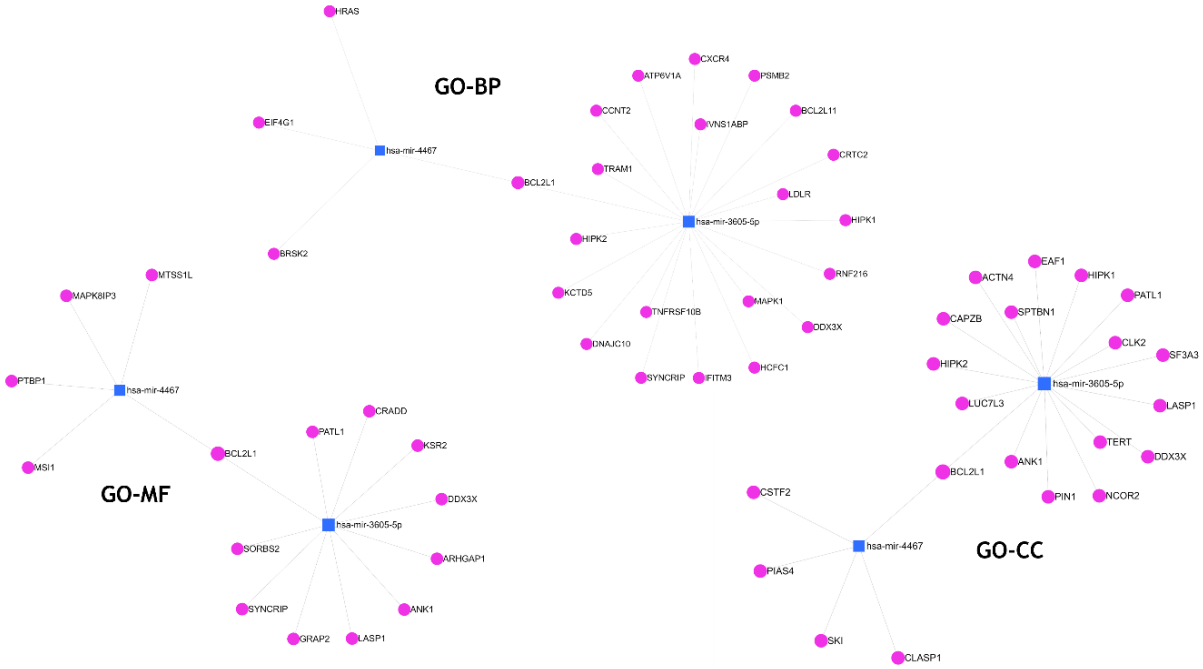

Figure 5-14. Gender-dependent GO enrichment pathway miRNA networks.

Overall, GO pathway enrichment analyses (Figure 5-14) provided limited results. GO-BP enrichment was observed in interactions with host (adj.P=0.02) and intrinsic apoptotic signalling pathways (adj.P=0.03). GO-MF demonstrated single-

stranded RNA binding, and protein binding, bridging pathway enrichment (adj.P=0.04). GO-CC demonstrated enrichment in nuclear body, and cortical cytoskeleton (adj.P=0.02). DisGeNET reported 100 enriched pathways associated with these differentially expressed miRNA relating largely to esoteric conditions, or indeed cosmetic traits, and not cardiovascular conditions *per se*. The top enriched pathways and miRNA networks are depicted in Figure 5-15. Common genes highlighted across these enrichment analyses included: *BCL2L1, DDX3X, MAPK1, HIPK2, BCL2L11, HRAS, HIPK2, HIPK1, SYNCRIP, PATL1, ANK1, LASP1, SKI, ZMPSTE24, TFAP2A, NSD2*, and *COL1A1*.

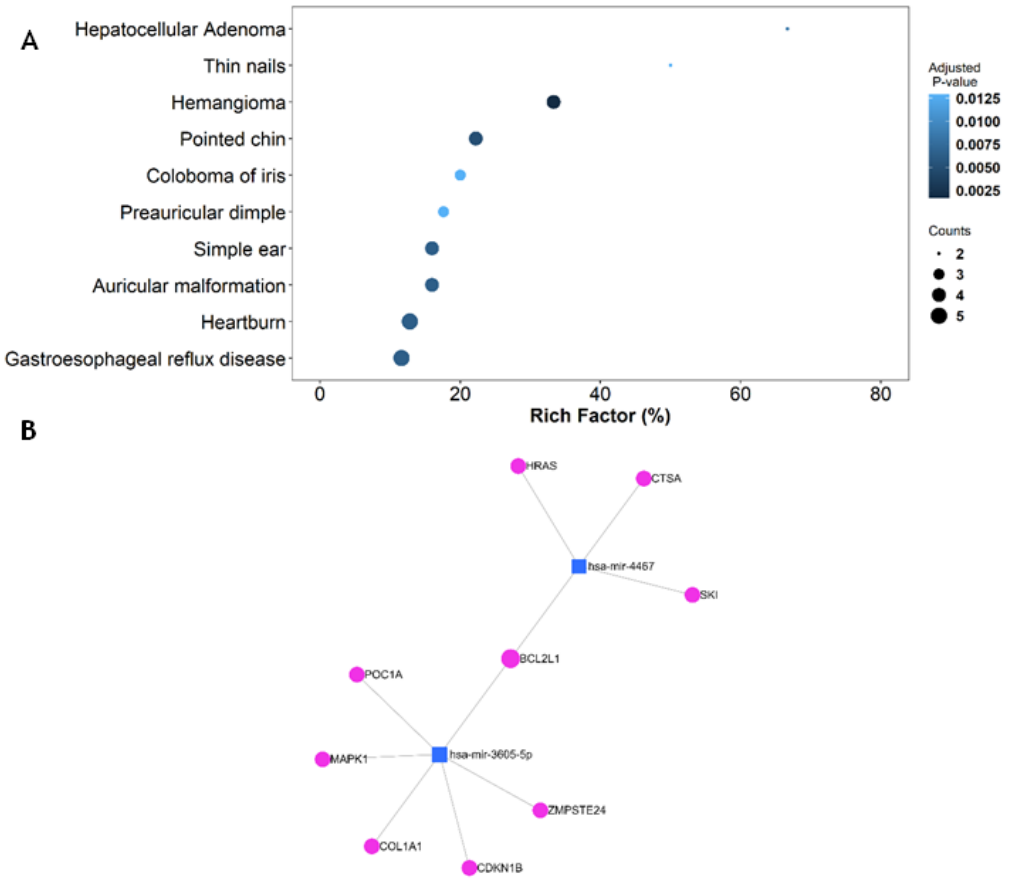

Figure 5-15. Gender-dependent DisGeNET pathway enrichment analysis

A) DisGeNET pathway enrichment of differentially expressed miRNA bubble plot. B) DisGeNET enrichment pathway differentially expressed miRNA networks

5.6 Discussion

In this chapter, multiple circulating miRNA were identified to be differentially expressed in ACS according to sex and gender. Sex differences in cardiovascular disease-related biomarkers have previously been observed and reflect distinct variation in disease pathophysiology (Lau *et al.*, 2019). Biomarkers that regulate inflammation and adipokine signalling (e.g. hemopexin, leptin) were found to be upregulated in females. Conversely, biomarkers related to coagulation homeostasis, including platelet function, and fibrosis were upregulated in men (e.g. matrix metalloproteinase-8, tissue inhibitor of metalloproteinases 1). Importantly, differential expression of these biomarkers was found to predict incident cardiovascular events and all-cause mortality, thereby highlighting their importance sex-mediated differences in cardiovascular pathology.

In this analysis the miRNA miR-3613-5p, miR-10b-5p, miR-885-5p, miR-32-5p were upregulated in females compared to males. miR-10b-5p has demonstrated multiple roles important to ACS pathophysiology including the regulation of vascular smooth muscle cell proliferation and advanced atherosclerosis progression (Yu *et al.*, 2015; Wang *et al.*, 2018). The levels of this miRNA typically fall following MI-induced hypoxia as a consequence of increases hypoxia-inducible factor 1α (HIF-1α) levels, which in turn decrease miR-10b-5p expression. However, overexpression of miR-10b-5p in murine models of MI significantly reduces infarct size, improves cardiac function and inhibits cardiomyocyte apoptosis (Wu *et al.*, 2019). Consequently, increases in this miRNA in females may provide a degree of cardioprotection compared to males.

The *mir-10* gene is located within the *Hox* gene clusters of developmental regulators, and its location is in close proximity to *Hox4* paralogues (Tehler *et al.*, 2011). miR-10 paralogues are anticipated to demonstrate the same regulatory elements and expression patterns as neighbouring *Hox* genes, of which many members are regulated via oestrogen and ERE interactions (Jin & Sukumar, 2016).

Accordingly, miR-10-b-5p expression in human T-cells are promoted by oestrogen exposure (Ramanujan *et al.*, 2021). This may therefore provide an important epigenetic link between established ACS pathophysiology and sex-dependent regulation.

Similarly, miR-885-5p appears to demonstrate sex-specific expression in response to pathological stimuli. In gestational diabetes, expression of this miRNA is increased in female primary human fetal hepatocytes extracted from second trimester amniotic fluid in response to maternal obesity (Joshi *et al.*, 2020). Conversely, increased expression is not observed in male sex hepatocytes. This miRNA is also differentially expressed in females with cystic fibrosis in comparison to males (Mooney *et al.*, 2020). Elevated miR-885-5p are associated with pre-eclampsia, potentially via the promotion of endothelial and placental apoptosis, whereby the former may have implications in myocardial ischaemia and reperfusion (Sandrim *et al.*, 2016).

miR-32-5p has been implicated in oxidised LDL-induced endothelial injury and inflammation in atherosclerosis, and direct myocardial injury and inflammatory responses in acute MI (Dai *et al.*, 2020; Zhang *et al.*, 2022). However, no data exists on sex-specific expression or actions of this miRNA. Limited data are available on the function of miR-3613-5p. Nonetheless, it is differentially expressed in endometriosis and therefore may demonstrate a degree of oestrogen sensitivity and vascular actions (Cosar *et al.*, 2016).

Within this analysis, a number of miRNA were also downregulated (i.e. miR-664a-5p, miR-382-5p, miR-134-5p and miR-206). miR-664a-5p has been implicated in the senescence of vascular smooth muscle cells (Nguyen *et al.*, 2021). It has also been shown that exosomal perivascular adipose tissue release of miR-382-5p may reduce macrophage foam cell formation, and therefore provide vascular protective effects (Liu *et al.*, 2022b).

miR-134-5p has been shown to be strongly expressed as a consequence of MI, alluding to its potential role in MI diagnostics (Wang *et al.*, 2016a). Importantly, silencing of miR-134-5p may inhibit ischaemic reperfusion cardiomyocyte apoptosis and promote myocardial angiogenesis (Li *et al.*, 2020; Wu *et al.*, 2021). This suggests that the downregulation of this miRNA in females may provide a relative advantage in restoring postinfarction coronary blood flow and salvaging myocardium.

Lastly, miR-206 expression is significantly decreased in ERα-positive human breast cancer tissues, and has been shown to be downregulated by 17β-estradiol in a dose-dependent fashion (Kondo *et al.*, 2008; Khalilian *et al.*, 2022). Importantly, miR-206 expression is inversely related to ERα but not ERβ transcript levels. In a rat model of MI, down-regulation of miR-206 increased cardiomyocytes apoptosis, while overexpression reduced infarct size and apoptosis, suggesting a potential cardioprotective role of this miRNA, which may be regulated via sex-specific mechanisms (Yan *et al.*, 2020).

Enrichment analysis also highlighted a number of processes important to ACS pathophysiology that may demonstrate sex-dependent mechanisms. Many of these pathways are implicated in the process and recovery of MI. KEGG analysis highlighted both circadian rhythm and Wnt signalling as potential mediators. Disruption of circadian rhythm may increase cardiovascular risk, and various elements of cardiovascular physiology, such as endothelial function and thrombus formation, are regulated by this biological clock (Crnko *et al.*, 2019). Moreover, emerging evidence indicates that circadian rhythms may influence MI pathophysiology, cardiac hypertrophy and blood pressure regulation (Glen Pyle & Martino, 2018). Similarly, it has been demonstrated that Wnt signalling is initiated during myocardial ischaemia and associated with including inflammation, angiogenesis and fibrosis (Fu *et al.*, 2019). Importantly, in several pathological and physiological processes, significant cross-talk has been demonstrated between oestrogen and Wnt signalling pathways (Wu *et al.*, 2015).

The GO-BP analysis suggested that these differentially expressed miRNAs may be involved in vascular development, which again may play a significant role in sex-specific ACS development. This may contribute to differences observed in epicardial coronary arteries, whereby females are more likely to experience pathological vasoreactivity and endothelial dysfunction, whereby males are predisposed to atherosclerosis (Regitz-Zagrosek & Kararigas, 2017). Collagen metabolism appears to be sex-specific and its synthesis is differentially regulated by oestrogen and testosterone in cardiac fibroblasts (Seeland & Regitz-Zagrosek, 2013; Grilo *et al.*, 2020). After the occurrence of a MI the collagen network is expanded via fibroblast mediated cardiac repair, remodelling and fibrosis (Hanna *et al.*, 2021). This may account for the sex differences observed in myocardial remodelling following a MI, whereas females exhibit concentric rather than eccentric hypertrophy evident in males (Crabbe *et al.*, 2003; Kessler *et al.*, 2019).

A number of gene-miRNA interactions were also highlighted via enrichment analysis with potential roles in sex-mediated ACS differences. *BMPR2* encodes Bone Morphogenetic Protein Receptor Type 2, which contains an ERE within its promoter and is downregulated via ERα (Austin *et al.*, 2012). *BMPR2* dysregulation is strongly associated with the development of pulmonary arterial hypertension via endothelial dysfunction, and oestrogen may modulate this relationship via epigenetic influences such as miRNA (Cuthbertson *et al.*, 2023). *BMPR2* is also downregulated during disturbed endothelial flow, which promotes inflammation and vascular remodelling (Andruska & Spiekerkoetter, 2018). Although data relating to non-pulmonary endothelial *BMPR2* are limited, they do again suggest a potential link by which the pathophysiology of ACS may differ in a sex dependent manner.

CCND1 encodes a cell cycle regulatory protein that modulates multiple nuclear receptors, including cyclin-dependent kinases, important to cellular proliferation and differentiation (Liu *et al.*, 2022a). Following MI, overexpression of CCND1 promotes cardiomyocyte proliferation and increases cardiac function (Mohamed

et al., 2018). CCND1 also influences myocardial ischemia–reperfusion injury, and its transcription is regulated via miRNA (Kang *et al.*, 2022). It has been demonstrated that 17β-estradiol alters *CCND1* expression via the protein kinase mTOR. This is achieved via ER binding, which promotes ERK1/2 and PI3K signalling pathways activation, which in turn facilitates altered mTOR mediated gene transcription (Yang *et al.*, 2015). This therefore highlights an additional indirect mechanism by which sex-related factors may facilitate differences in ACS pathophysiology.

The role of social determinants of health, and their potential impact upon developmental plasticity is well recognised. For instance, it has been demonstrated that socio-economic disadvantage is associated with higher all-cause and cardiovascular mortality in patients who have experienced a MI (Berman *et al.*, 2021). However, limited data exist on how such factors directly influence biology, for example through the modulation of miRNA expression, to alter cardiovascular pathophysiology. In line with inherent biological differences evident in sex, environmental factors, with underlying psychosocial aetiologies, may drive pathophysiological processes. For instance, significant noise exposure is associated with arterial hypertension, coronary artery disease, and heart failure (Münzel *et al.*, 2021). Excess noise has been shown to regulate miRNA expression, which modulate genes involved in oxidative stress and apoptosis that promote sensorineural hearing loss (Miguel *et al.*, 2018). These epigenetic mediators may then facilitate increased cardiovascular risk as collateral damage, or the index stress-response mechanism may promote cardiovascular specific deleterious miRNA as a parallel response. Consequently, complex psychosocial interactions (e.g. urban living, deprivation) may promote disease via these mechanisms, and provides the opportunity to identify the biological influences of gender in cardiovascular disease.

This analysis identified two differentially regulated miRNA (i.e. miR-3605-5p and miR-4467) in males with feminine versus masculine gender characteristics, as

determined via the GGI. However, minimal data are available on the biological function of either miRNA, or how these may influence target genes to regulate cardiovascular health and disease.

miR-3605-5p has been found to be downregulated in females compared to males adolescents with obesity, however, this was only found to be of borderline significant following FDR P-value adjustment (Karere *et al.*, 2021). Upregulation of miR-3605-5p may also be of importance in hypopharyngeal squamous cell carcinoma related survival following post-operative radiotherapy (Xu *et al.*, 2019).

Upregulation of circulating miR-4467 has been identified as a potential biomarker in the early diagnosis of schizophrenia, and may play a role in neurodevelopment and synaptic transmission (Jin *et al.*, 2022). Moreover, this miRNA is differentially expressed in Alzheimer`s Disease and vascular dementia, which again suggests a neurophysiological role of this miRNA, which may be in keeping with potential gender-related influences in disease (Denk *et al.*, 2015; Liu *et al.*, 2021). However, no data relating to the influences of this miRNA on cardiovascular disease or physiology have been published to date.

Ultimately, the role of miR-3605-5p and miR-4467 and their associations with gender in ACS remains unclear. However, gene prediction analysis may provide novel insight into putative gene targets. *DDX3X* is located on the X-chromosome and encodes DEAD-box helicase 3 X-linked (*DDX3X*) and modulates a number of cellular processes such as cell cycle progression, cellular differentiation, cell survival, and apoptosis. In patients with diabetes it has demonstrated to have a role in endothelial injury (You *et al.*, 2023). Overexpression of *DDX3X* has also shown to promote the incidence of aortic aneurysms, vascular inflammation and smooth muscle transformation, and oxidative stress levels in a murine model (Zhou *et al.*, 2022). Interestingly, *DDX3X* mutations are a common X-linked genes associated with intellectual disability and behavioural abnormalities, thereby

potentially providing a link between neurodevelopment and cardiovascular disease (Sun *et al.*, 2022).

HIPK 1/2 encode Homeodomain-Interacting Protein Kinase 1/2 (HIPK1/2), a protein kinase regulates cell proliferation, apoptosis, and mitochondrial function (Phrommintikul *et al.*, 2022). Factors such as exercise have been shown to downregulate HIPK1/2 via altered miRNA expression, which in turn reduces cardiomyocyte apoptosis and preserves cardiac function post-MI in animal models (Zhou *et al.*, 2021). Consequently, gendered-factors that modulate physical activity could putatively alter the risk of MI and recovery.

MAPK1 encodes mitogen-activated protein kinase 1, which is also known as extracellular signal-regulated kinase 2 (ERK2). This is a highly conserved serine/threonine kinase that regulate myocyte survival following ischemic reperfusion injury (Lips *et al.*, 2004). This had been shown to be differentially regulated in females with acute MI (Jiao *et al.*, 2023). Consequently, it is possible that this may be mediated via gender rather than sex.

Enrichment analysis provided some insight into mechanism by which gender may influence cardiovascular pathophysiology in males. GO enrichment analysis demonstrated a potential role of intrinsic apoptotic signalling. The latter is activated in response to intracellular stressors, and promotes cell death may have particular importance in the context of ACS (Wu & Bratton, 2013). Interestingly, a number of pathways in the DisGeNET database relating to physical appearance (e.g. thin nails, pointed chin, simple ear, and auricular malformation) were enriched. Adherence to masculine gender norms has been shown to be associated with physical traits, and therefore associations with these genetic phenotypes may be associated with feminine gender also (Amos & McCabe, 2016). Ultimately, the mechanism by which feminine and masculine genders influence transcriptomic regulation requires further elucidation.

Surprisingly, only a limited number of studies have been conducted addressing sex and gender differences in miRNA expression in ACS. In response to ischemia sex differences have been observed in human myocardial cells, whereby *FAM5C*, *PLA2G4E* and *CYP1A1* were upregulated and *DIO3*, *MT1G* and *CMA1* were downregulated in females compared to males (Stone *et al.*, 2019). In addition, three miRNA have been demonstrated to be differentially expressed in ischaemic cardiomyopathy (miR-3615, miR4223-5p,and miR-4709-3p) in a sex-dependent manner (Tsuji *et al.*, 2020). Importantly, although 13 miRNA were differentially expressed in between males and females in healthy controls, none of these featured within this analysis. This suggests the differentially expressed miRNA in this analysis are not purely a reflection of baseline sex differences.

A major strength of this analysis is the well-phenotyped sample in terms of sex and gender stratification. This provided an excellent resource in terms of identifying potential miRNA and gene interactions evident in ACS. Moreover, the utilisation of next-generation sequencing is both sensitive and accurate in the analysis of miRNA expression (Tam *et al.*, 2014). Given the absence of previous analyses of this nature, this permitted the identification of novel miRNA that may not have been possible using microarray methodologies. Although this study is limited by sample size, it demonstrates the important role of miRNA and sex and gender differences in ACS. Applying this analysis across the wider GENESIS-PRAXY population would permit a broader assessment of miRNA interactions and potential roles across the spectrum of gender scores evident in males and females.

This analysis had however a number of limitations. Data were not provided as to whether the females in this study were pre-, peri- or postmenopausal or were exposed to exogenous hormone preparations. Such factors would be expected to regulate oestrogen-sensitive miRNA and confounds the interpretation of these results (Ferraro *et al.*, 2012). Similarly, data were not provided on the subtype of ACS experienced by individuals included in this study. ACS encompasses a spectrum of conditions, including myocardial infarction with or without ST-

segment elevation, and unstable angina. Consequently, factors pertaining to the pathophysiology of these conditions, such as the presence of myocardial infarction, may have influenced miRNA expression, especially if these ACS subtypes were unevenly distributed among groups.

Furthermore, this sample was limited to individuals who has experienced ACS, and therefore there is the potential that some differentially expressed miRNA may be related to sex or gender rather than ACS. This is a limitation of the GENESIS-PRAXY study, by which gender stratification was only undertaken in a patient population. As demonstrated in Chapter 4, the results obtained via the GGI model are dependent upon the population sampled and therefore comparisons between the GENESIS-PRAXY and other cohorts may not be directly applicable.

Importantly, the nature of this study is that of a cross-sectional analysis of cases (i.e. a case series study), which lacks a control population. Therefore it is not possible to delineate whether differentially regulated miRNA are related to ACS pathophysiology or merely related to sex and gender. However, as mentioned previously, the miRNA detected in this analysis have not been found in analyses in healthy subjects stratified by sex, therefore suggesting that miRNA isolated in this analysis are likely to be ACS specific (Tsuji *et al.*, 2020). Regardless, a further analysis of age, sex and ideally gender score matched individuals should be undertaken to ensure this relationship holds true. In terms of future perspectives and research, the relationships that have been demonstrated are associative and therefore functional studies are required to determine the role of differentially expressed miRNA and related gene interactions in the pathophysiology of ACS. Moreover, this bioinformatic analysis requires future validation via reverse transcription quantitative PCR analysis.

To conclude, this chapter has identified sex and gender differences in circulating miRNA in ACS via NGS. In this chapter a number of miRNA and related gene networks with a potential biological role in ACS are differentially expressed in

females versus males, and masculine versus feminine males. Importantly, this is the first study to directly assess differentially expressed miRNA in individuals stratified by gender, and in combination with sex. This research provides novel insight into our understanding of the potential underlying pathophysiological processes associated with sex and gender in ACS.

Studies that embark upon the investigation of differentially expressed miRNA in ACS, in addition to other cardiovascular diseases, should consider the potential influence of sex and gender upon their analyses. Moreover, further research is required to identify whether these miRNA are regulated by sex hormone expression, which in turn may be influenced by reproductive stage of life and the use of sex hormone therapies, such as contraception or post-menopausal hormonal replacement therapy (Regitz-Zagrosek & Kararigas, 2017). Lastly, the development of adequate tools to assess gender are required to better understand the role of these characteristics in cardiovascular disease, including epigenetic modification.

Chapter 6 Vascular Effects of Sex Steroids in Transgender Adults: A Preliminary Analysis

6.1 Chapter overview

In the previous chapter, sex and gender differences in circulating microRNA (miRNA) in acute coronary syndrome (ACS) were identified. This chapter aims to continue this theme by investigating the influences of being transgender (i.e. a person whose gender identity differs from their natal sex), and in particular the influences of gender-affirming hormone therapy (GAHT), upon vascular health. To meet this aim, this chapter includes preliminary descriptive analysis for the Vascular Effects of Sex Steroids in Transgender Adults (VESSEL) study, which assessed the long-term effects of GAHT upon vascular function, however, was discontinued due to the impact of Coronavirus disease 2019 (COVID-19). The data presented demonstrates the feasibility of recruitment of transgender individuals and descriptive data relating to the impact of GAHT upon blood pressure measurements, flow mediated dilatation, peripheral arterial tonometry, and pulse wave analysis and velocity.

6.2 Introduction

The influence of exogenous and endogenous sex steroids upon vascular function and physiology is a topic of great interest. Understanding the influence of GAHT upon cardiovascular pathophysiology in transgender people is imperative to improving the management of cardiovascular risk in this population and more broadly advance our insight of the role of sex and gender in vascular health and disease.

GAHT is central in the management of many transgender individuals yet our appreciation of the effects of such hormones upon vascular health is not fully understood (Streed *et al.*, 2021). The studies addressing cardiovascular risk in this population are often retrospective and fail to identity pathophysiological

mechanisms by which sex steroids interact with vasculature (Streed *et al.*, 2017; Connelly *et al.*, 2019).

Consequently, it is necessary to conduct research of the vascular health in transgender individuals receiving GAHT to establish the presence of any risks and the mechanisms responsible. Increasing our understanding of the pathophysiology of cardiovascular diseases in transgender people will facilitate improvements in clinical care and guidance. Furthermore, it will offer insight into the role of sex steroids within the vasculature and the well-established epidemiological, pathophysiological and clinical differences in cardiovascular disease between cisgender men and women.

This chapter therefore reports preliminary findings from the VESSEL study. VESSEL began recruitment in August 2019 but unfortunately the impact of the COVID-19 pandemic on this study was significant and it was required to be discontinued. Consequently, the results reported in this chapter remain preliminary and descriptive, however, do provide insight into the feasibility of such a study and highlight potential avenues for future research in this field.

6.3 Hypothesis & Aims

6.3.1 Hypothesis

- Differences exist between measures of blood pressure, endothelial function and arterial stiffness between transgender and cisgender individuals.

6.3.2 Aims

- To describe the blood pressure measurements, including mean arterial blood pressure and pulse pressure, of transgender versus cisgender individuals.

- To assess endothelial function in transgender versus cisgender individuals via flow mediated dilation (FMD) and peripheral arterial tonometry (PAT).

- To assess arterial stiffness in transgender versus cisgender individuals via pulse wave analysis (PWA) and velocity (PWV).

6.4 Methods

6.4.1 Study participants

Participants were suitable for inclusion in this study if they were between the ages of 18 to 50 years, able to provide informed consent, and able to comply with all procedures, either alone or with the aid of a responsible caregiver (Appendix viii). Transgender participants were required to be diagnosed with gender dysphoria by a specialist at a recognised gender identity clinic and receiving GAHT. Participants were excluded if: they had a history of Raynaud's disease, atrial fibrillation, or other cardiac arrhythmia; were receiving anticoagulation treatment; were transgender and not receiving standard hormone regimes as set out the National Gender Identity Clinical Network for Scotland (NGICNS) Endocrine Management of Adult Transgender Patients; pregnant; transgender men prescribed GnRH analogues (goserelin, leuprorelin, triptorelin); cisgender and receiving exogenous sex hormones, spironolactone or had undergone an orchiectomy or oophorectomy.

6.4.2 Participant recruitment

Participants were recruited from the Glasgow Sandyford Gender Identity Service and Endocrinology clinics, Queen Elizabeth University Hospital, Glasgow. The study was also promoted via posters (Appendix vi) displayed in clinical areas relevant to gender identity services, GP practices, public areas including the University, transgender social groups and published online on relevant websites

(e.g. https://www.scottishtrans.org, social media sites and websites affiliated with the University of Glasgow). The control group was identified from GP surgeries affiliated with the University of Glasgow or via publication of this study via the aforementioned posters.

6.4.3 Study visits

All vascular assessments took place at the Queen Elizabeth University Hospital Clinical Research Facility in temperature controlled rooms (22-24^{o}C). Participants attended in the morning, refraining from caffeine, alcohol, cigarettes and E-cigarettes and after an overnight fast. Following arrival the patient information sheet (Appendix vii) was discussed and any questions about the study were answered prior to obtaining informed consent (Appendix viii). Study procedures were then performed as described below.

In order to overcome the potential fluctuation of sex hormones upon vascular analysis all studies were undertaken at 9am. Cisgender women were assessed within the follicular phase of their menstrual cycle (days 1-14). Transgender men prescribed testosterone therapy were assessed midway between injections if prescribed testosterone esters (i.e. Sustanon) or enantate, as close to their next injection of testosterone undecanoate (i.e. Nebido) as possible, or 2-8 hours following the application of testosterone gels. Transgender women were assessed on any day if they are prescribed oestrogen tablets or 2 days after changing their oestrogen transdermal patches. There were no restrictions as to when cisgender men could be assessed.

6.4.4 Basic clinical parameters

Study visits commenced with a brief medical history and a list of concurrent medications were obtained from the participants. Body mass index (BMI) was obtained via measuring height and weight. Following a 10-minute seated rest,

brachial artery blood pressures were measured using a calibrated Omron MX2 automated device. This was repeated a total of three times, and the average blood pressure was used in the analysis of this study. Mean blood pressure was calculated as (0.33 × systolic blood pressure) + (0.67 × diastolic blood pressure). Pulse pressure was calculated as the difference between systolic and diastolic blood pressures.

6.4.5 Vascular phenotyping

6.4.5.1 Flow mediated dilatation

FMD of the brachial artery was then assessed as a surrogate marker of endothelial function with an ultrasonographic semi-automated device (UNEX; Unex Co. Ltd., Nagoya, Japan). This is an established predictor of future cardiovascular events and a widely utilised non-invasive tool for the assessment of endothelium-dependent dilatation (Shechter *et al.*, 2009; Thijssen *et al.*, 2019). This technique exploits reactive hyperaemia, achieved via brachial occlusion, to promote endothelial shear stress and vasodilation. To achieve this, a 10-MHz linear array transducer continuously records the diameter of the brachial artery during periods of rest, occlusion and during the post-occlusive phase (Takase *et al.*, 2013). With the participant supine, the brachial artery was obtained in the short axis of the ultrasound probe over a period of 3 minutes. Automatic tracking of the arterial was achieved by automated edge-detection software and a vessel tracking system that permits the correction of probe position to ensure accurate images and measurements are obtained in the presence of involuntary arm movements. A blood pressure cuff over the brachial artery was then occluded for a period of 5 minutes then deflated. FMD was then calculated as the maximum change in diameter after cuff release normalized to the baseline diameter. All automated outputs were manually reviewed and analysed with UNEX software if considered non-diagnostic.

6.4.5.2 Peripheral arterial tonometry

PAT then provided an assessment of peripheral blood flow and endothelial function. This was conduced using the Endo-PAT2000 system (Itamar Medical Ltd, Caesarea, Israel). Using pneumatic probes this system measures endothelium-mediated changes in the digital pulse waveform (Axtell *et al.*, 2010). A blood pressure cuff was applied to the dominant arm and pneumatic fingers probes are applied to the index finger of each hand and inflated. A baseline PAT signal (i.e. pulse amplitude) was then obtained over 5 minutes of rest. The brachial artery was then occluded via the blood pressure cuff reaching suprasystolic pressures (60 mmHg above systolic pressure, ensuring this was not less than 200 mmHg or greater than 300 mmHg) for 5 minutes, then released. The PAT signal was then recorded for up to 10 minutes. The remaining arm does not experience occlusion and acts as a control and helps to correct for non-endothelial dependent factors such as room temperature. The digital pulse volume was then measured during post-ischaemic reactive hyperaemic phase and allows the calculation of the reactive hyperaemia index (RHI) by the ENDO-PAT software, which was calculated by dividing the mean PAT signal amplitude of the following deflation by the mean PAT amplitude prior to brachial occlusion. The RHI that has been demonstrated correlate with endothelial function in coronary arteries, and is associated with multiple cardiometabolic risk factors (Bonetti *et al.*, 2004; Hamburg *et al.*, 2008). Furthermore, this system permits the measurement of the augmentation index (AIx), which provides an assessment of medium to larger arterial wall elasticity, and will be described in the next section addressing PWA.

6.4.5.3 Pulse wave analysis & velocity

Lastly, PWA and PWV was undertaken using the SphygmoCor XCEL (AtCor Medical, Sydney, Australia) system. The predictive value of arterial wave reflection, central pulse pressure and arterial stiffness in cardiovascular risk prediction has

previously been demonstrated and provides as assessment of overall vascular health (Laurent *et al.*, 2001; Weber *et al.*, 2005).

In PWA, a brachial blood pressure cuff assesses systolic and diastolic pressures and captures a sub-diastolic volumetric displacement signal to provide a central aortic waveform (Butlin & Qasem, 2017). The cuff was placed on the upper arm of the patient, cantered on the brachial artery, and the analysis was performed in the supine position following 5 minutes of rest. PWA assessment was obtained following the measurement of two brachial blood pressures with a minute interval. The SphygmoCor XCEL software provides a quality control assessment to ensure the validity of PWA measurements based on variability parameters. This provides an assessment of central aortic systolic and diastolic pressure and AIx, which evaluates arterial stiffness and was calculated from the ascending aortic pressure waveform. This parameter is significantly influenced by heart rate, therefore the AIx corrected to heart rate of 75 beats per minute (AIx@75), which is performed automatically via the SphygmoCor software. As duplicate waveforms were assessed via this system an average measurement of these parameters is reported.

Volumetric displacement waveforms measured via a femoral cuff, combined with a carotid artery tonometry, provides an assessment of PWV. PWV is a measure of arterial stiffness, whereby the velocity of the arterial waveform increases as a consequence of aberrations in arteriolar constriction or elasticity (Milan *et al.*, 2019). A blood pressure cough was applied to the right thigh in close proximity to the femoral artery with the participant in a supine position. Concurrently, a central pulse wave was detected by placing a probe over the right common carotid artery. Carotid-femoral distance was then calculated via the SphygmoCor software via a subtraction method from the participants carotid measurement to the sternal notch and the sternal notch to femoral blood pressure cuff. PWV is then calculated by dividing the distance by the pulse transit time and a quality control assessment of waveform variability was provided by the software. Duplicates of this assessment were taken and averaged for assessment.

6.4.6 Ethical approval

This study was approved by the West of Scotland Research Ethics Committee 4 (19/WS/0084) and NHS Greater Glasgow and Clyde Research and Development (GN19CA257) in June 2019.

6.4.7 Patient & public involvement

This research study was supported by the Scottish Transgender Alliance, an Equality Network project aimed at improving gender identity and gender reassignment equality, rights and inclusion in Scotland. Study participants were invited to provide feedback with regards to the acceptability of this study and its procedures.

6.4.8 Statistical analysis & sample size

Continuous variables were expressed as mean (SD). Power analysis for a one-way ANOVA with 4 groups indicated that a sample size of 180 will be required to detect effect sizes of f=0.25 using an α of 0.05 and a power of 0.80. The primary outcome of this analysis was carotid-femoral PWV. It was planned that 200 participants should be recruited into this study to allow for any incomplete data. Predicted participant enrolment forecasts were calculated using weighted linear regression, based upon initial study recruitment. All statistical analyses were completed using R version 4.2.2 (R Core Team, Vienna, Austria).

6.5 Results

6.5.1 Participant recruitment

Recruitment into this study was halted due to the COVID-19 pandemic. Predicted enrolment was determined via weighted linear regression model, taking into account initial enrolment rates. This demonstrated that this study was on track to meet recruitment targets by November 2021 (i.e. within the allotted study period). As this analysis was consequently deemed to be underpowered due to limited recruitment, the results from this analysis remain preliminary and descriptive. Moreover, they provide insight into the feasibility of a study of this nature.

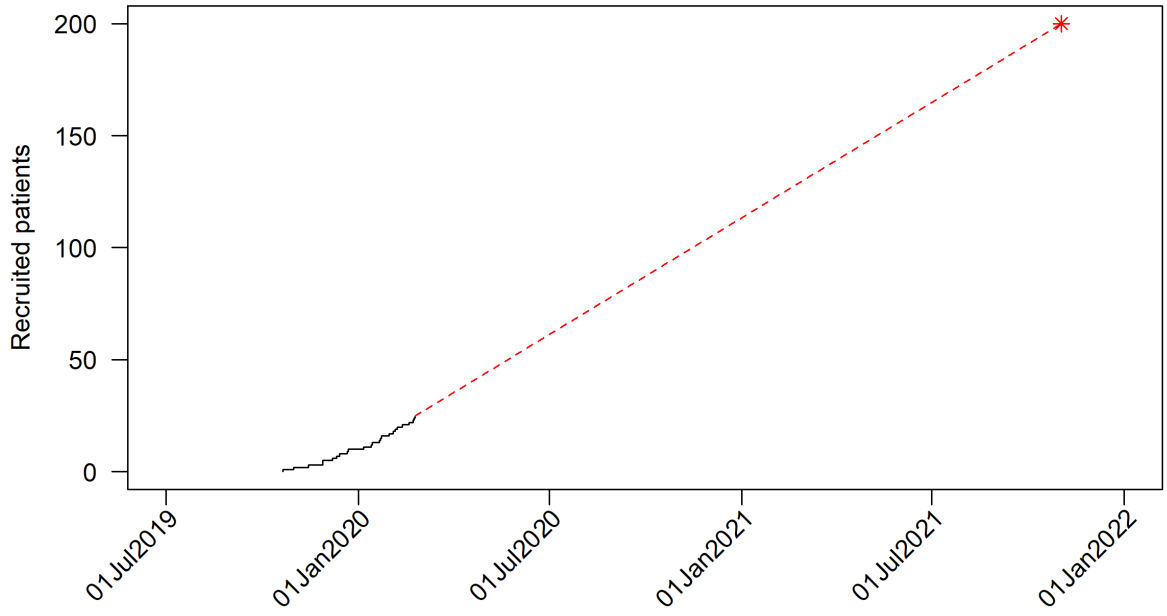

Figure 6-1. Predicted recruitment of the VESSELS study.

The black line denotes initial study recruitment. The red dashed line represents predicted recruitment determined via a weighted linear regression analysis, taking into account initial enrolment rates. Recruit targets were predicted to be met by November 2021, as represented by the red asterisk symbol.

The most successful means of recruitment was found to be posters, by which almost all transgender individuals included this study were recruited (n=5). Three participants were recruited via physical poster, whereas the remaining two participants were recruited via online publication.

Recruitment via clinics was found not to be successful. The Sandyford typically reviews patients within the first three years of commencing hormonal therapy. As a consequence, the majority of individuals that attended these clinics did not meet the inclusion criteria of exposure to GAHT for a minimum of five years. Similarly, endocrinology clinics did not prove to be a successful source of recruitment of transgender individuals. This was deemed to be a consequence of the older age of these patients, who were often excluded as they were over 50 years of age, and the low rate of transgender individuals attending this clinic.

Additionally, under section 22 of the Gender Recognition Act 2004 it is an offence for a person who has acquired protected information in an official capacity to disclose this information to any other person (UK Government, 2004). Consequently, for the purposes of this study, and in according with the Gender Recognition Act and GMC guidance, no potential participant's gender history could be communicated with the research team without first obtaining consent from the potential study participant to share this information. This acted as a barrier for recruitment in clinical settings.

Lastly, transgender study participants were invited to provide feedback with regards to the acceptability of this study and its procedures. No negative feedback was received with respect to the study visit or procedures, and no adverse events were recorded.

6.5.2 Study cohort

Between August 2019 and March 2020, a total of 18 participants were recruited to this study including 5 cisgender men, 7 cisgender women, 3 transgender men and 3 transgender women. Baseline characteristics are summarised in Table 6-1. The mean age of participants was 33.1 years (SD 5.3) with a mean BMI of 26.1 (SD 3.9). The majority of participants recruited were of Caucasian ethnicity (88.8%). Only 2 participants (11.1%) within this cohort were ex or current smokers. There was no history of hypertension, dyslipidaemia, myocardial infarction (MI), ischaemic heart disease, stroke, transient ischaemic attack (TIA) or venous thromboembolism in any participants.

	Cisgender Men	Cisgender Women	Transgender Men	Transgender Women
n	5	7	3	3
Age, years (SD)	31.8	31.3	36.7	35.7
BMI (SD)	25.9 (1.7)	25.4 (3.8)	25.4 (4.9)	29 (6.2)
Smoker, n (%)	0 (0%)	1 (14.3%)	0 (0%)	1 (33.3%)
Caucasian, n (%)	5 (100%)	5 (71.4%)	3 (100%)	3 (100%)
Systolic Blood Pressure, mmHg (SD)	118.1 (9.9)	115.2 (5.0)	118.2 (6.4)	126.9 (5.6)
Diastolic Blood Pressure, mmHg (SD)	78.0 (11.1)	73.1 (4.9)	75.0 (82.1)	82.1 (5.7)
GAHT exposure, years (SD)	-	-	11.7 (6.8)	6.3 (1.2)

Table 6-1. Participant demographics stratified by gender identity groups.
Data expressed as mean (SD) unless otherwise stated. BMI: Body mass index; GAHT: Gender-affirming hormonal therapy.

The mean exposure to GAHT in either transgender men or women was 9 (SD 5.6) years. All participants utilising transfeminine therapies utilised estradiol valerate at doses of 5-8 mg/day with mean exposure of 6.3 (SD 1.2) years. Doses of cyproterone acetate (12.5 – 25mg/day) were used in two participants. Participants within the transgender men group used intramuscular (IM) testosterone undecanoate (i.e. Nebido) 1 g every 12 weeks (n=1) or an ester formulation (i.e. Sustanon) 250 mg every 3-4 weeks (n=2). The mean GAHT exposure of transmasculine therapies was 11.7 (SD 6.8) years.

6.5.3 Blood pressure measurements

In this cohort the mean systolic blood pressure was 118.4 (SD 7.6) mmHg and diastolic blood pressure was 76.3 (SD 8.5) mmHg (Figure 6-2). In those using transmasculine therapies, systolic blood pressures were 118.2 (SD 6.4) mmHg and diastolic blood pressures were 75.0 (82.1) mmHg. Individuals using transfeminine GAHT were found to have a mean systolic blood pressure of 126.9 (SD 5.6) mmHg and a diastolic blood pressure of 82.1 (SD 5.7) mmHg.

Systolic blood pressures measured between cisgender men and transgender men were equivalent (118.0 mmHg), while the lowest mean systolic blood pressure was in the cisgender women group (115.2 mmHg (SD 5.0)). The mean systolic blood pressures of transgender women were found to be 12 mmHg higher than cisgender women, and 9 mmHg higher than cisgender men. Similarly, the highest diastolic blood pressure (82.1 mmHg (SD 5.7)), mean arterial blood pressure (97 mmHg (SD 5.4)) and pulse pressure (44.8 mmHg (SD 4.1) were identified within the transgender women group (Table 6-2). One transgender woman demonstrated high-normal blood pressure (130–139 mmHg), while one cisgender man had a blood pressure consistent with Grade 1 hypertension, as defined the 2023 European Society for Hypertension guidelines for the management of arterial hypertension guidelines (Mancia et al., 2023). There were no recorded issues with respect to the tolerability of this measurement in either the cisgender or transgender groups.

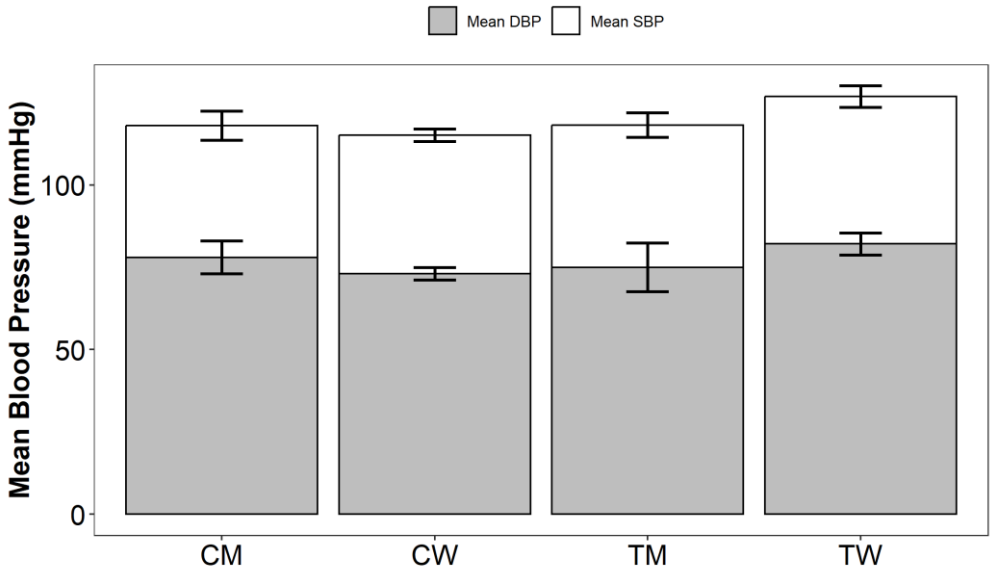

Figure 6-2. Blood pressure measurements in VESSEL transgender participants.
Data presented as means with standard error bars. Blood pressure measured in the sitting position. SBP: systolic blood pressure; DBP: diastolic blood pressure; CM: Cisgender Men; CW: Cisgender Women; TM: Transgender Men; TW: Transgender Women.

6.5.4 Flow mediated dilatation

Endothelium-dependent brachial artery FMD was determined to be 5.8% (SD 2.8) in cisgender men and 6.9% (SD 2.0) in cisgender women. Within the transgender groups, FMD was found to be 6.2% (SD 0.7) in transgender men, and 7.0% (SD 2.1) in transgender women. Figure 6-3 demonstrates the endothelium-dependent brachial artery FMD values obtained from these participants, whereby transgender women demonstrated the highest mean FMD across all groups. There were no recorded issues with respect to the tolerability of this measurement in either the cisgender or transgender groups.

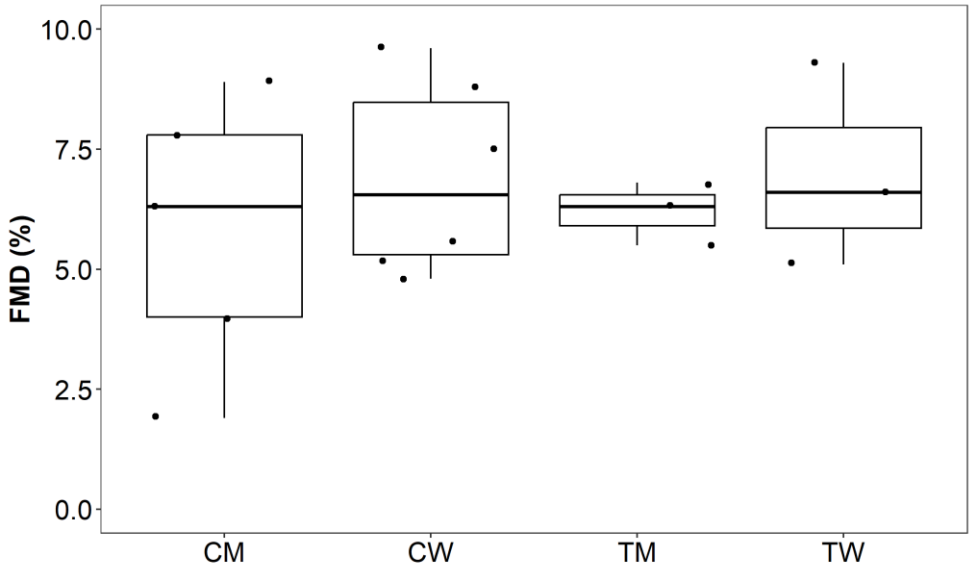

Figure 6-3. Endothelium-dependent brachial artery flow-mediated dilatation.
FMD, expressed as percentage change from baseline. CM: Cisgender Men; CW: Cisgender Women; TM: Transgender Men; TW: Transgender Women.

6.5.5 Peripheral arterial tonometry

Using the ENDO-PAT 2000 system, the natural logarithmic transformation of reactive hyperaemia index (LnRHI) was calculated for each group (Table 6-2). This was found to be 0.4 (SD 0.4) in cisgender men, 0.6 (SD 0.3) in cisgender women, 0.6 (SD 0.4) in transgender men and 0.9 (SD 0.4) in transgender women. The RHI was 1.5 (SD 0.5) in cisgender men 2.0 (SD 0.6) in cisgender women, 1.8 (SD 0.7) in transgender men and 2.7 (SD 0.9) in transgender women.

The AIx@75 was also derived from the ENDO-PAT 2000 system, and will be later compared with data derived from PWA studies. This was demonstrated to be -3.6% (SD 14.8) in cisgender men, -7.4% (SD 17.2) in cisgender women, -7.0% (SD 9.2) in transgender men and -18.7% (SD 8.6) in transgender women. Consequently, the transgender women group demonstrated the highest values in LnRHI, RHI and Aix@75 values across these groups. There were no recorded issues with respect to

the acceptability of this measurement in either the cisgender or transgender groups.

6.5.6 Pulse wave analysis & velocity

Aortic systolic blood pressures in each group were as follows: cisgender men: 111.9 mmHg (SD 3.0); cisgender women: 106.5 mmHg (SD 5.8); transgender men: 110.3 mmHg (SD 5.8); and transgender women: 112.3 mmHg (SD 7.8). Mean aortic diastolic blood pressures were broadly equivalent in each group and ranged between 76.3 and 79.2 mmHg. Mean aortic pulse pressures ranged between 32.2 and 36.0 mmHg, with the highest values being demonstrated in the transgender women group.

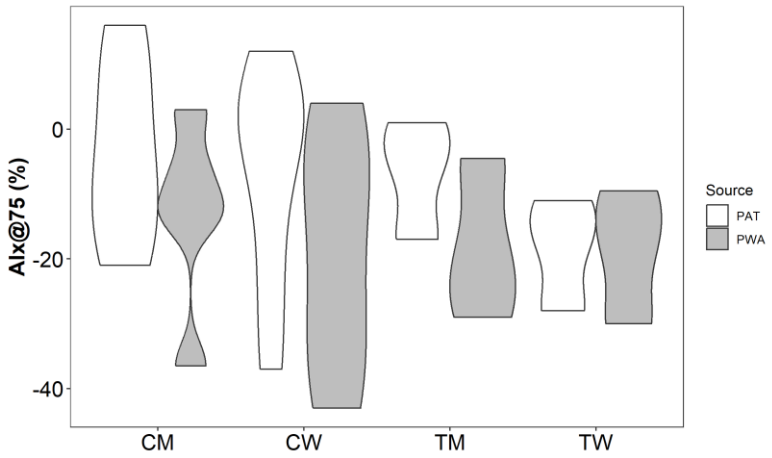

Figure 6-4. AIx@75 Violin plot.

AIx@75 values recorded by peripheral arterial tonometry (PAT) and pulse wave analysis (PWA) procedures. CM: Cisgender Men; CW: Cisgender Women; TM: Transgender Men; TW: Transgender Women.

Aix@75 derived from PWA was 13.0 (SD 14.7) in cisgender men, -18.8 (18.6) in cisgender women, -17.8 (SD 19.0) in transgender men and -19.0 (SD 10.3) in transgender women. These results were compared between PAT and PWA techniques (Figure 6-4). Although broadly of greater value in PWA compared to PAT, there is agreement between these two measurements of Aix@75, as

demonstrated in the Bland-Altman plot in Figure 6-5. This demonstrates limited systemic differences and good agreement between methods.

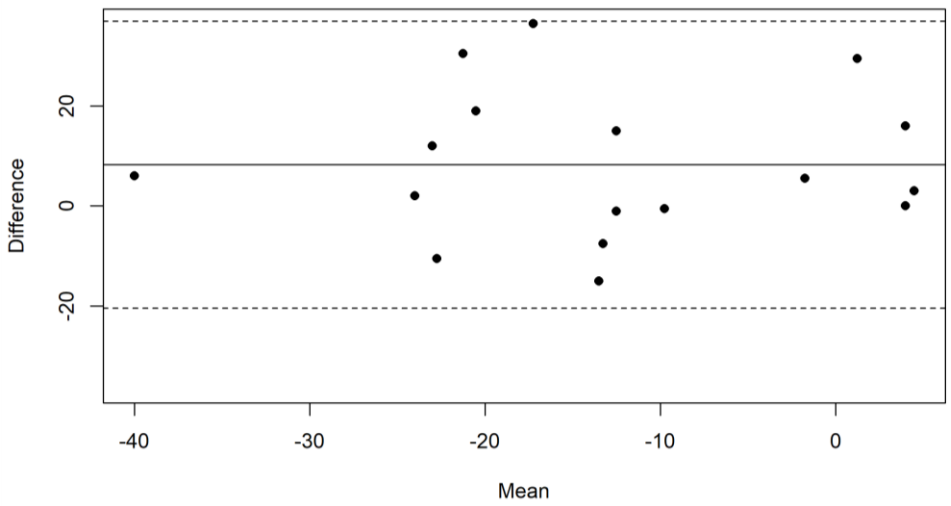

Figure 6-5. Aix@75 Bland-Altman plot.

This Bland-Altman plot demonstrates the mean difference in Aix@75 between PAT and PWA procedures. The dashed lines represent 95% confidence intervals. The solid line represents the mean difference (i.e. mean bias or systematic difference) between instruments. The x-axis represents the mean and the y-axis represents the difference between the two methods.

Lastly, PWV was recoded in each group. This was demonstrated to be 7.0 m/s (SD 1.8) in cisgender men, 6.2 m/s (SD 0.5) in cisgender women, 6.9 m/s (SD 1.4) in transgender men and 7.7 m/s (SD 0.6) in transgender women. Consequently, those utilising feminising endocrine therapies, demonstrated the most elevated PWV within the groups measured in this analysis. There were no recorded issues with respect to the tolerability of these measurements in either the cisgender or transgender groups.

	Cisgender Men	Cisgender Women	Transgender Men	Transgender Women
N	5	7	3	3
Sitting Blood Pressure				
Systolic Blood Pressure, mmHg (SD)	118.1 (9.9)	115.2 (5.0)	118.2 (6.4)	126.9 (5.6)
Diastolic Blood Pressure mmHg (SD)	78.0 (11.1)	73.1 (4.9)	75.0 (82.1)	82.1 (5.7)
Mean Arterial Blood Pressure mmHg (SD)	88.4 (15.6)	87.2 (4.6)	89.4 (10.6)	97.0 (5.4)
Pulse Pressure mmHg (SD)	31.2 (16.6)	42.1 (4.1)	43.2 (6.8)	44.8 (4.1)
FMD & PAT				
Brachial Artery Flow Mediated Dilatation (%)	5.8 (2.8)	6.9 (2.0)	6.2 (0.7)	7.0 (2.1)
LnRHI	0.4 (0.4)	0.6 (0.3)	0.6 (0.4)	0.9 (0.4)
RHI	1.5 (0.5)	2.0 (0.6)	1.8 (0.7)	2.7 (0.9)
AIx (%)	5.6 (14.7)	3.3 (19.9)	7.3 (12.0)	-9.3 (12.2)
AIx@75 (%)	-3.6 (14.8)	-7.4 (17.2)	-7.0 (9.2)	-18.7 (8.6)
PWA & PWV				
Central Aortic SBP (mmHg)	111.9 (3.0)	106.5 (5.8)	110.3 (5.8)	112.3 (7.8)
Central Aortic DBP (mmHg)	79.2 (4.2)	74.3 (2.9)	76.3 (8.4)	76.3 (2.1)
Central Aortic PP (mmHg)	32.7 (5.6)	32.2 (3.7)	34.0 (2.8)	36.0 (9.1)
AIx@75 (%)	-13.0 (14.7)	-18.8 (18.6)	-17.8 (19.0)	-19.0 (10.3)
Pulse Wave Velocity (m/s)	7.0 (1.8)	6.2 (0.5)	6.9 (1.4)	7.7 (0.6)

Table 6-2. Vascular phenotyping parameters.

Data presented as means (SD). SBP, Systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; LnRHI, natural logarithmic transformation of reactive hyperaemia peripheral arterial tonometry index; RHI; reactive hyperaemia peripheral arterial tonometry index AI, augmentation index; AI@75, augmentation index corrected for heart rate of 75 bpm.

6.6 Discussion

In this chapter, a total of 18 individuals, including 6 people who are transgender and 12 people who were cisgender were recruited to the VESSELS study. This analysis demonstrated the feasibility of conducting a study within this cohort, as determined by predicted recruitment forecasts. In particular, there was successful recruitment of transgender populations, largely via the physical and online publication of study posters, and broad acceptability of the study visit interventions. Although this preliminary descriptive analysis was underpowered to detect meaningful differences between groups, it should be noted that the transgender women group demonstrated the most extreme values in almost all vascular measurements, including systolic blood pressure, diastolic blood pressure, MAP, pulse pressure, FMD, LnRHI, RHI, Aix@75, central aortic systolic blood pressure, central aortic pulse pressure and PWV.

As demonstrated in Chapter 2, the evidence for altered blood pressures in transgender individuals is limited. Within this systematic review of 14 studies comprising 1,309 transgender individuals, transgender men did not experience any significant change in systolic or diastolic blood pressures (Connelly *et al.*, 2021c). Conversely, blood pressure responses in transgender women were inconsistent with both increases and decreases observed. However, data utilised within this analysis had significant heterogeneity with respect to gender-affirming hormones utilised and often did not provide control subjects for comparison. Consequently, it could not provide clinical recommendations for either the impact of gender-affirming hormones on blood pressure, or when this important determinant of vascular health should be regularly monitored in this population.

More recently, a study of 470 transgender individuals with a follow up 57 months demonstrated an increase in systolic blood pressure of 2.6 mmHg in transgender men and decreases in systolic blood pressure of 4 mmHg in transgender women (Banks *et al.*, 2021). Therefore, the results obtained for such analyses remain

inconsistent and the field requires prospective longitudinal observational cohort studies of blood pressure measurement, ideally including ambulatory blood pressure monitoring, to determine whether hormonal therapy promotes differences in blood pressure, and whether these translate into adverse cardiovascular outcomes (Connelly *et al.*, 2021c).

Both FMD and PAT have been shown to significantly predict cardiovascular events, whereby a decrease in these assessments by 1 standard deviation is associated with a doubling of cardiovascular risk (Matsuzawa *et al.*, 2015). However, limited data are available on the effect of GAHT upon endothelial function in transgender individuals.

Previously, no differences in FMD were observed between transgender men and cisgender women controls, although a reduction in nitrate induced vascular responses was observed (McCredie *et al.*, 1998). In a prospective observational study of 20 transgender men receiving testosterone therapy, no difference in FMD was demonstrated at 6 or 12 months following commencement of these therapies (Aranda *et al.*, 2019). However, in a cohort of 11 transgender men, FMD was found to be significantly lower compared to 20 cisgender women during the follicular phase of their menstrual cycle (Gulanski *et al.*, 2020). Conversely in 14 transgender women, FMD was elevated compared to cisgender men but non women, suggesting a potential role of oestrogen mediated-dilatation in this cohort (New *et al.*, 1997). With respect to PAT, to date no studies have been published relating to endothelial function in transgender individuals. Taken together, there are limited and inconsistent data regarding the effects of these feminising or masculinising gender-affirming therapies upon endothelial function and further research is merited to better understand the interaction between exogenous sex steroids and endothelial function.

Arterial stiffness has previously been assessed in 48 transgender men reiving transmasculine therapy and 63 untreated transgender men (Emi *et al.*, 2008). This

demonstrated that those receiving transmasculine therapies demonstrated a higher brachial-ankle PWV. Similarly, in a cross-sectional case–control study of 33 transgender men receiving IM testosterone esters for a mean of 14 years, carotid-femoral PWV was significantly higher compared to both cisgender men and women, potentially suggesting longer-term cardiovascular risk associated with transmasculine therapies in this population (Cunha *et al.*, 2023). In an analysis of 56 transgender women using oral conjugated equine oestrogen, brachial-ankle PWV was lower compared to 22 transgender women not receiving treatment (Sharula *et al.*, 2012). In this study, the carotid Aix was similarly found to be lower in those exposed to transfeminine therapies.

In the study described in this chapter, although statistical analysis was precluded as a consequence of being underpowered, transgender women had a higher carotid-femoral PWV compared to any other cohort. This suggests that long-term oestrogen exposure in transfeminine individuals may facilitate arterial stiffness. This differs from the analysis by Sharula et al, however, differences exist between methodologies (Sharula *et al.*, 2012). In particular, carotid-femoral PWV is considered the gold-standard compared to brachial-ankle PWV (Townsend *et al.*, 2015; Van Bortel *et al.*, 2016). Furthermore, conjugated equine oestrogens were utilised in the previous analysis, which are generally considered more potent and at a higher risk of promoting cardiovascular disease when compared to oestrogen valerate used by VESSEL participants (Seal *et al.*, 2012; Nolan & Cheung, 2020).

If this relationship were to hold true, it may occur via a number of mechanisms including the promotion of arterial calcification via oestrogen mediated-vascular smooth muscle cells into osteoblast like cell (McRobb *et al.*, 2017). Similarly, such effects may be a consequence of a legacy effect of natal sex, whereby males typically exhibit higher systolic blood pressures and PWV compared to age-matched females (Weng *et al.*, 2013; Ji *et al.*, 2020). However, due to the significantly limited power of this analysis it would not be appropriate to draw firm conclusions without further research.

A strength of this analysis is that is demonstrates a degree of feasibility with respect to the local recruitment of transgender participants. This study was well within target to reach the recruitment targets of 50 transgender men, women and cisgender controls within the study period. Indeed, recruitment forecasts determined via a weighted linear regression model of initial enrolment rates suggested that study recruitment should have concluded well within the study period. It is recommended that future studies engage with transgender community groups, charities and social media to publicise research given the relative success of recruitment of transgender individuals compared to clinical environments. Furthermore, as a methodological approach, this analysis utilised a broad range of well-tolerated vascular phenotyping techniques to provide a comprehensive assessment of the vascular function of this cohort, which would have offered significant insight into the effects of GAHT. Moreover, this analysis aimed to look at the effect of hormone therapy after 5 years of therapy. Many studies of this nature are limited to the short-term and do not provide assessments of longer-term use and this has been identified as a key priority for future research (Streed et al., 2021).

The most important limitation of this analysis was its premature discontinuation as a result of the COVID-19 pandemic. This was a consequence of limited clinical activity of endocrinology and gender identity services, which impacted significantly the recruitment of participants. Furthermore, a number of study participants cancelled study appointment due to concern regarding COVID-19 transmission and infection. Lastly, there was restricted access of the Glasgow Clinical Research Facility to complete this study as a consequence of COVID-19 precautions and the adoption of a number of high priority vaccine and COVID-19 studies. Following clinical deployment as a medical registrar to Glasgow Royal Infirmary to provide medical support in the pandemic, and the following increased on-call and clinical commitments thereafter, it was not feasible to recommence this analysis. However, the preliminary data provided in this descriptive analysis will no doubt act as a springboard to facilitate future research in this field.

To conclude, this chapter presents preliminary data from the VESSEL study demonstrating the potential feasibility of conducting research of this nature within a cohort of transgender individuals. Moreover, across a number of vascular phenotyping measurements, transgender women had values consistently higher than other cohorts, albeit within the context of a limited sample size. As highlighted in a recent systematic review, the results obtained from vascular phenotyping studies in transgender individuals remain inconclusive due to limitations of sample size and heterogeneity in outcomes and interventions (Moreira Allgayer *et al.*, 2023). Thorough vascular phenotyping studies of this nature, with homogeneous interventions, are crucial to understanding the cardiovascular risk of this population, and the associated underlying pathophysiological mechanisms. Furthermore, the study of vascular function in the transgender population could more broadly advance our understanding of the role of sex and gender in vascular health and disease. Therefore, further research is merited to elucidate the potential effects of gender-affirming care upon endothelial function and arterial stiffness in this population. In particular, comprehensive prospective data are required in order to inform evidence based guidance and practice.

Chapter 7 General Discussion

7.1 Chapter overview

This concluding chapter begins with an introduction and recapitulation of the aims of this thesis. This is followed by a summary of the findings of this research. Next, the impact of the Coronavirus disease 2019 (COVID-19) pandemic upon this thesis is discussed before outlining the strengths and limitation of this piece of work. Lastly, the future directions of this research are considered.

7.2 Introduction

Appreciation of the role of sex and gender in the development of cardiovascular disease has expanded rapidly in recent years. Emerging data have demonstrated differences in the mechanisms, prevention, management and outcomes of cardiovascular disease between males and females and support the implementation of sex-specific guidelines (DeFilippis & Van Spall, 2021). Moreover, our understanding of the psychosocial influences of gender upon cardiovascular health has evolved in the last decade permitting the identification and analysis of gender-related mechanisms in health and disease (Regitz-Zagrosek & Gebhard, 2023). Such factors are of upmost importance to transgender populations, where they are uniquely exposed to a combination of novel sex and gender mediators that impact upon cardiovascular health (Caceres & Streed, 2021). Importantly, the population of individuals identifying as transgender and accessing gender-affirming healthcare are both increasing (Dhejne *et al.*, 2014; Meerwijk & Sevelius, 2017), which may have significant implication for equitable health care access and resource. Consequently, it is vital that we continue to progress our understanding of how sex and gender influence cardiovascular disease, particularly in minority populations, in order to mitigate health inequalities and improve the care of our patients.

This thesis aimed to explore the relationship between sex and gender in cardiovascular disease through a range of methodological approaches that could be utilised in future research. This was accomplished by: 1) reviewing the literature relating to sex, gender and cardiovascular disease; 2) performing a systematic review of the blood pressure effects of gender-affirming hormone therapy (GAHT) in transgender individuals; 3) adapting a gender-stratification questionnaire for use in clinical research; 4) identifying differentially expressed miRNA according to sex and gender phenotypes in individuals with ACS; and 5) conducting an analysis of vascular phenotypes in transgender individuals.

7.3 Summary of findings

In Chapter 2, the context for the means by which sex and gender factors may influence cardiovascular health of cisgender and transgender populations is outlined in a comprehensive literature review. This provides an overview of the complexity of sex and gender, and how variations in these traits may facilitate the development of cardiovascular disease, thereby establishing a foundation of understanding for future chapters.

In Chapter 3 a systemic review of the effect of GAHT on the blood pressure of transgender individuals was undertaken. A total of 14 studies were identified including 1,309 transgender individuals receiving GAHT who demonstrated blood pressure measurements before and after the introduction of these therapies. This systematic review demonstrated that in the majority of studies of transmasculine individuals using testosterone, no significant differences in either systolic or diastolic blood pressures were identified. In transfeminine individuals using oestrogen therapies, increases and decreases in systolic blood pressure were demonstrated.

Importantly, this systematic review highlighted many methodological issues with research in this domain: 1) studies were quasi-experimental uncontrolled pre-post

analyses; 2) hormonal therapies in cohorts were largely heterogenous, resulting in a lack of consistency in results; 3) there was inadequate follow up periods and small sample sizes; and 4) a general failure to standardise blood pressure measurements or use validated devices. The majority of studies were consequently rated as poor to fair in the quality assessment undertaken. Therefore, this analysis was unable to provide clinical recommendations of the blood pressure effects of GAHT in transgender individuals.

Chapter 3 sets forth recommendations for future blood pressure research in this population with a particular focus upon prospective longitudinal observational cohort studies to identify whether blood pressure aberrations occur within this population, and randomised controlled trials (RCTs) comparing intensive versus non-intensive blood pressure targets upon composite cardiovascular outcomes. Since the publication of this research (Connelly *et al.*, 2021c), the most recent version of the World Professional Association for Transgender Health (WPATH) Standards Of Care guidance, in which this systematic review is cited, recommends blood pressure monitoring prior to and following the commencement of testosterone therapy, particularly within the first two to four months of treatment (Coleman et al., 2022), thereby demonstrating the real-world impact of this research.

In Chapter 4, the Gender and Sex Determinants of Cardiovascular Disease: From Bench to Beyond-Premature Acute Coronary Syndrome (GENESIS-PRAXY) questionnaire was successfully adapted and applied to a healthy UK University cohort in order to assess its utility and analysis methods for future use in cardiovascular research. This demonstrated a spectrum of overlapping gender scores in 446 cisgender men and women. The model used to develop this score derived from instruments relating to whether someone was available to provide good advice; where they felt they stood in their community, personal income; and Bem sex role inventory (BSRI) masculinity and femininity scores. Furthermore, a simple scale of self-assigned masculinity and femininity was applied within this

cohort that demonstrated a gender continuum and was found to be a more sensitive predictor in this setting.

Interestingly, these results differed somewhat from the original GENESIS-PRAXY analysis, whereby more separate gender score distributions between men and women were observed (Pelletier *et al.*, 2015). These differences may have arisen by differing components used in the GGI model, where the GGI relied upon household primary earner status, personal income, the number of hours per week dedicated to housework, the level of stress at home, and BSRI masculinity and femininity scores. Furthermore, this may be a consequence of the populations sampled, whereby GENESIS-PRAXY was derived from older individuals that had experienced ACS conducted between 2009-2013 in North America (Pelletier et al., 2016). This therefore highlights that gender and its related factors are evolving, dynamic and context dependent (Connelly *et al.*, 2021a). Research utilising such components should incorporate gendered-factors relevant to the population sampled to ensure validity. This concept was recently exemplified in a cross-temporal meta-analysis (i.e. a meta-analysis of published data across different time periods) of U.S. college students BSRI evaluations, whereby femininity scores have decreased significantly across the last three decades, which may indicate cultural changes in expected feminine gender norms (Donnelly & Twenge, 2017). Moreover, as the majority of quantitative tools to measure gender have been conducted in Caucasian populations further research is required to determine the impact of race and ethnicity on these measures (Nielsen *et al.*, 2021).

Chapter 5 identified multiple differentially expressed circulating miRNA in a study of sex and gender stratified participants who has experienced ACS from the original GENESIS-PRAXY cohort. In this analysis the miRNA miR-664a-5p, miR-3613-5p, miR-382-5p, miR-134-5p, miR-10b-5p, miR-885-5p, miR-206, and miR-32-5p were demonstrated to be differentially expressed in females compared to males in a next generation sequencing (NGS) bioinformatic analysis. Many of these miRNA and their gene networks demonstrate multiple important functions to ACS

pathophysiology including the regulation of vascular smooth muscle cell proliferation, endothelial injury and inflammation, atherosclerosis progression (Yu *et al.*, 2015; Wang *et al.*, 2018; Dai *et al.*, 2020; Zhang *et al.*, 2022). Notably, many of these miRNA may be modulated via oestrogen and oestrogen response element (ERE)-mediated interactions or demonstrate sex-dependent expression (Mooney *et al.*, 2020; Ramanujan *et al.*, 2021).

Additionally, this analysis detected two differentially regulated miRNA (i.e. miR-3605-5p and miR-4467) in males with feminine versus masculine gender characteristics. It has previously been recognised that epigenomic processes, such as environmental or psychosocial factors, may influence pathophysiological processes. A prime example of this is the epigenetic response to excess noise, which has been shown to modulate miRNA expression, and is associated with hypertension and coronary artery disease (Miguel *et al.*, 2018; Münzel *et al.*, 2021). Although data are limited on the functionality of these differentially expressed gender-related miRNA with respect to the development of ACS, this research provides a springboard for future research into the social epigenomic regulation of cardiovascular disease and its associations with gendered-factors.

Chapter 6 presents preliminary analysis from the Vascular Effects of Sex Steroids in Transgender Adults (VESSEL) study. This study aimed to conduct vascular phenotyping procedures, including flow-mediated dilatation (FMD), peripheral artery tonometry (PAT), and pulse wave analysis (PWA) and velocity (PWV) in transgender individuals on long-term GAHT compared to cisgender individuals. This study was disrupted and eventually discontinued as a result of the COVID-19 pandemic (Section 7.5). Consequently, the results of this chapter are limited to a descriptive analysis, however, do demonstrate that across a number of vascular phenotyping measurements (i.e. systolic blood pressure, diastolic blood pressure, mean arterial blood pressure, pulse pressure, FMD, reactive hyperaemia index (RHI), augmentation index corrected for 75 beats per minute (Aix@75), central aortic systolic blood pressure, central aortic pulse pressure and PWV) transgender

women elicited the uppermost values. Although it is regrettable that this study is underpowered and could not be completed, it does demonstrate feasibility with respect to the local recruitment of transgender participants and acceptability of the study protocol. In particular, using a weighted linear model of initial study recruitment, predictions of study completion fell within the study allotted study period, suggesting research of this nature is possible and realistic. Importantly, this highlights the importance of engagement with transgender communities in successful recruitment. Moreover, this chapter reviews the available data relating to vascular phenotyping studies in transgender individuals, and highlights the paucity of data available and the potential utility of such research with the aim of understanding the pathophysiological mechanisms responsible and cardiovascular health of transgender individuals.

7.4 Strengths & limitations

One of the main strengths of this thesis is the utilisation of multiple methodological approaches utilised to gain a better understanding of the role of sex and gender in cardiovascular disease, and in particular the impact of being transgender upon cardiovascular risk. Included methodologies included: 1) systematic review; 2) the implementation and analysis of a questionnaire via principal component analysis; 3) the utilisation of a bioinformatics approach to identify novel differentially expressed miRNA expressed according to both sex and gender; and 5) a series vascular phenotyping procedures such as FMD, PAT, PWA and PWV. The diversity of methods used within this thesis has been challenging and rewarding in equal measure, and provides a novel exploration of the factors by which sex and gender may influence cardiovascular disease, but also how such traits could be measured in future research.

Another strength of this thesis is that it provides clear descriptions of the concepts of biological sex and psychosocial gender, and how these related but distinct components may modulate the development of cardiovascular disease.

Ultimately, a clear understanding of these factors is imperative to conducting higher quality research in this field and delivering better evidence-based guidance for our patients. Across this thesis, many attempts have been made to facilitate this understanding including: an in depth literature review in Chapter 2; the construction of a gender score to facilitate future cardiovascular research in Chapter 4; and the combined assessment of sex and gender-related differential miRNA expression in Chapter 5. Only through the prism of both sex and gender can we fully understand their combined contribution to the development of cardiovascular disease, and optimise our research efforts to improve health care for all patients.

A further strength of this thesis is that it seeks to highlight and address significant gaps in our knowledge of the effects of sex and gender upon the cardiovascular health of transgender populations, who have been historically underserved with respect to research and evidence based practice. (Rytz *et al.*, 2023). Undertaking research aimed at improving and understanding the cardiovascular health of this population requires a versatile approach (Streed *et al.*, 2021b). Consequently, the methodologies demonstrated within this thesis can be utilised to gain insight into the complex relationship between sex, gender and cardiovascular disease within this population. Further research is required to clarify this association and the mechanisms responsible.

The research conducted in this thesis is of high relevance within a rapidly evolving field. Publications originating from this thesis have thus far had significant impact upon the available evidence base available for the understanding of cardiovascular disease in people who are transgender. In particular, components of this thesis have informed the cardiovascular section of the WPATH Standards of Care for the Health of Transgender and Gender Diverse People, Version 8 guideline (Coleman *et al.*, 2022), a recent scientific statement from the American Heart Association relating to assessing and addressing cardiovascular health in LGBTQ Adults (Caceres *et al.*, 2020), an Endocrine Society scientific statement outlining

Endocrine Health and health care disparities in gender minority populations (Diaz-Thomas *et al.*, 2023), and the 2023 European Society of Hypertension Guidelines for the management of arterial hypertension (Mancia *et al.*, 2023).

Moreover, the focus of this thesis has permitted the opportunity to disseminate this expertise to wide range of audiences including the Faculty of Liaison Psychiatry Conference, Royal College of Psychiatrists, the European Council for Cardiovascular Research, the 7th Annual Healthy Hearts for Women Virtual Symposium hosted by Kentucky University, and even be interviewed by Heart Matters, the British Heart Foundation magazine. Importantly, it has provided me with the opportunity to taken on the role of a Special Issue Editor of the *Journal of Human Hypertension* special issue on sex and gender differences in hypertension, and compile review and research articles on this topic. Moreover, in collaboration with Professor Delles and Professor Katikireddi, we have been successful in obtaining funding from the Chief Science Office to host a call to fund research on the long-term health outcomes for those accessing gender identity healthcare in Scotland.

This expertise has permitted me to become a member of Health Improvement Scotland Gender Identity Healthcare Services Standards Development Group, and National Gender Identity Healthcare Reference Group, which aim to develop the standards and progress improvement for gender identity healthcare services in Scotland. Consequently, the knowledge I have obtained from this period of research will be used to improve the quality of care delivered to this population at a national level.

The work presented in this thesis also demonstrates a number of limitations. In Chapter 3, the systematic review focused upon the influence of gender-affirming hormones upon alterations in blood pressure rather than the development of hypertension. This potentially limited the scope of the analysis by not including clinically meaningful outcome. However, data relating to this outcomes remains

sparse and is constrained by many of the methodological limitations described in this thesis (Irwig, 2022).

With respect to the Chapter 4 gender-stratification questionnaire, a number of limitations are also evident. In this analysis gender is confined to a one-dimensional spectrum, which may not fully reflect the complexity of this construct. Researchers have proposed the use of the Stanford Gender-Related Variables for Health Research tool for gender assessment, which embraces a multi-dimensional approach (Nielsen *et al.*, 2021). However, this methodology does not accommodate the for the dynamic temporal or societal influences on gender, and as a consequence more responsive approaches will be required. Importantly, these methods should be validated within transgender populations to quantify the relationship between gender aspects and health outcomes.

There are also a number of limitations evident in the miRNA analysis conducted in Chapter 5. In particular, this cohort was limited to those who had experienced ACS and therefore the differentially expressed miRNA identified may reflect sex- and gender-specific differences rather than being ACS related per se. However, given the established role of many of these epigenetic modifications in vascular pathophysiology it is considered that this is less likely (Tsuji *et al.*, 2020). Regardless, validation in a gender stratified cohort which have not experienced ACS is required. Lastly, this chapter was limited to a bioinformatic analysis, and therefore required validation reverse transcription quantitative PCR and functional analysis. The latter would be of particular interest given the unknown influences of gender-specific differentially expressed miRNA in males.

Additionally, the vascular phenotyping study undertaken in Chapter 6 was significantly limited by recruitment challenges. The factors responsible for the difficulties this study faced are discussed in section 7.5. As a consequence, this is presented as a preliminary descriptive analysis.

A general limitation of this thesis is that the scope of its purpose is broad. As a consequence multiple components of sex and gender, their measurement and influence upon cardiovascular disease are addressed. Although this multi-method approach has also been considered a strength of this thesis, such an approach precludes a more in depth analysis of the investigated components. Although, this was a necessary response to the impact of COVID-19, it has resulted in a degree of disjointedness between chapters. Along with the diverse populations included within this thesis (e.g. transgender, cisgender, students, those who have experience ACS), the cardiovascular conditions assessed (e.g. hypertension, ACS, vascular phenotypes) is also broad. Consequently, the thesis would have benefited from greater focus. However taking into account the significant disruption caused by the COVID-19 pandemic, every effort has been made to present this thesis as a coherent work of novel research.

7.5 Impact of the COVID-19 pandemic

With regret, the impact the pandemic of COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) on this thesis has been substantial. Having commenced research in February 2019, the original focus of this thesis, which aimed to perform vascular phenotyping in those using long-term GAHT, was well underway (Chapter 6). However, in the UK a national lockdown was commenced in March 2020, which as a consequence resulted in a significant impact upon clinical research (Finn *et al.*, 2022). As a consequence, non-essential research activity at the University of Glasgow was suspended. Moreover, in the approach to this period, a number of study participants cancelled their study visits due to concern regarding the potential infection of COVID-19. Moreover, during this period I was deployed to Glasgow Royal Infirmary for three months in order to provide general internal medicine support as a medical registrar. This period and the subsequent escalated on call commitments impacted significantly the time available to undertake research, including limited access to the laboratory.

Following the national lockdown, the impact of the pandemic upon this research was pervasive. There was limited activity of endocrinology and gender identity services, which impacted recruitment. Reports suggesting an association between COVID-19, hormonal therapy and thrombosis may have also acted as a barrier to recruitment during this time (LaVasseur *et al.*, 2022). Furthermore, ongoing limited access was available at the Glasgow Clinical Research Facility to complete this study as a consequence of COVID-19 precautions and the adoption of a number of vaccine studies. Consequently, this study was deemed no longer feasible, and this study was halted indefinitely. Following the adaptation of the gender stratification questionnaire (Chapter 4) we had planned to amend the VESSELS protocol to undertake a pilot study in a transgender cohort. However, this was abandoned given concerns with regards to recruitment and engagement with this population during this time.

In order to mitigate the impact of COVID-19, I was required to adapt this thesis and explore further avenues of research. This resulted in significant delays and a steep learning curve required to produce the submitted thesis. This also meant that planned laboratory activity, including wire myography of facial vessels derived from transgender women undergoing facial feminisation surgery could not be completed.

Furthermore, in attempts to better understand the clinical impact of being transgender upon cardiovascular outcomes, I have also had the opportunity to work on the Clinical Practice Research Datalink (CPRD), which comprises one of the largest databases of longitudinal primary care electronic medical records in the world (Wolf *et al.*, 2019). The utilisation of linked primary and secondary longitudinal care records provides the opportunity to study the health of transgender people. However, this requires the development of a valid and reproducible transgender phenotype, which is not without challenges (Thomson & Katikireddi, 2019). Therefore this work has aimed to develop and validate an electronic phenotype for transgender identity in the UK based on a range of

ascertainment approaches using administrative health data utilising clinician codes in primary and secondary care indicating transgender identity. This process has involved developing a list of diagnostic terms indicating transgender identity, identifying a means of defining masculine and feminine transgender identity via administrative data and comparing the completeness of data for socio-demographic variables in the transgender and cisgender cohorts. Once developed this electronic phenotype would permit analysis of the incidence of cardiovascular disease, stroke, and thrombosis among transgender people compared to cisgender people. However, due to significant time constraints as a consequence of the impact of COVID-19, this research could not be completed prior to the submission of this thesis. However, funding has been granted by the Chief Scientist Office to support a PhD project in completing this important work in near future.

7.6 Future research directions

There are multiple aspects this thesis that merit future research. With respect to Chapter 3, further research is required to determine whether GAHT alters blood pressure in transgender individuals. Given the significant methodological limitations evident in established research, potential analyses could adopt an interrupted time series or self-controlled case series design. Ideally longitudinal observational cohort studies could be employed to identify the impact of these potential blood pressure alterations upon the cardiovascular risk (Connelly *et al.*, 2021c). RCTs are required to determine whether blood pressure interventions targeted towards an intensive or standard blood pressure regimes provide clinical benefit in this population. Moreover, there is an absence of cardiovascular guidelines for the investigation and management of individuals who are transgender. For instance, it is uncertain what sex specific high-sensitivity troponin thresholds should be used for the diagnosis of myocardial injury in this population (Lee *et al.*, 2019).

In Chapter 4, a gender stratification questionnaire was applied in a cohort of young people. Given the differences evident in gender score distributions between this research and the original GENESIS-PRAXY analysis, an examination of health outcomes associated with gender traits would be required to demonstrate clinical utility. Similarly, as gender scores may be affected by societal influences over time (Donnelly & Twenge, 2017), a longitudinal evaluation of gender scores within a cohort would permit a better understanding how these measures may alter through the lifespan and in response to societal changes, and how gender score variability may alter health outcomes.

In Chapter 5 the relationships demonstrated between sex, gender and differential miRNA expression are associative and therefore functional studies are required to determine the role of these miRNA and related gene interactions in the pathophysiology of ACS. Given the small sample size utilised in this analysis, expanding this cohort will be beneficial in the evaluation between miRNA gene dosage and the spectrum of gender scores obtained in the GENESIS-PRAXY cohort.

A priority for future research will be to better engage with the transgender community. Vascular phenotyping studies, such as those examining endothelial function and arterial stiffness described in Chapter 6, are imperative to broadening our understanding of cardiovascular risk in this population, and the role of sex and gender in vascular disease more generally. However, multiple barriers to transgender people participating in research have previously been identified including distrust, concerns regarding exploitation, and research studies not being accessible to transgender individuals (Asquith *et al.*, 2021).

To improve inclusion and engagement, Rytz *et al* have proposed a strategic framework (Figure 7-1) aimed at promoting engagement with transgender individuals in the development, execution, and dissemination of cardiovascular research (Rytz *et al.*, 2023). This article, of which I was a co-author, provides a roadmap that encourages researchers to recognise the bias towards cisgender, and

largely male, focused research, and the importance of accurate and safe data acquisition. Furthermore, to provide outcomes of clinical and societal value, transgender people must be involved in all aspects of research, including incorporation of individuals with lived experience into the research team. Given the recognised increase in the population of transgender individuals and the inequities evident in healthcare engagement and resource, it is imperative that such frameworks are adopted to improve the quality and quantity of research involving this population.

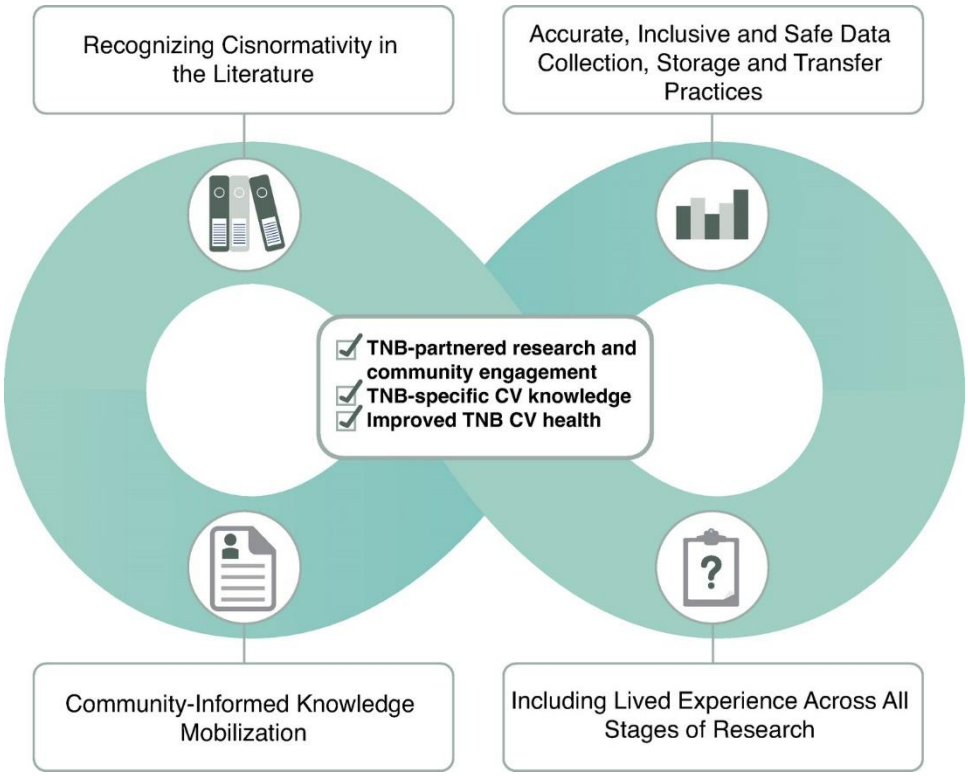

Figure 7-1. Improving inclusion of transgender participants in cardiovascular research.
A proposed road map to enhance the inclusion of transgender individuals in the planning, completion, and mobilisation of cardiovascular research. Reproduced with permission (Rytz *et al.*, 2023). TNB: transgender and nonbinary; CV: cardiovascular.

7.7 Conclusions

To conclude, through a range of investigational approaches, this thesis expands our appreciation and understanding of the role of sex and gender in cardiovascular disease. It presents a means by which gender can be measured in clinical research, and demonstrates the potential influence of both sex and gender upon the epigenomic regulation of miRNA in ACS. Additionally, this thesis improves our understanding of limitations and barriers in conducting research in people who are transgender, which must be overcome to provide better evidence based guidance for this underserved population. Overall, this thesis provides valuable insight into the methodological approaches used the investigation of sex and gender in cardiovascular disease, which can be applied in future cardiovascular research in cisgender and transgender populations.

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Appendix

Appendix i. Modified GENESIS-PRAXY questionnaire

Sex is recognized as an important demographic detail in the conduct of clinical research. Yet the terms sex and gender are often confused or miscategorised. Sex is assigned at birth and is based on biological factors. On the other hand gender is shaped by social context. Consequently, our gender can lie along a spectrum of masculinity, femininity or both and this might not match with the sex we are given at birth.

A research study in Canada has demonstrated that people with feminine genders, which were assessed using a gender questionnaire, were more likely to have recurrent heart attacks, regardless of what sex they were. The purpose of this study is to validate a modified version of this questionnaire in the UK so that it can be used in future cardiovascular research.

1. What sex were you assigned at birth? Male ▫ Female ▫
2. What gender do you identity as Man ▫ Woman ▫ Other ▫ (please specify_____)
3. What is your ethnicity?

White British ▫ White Irish ▫ White Other ▫ Mixed Race ▫

White & Black Caribbean ▫ White & Black African ▫ White & Asian ▫

Other mixed background ▫ Asian or Asian British ▫ Indian ▫ Bangladeshi ▫ Pakistani ▫ Other Asian background ▫ Black or Black British Caribbean ▫ African ▫ Black Other ▫ Chinese or other ethnicity Chinese ▫

Other ▫ (please specify) _____

4. What age are you? _____

5. What stage of study are you? Undergraduate□ Postgraduate□

6. What college of school/programme do you attend?

- Life sciences
  - Marine and Freshwater Biology□
  - Zoology□
  - Immunology□
  - Microbiology□
  - Biochemistry□
  - Genetics□
  - Molecular & Cellular Biology□
  - Anatomy□
  - Human Biology□
  - Neuroscience□
  - Pharmacology□
  - Physiology□
- Medicine, Dentistry, Nursing
  - Dentistry□
  - Medicine□
  - Nursing□
  - Intercalated□
- Veterinary Medicine
  - Veterinary Biosciences□
  - Veterinary Medicine & Surgery□
- Graduate School
  - Postgraduate taught programme □
  - Postgraduate research programme □
- Other □ (please specify school and programme) _____

7. Where do you currently live?

Halls of residence □

Rented studio or one bed flat ¤

Rented shared flat or house ¤

Rented room in landlord's home ¤

Parental home ¤

Property owned by a relative ¤

Own home ¤

Other _____

8. On a scale of 1 to 7 how masculine do you feel?

(1 being not masculine at all and 7 being extremely masculine)

1¤ 2¤ 3¤ 4¤ 5¤ 6¤ 7¤

9. On a scale of 1 to 7 how masculine do you think other people perceive you?

(1 being not masculine at all and 7 being extremely masculine)

1¤ 2¤ 3¤ 4¤ 5¤ 6¤ 7¤

10. On a scale of 1 to 7 how feminine do you feel?

(1 being not feminine at all and 7 being extremely feminine)

1¤ 2¤ 3¤ 4¤ 5¤ 6¤ 7¤

11. On a scale of 1 to 7 how feminine do you think other people perceive you?

(1 being not feminine at all and 7 being extremely feminine)

1▫ 2▫ 3▫ 4▫ 5▫ 6▫ 7▫

GENESIS PRAXY Gender Questionnaire

1. Which statements describe your current work situation? (Please check all that apply)

▫ Currently working

▫ Student

▫ Homemaker

▫ Unpaid Volunteer

▫ Unemployed, looking for work

▫ On leave of absence

▫ Other (specify): _____

2. What is the highest level of education that you completed?

▫ No degree, certificate or diploma

▫ Completed High School

▫ Some college/university

▫ Completed post secondary school (college/university)

▫ Completed registered apprenticeship/or other trades certificate

3. What is your current job?_____

4. How many hours per week do you usually work in your job, including paid and unpaid overtime hours? _____ Hours per week

5. Do you or your spouse/ partner receive any of the following benefits through your/their jobs? (check all that apply)

▫ Private dental/ health insurance

▫ Paid maternity or parental leave (beyond statutory entitlements)

▫ A pension plan (above what's provided by the state)

6. For the children or other people living with you, to what level are you directly responsible for caring for them? (circle one number)

No Total

responsibility responsibility N/A

1▫ 2▫ 3▫ 4▫ 5▫ 6▫ ▫

7. For the children or other people living with you, to what level are you directly responsible for disciplining them? (circle one number)

No Total

responsibility responsibility N/A

1□ 2□ 3□ 4□ 5□ 6□ □

8. If any of your children or other people living with you get sick, compared to their other parent, how likely is it that you will be the one to go get them (circle one number)

Very unlikely Very likely N/A

1□ 2□ 3□ 4□ 5□ 6□ □

9. On average, how many hours a week do you usually spend doing housework (e.g., cleaning, cooking, washing, etc.)?_____

10. Are you the primary person responsible for doing housework in your home?

□ Yes □ No

11. On a scale of 1-10 with 10 being the most stressed, how do you rate the following?

	No stress								Most Stress
Stress level at work (□ I do not work)	1	2	3	4	6	7	8	9	10
Stress level at home									
Overall stress level									

12. During the last 2 weeks, have you felt sad, blue, or depressed for most days of the week?

□ Yes □ No □ Don't know

If yes, for 2 weeks or more in a row…	Yes	No	Don't know
Did you lose interest in things?			
Did you feel tired or low on energy?			
Did you gain or lose weight?			
Did you have trouble falling asleep?			
Did you have trouble concentrating?			
Did you think of death?			
Did you feel worthless?			

13. Please read the following questions and circle the response that most closely describes your current situation

	None of the time	A little of the time	Some of the time	Most of the time	All of the time
Is there someone available to you whom you can count on to listen when you need to talk?	1	2	3	4	5
Is there someone available to give you good advice about a problem?	1	2	3	4	5
Is there someone available to you who shows you love and affection?	1	2	3	4	5
Do you have as much contact as you would like with someone you feel close to, someone in whom you can trust and confide?	1	2	3	4	5

14. Please tick the box that most reflect your feelings to the following statements

I felt tense or 'wound up':

□ Most of the time

□ A lot of the time

□ Time to time, occasionally

□ Not at all

I felt as if I am slowed down:

□ Nearly all of the time

□ Very often

□ Sometimes

□ Not at all

I still enjoyed the things I used to enjoy:
□ Definitely as much
□ Not quite so much
□ Only a little
□ Hardly at all

I got a sort of frightened feeling like ‘butterflies in the stomach’:
□ Not at all
□ Occasionally
□ Quite often
□ Very often

I got a sort of frightened feeling like something awful is about to happen:
□ Very definitely and quite badly
□ Yes, but not too badly
□ A little, but it doesn’t worry me
□ Not at all

I had lost interest in my appearance:
□ Definitely
□ I don’t take as much care as I should
□ I may not take quite as much care
□ I take just as much care as ever

I could laugh and see the funny side of things:
□ As much as I always could
□ Not quite so much now
□ Definitely not so much now
□ Not at all

I felt restless as if I have to be on the move:
□ Very much indeed
□ Quite a lot
□ Not very much
□ Not at all

Worrying thoughts went through my mind:
□ A great deal of the time
□ A lot of the time
□ From time to time but not too often
□ Only occasionally

I looked forward with enjoyment to things:
□ A much as I ever did
□ Rather less than I used to
□ Definitely less than I used to
□ Hardly at all

I felt cheerful:
□ Not at all
□ Not often
□ Sometimes
□ Most of the time

I got sudden feelings of panic:
□ Very often indeed
□ Quite often
□ Not very often
□ Not at all

I could sit at ease and feel relaxed:
□ Definitely
□ Usually
□ Not often
□ Not at all

I could enjoy a good book or radio or TV program:
□ Often
□ Sometimes
□ Not often
□ Very seldom

15. Rate yourself on each item, on a scale (1 - Never or almost never true; 7 - Almost always true)

	1	2	3	4	5	6	7
Defend my own beliefs							
Have leadership abilities							
Affectionate							
Eager to soothe hurt feelings							
Conscientious							
Secretive							
Independent							
Willing to take risks							
Sympathetic							
Warm							
Moody							
Adaptable							
Assertive							
Dominant							
Sensitive to the needs of others							
Tender							
Reliable							
Conceited							
Strong personality							
Willing to take a stand							
Understanding							
Loves children							
Jealous							
Tactful							
Forceful							
Aggressive							
Compassionate							
Gentle							
Truthful							
Conventional							

Think of the ladder representing where people stand in their communities.

People define community in different ways; please define it in whatever way is most meaningful to you. At the top of the ladder are people who have the highest standing in their community. At the bottom are the people who have the lowest standing in their community

16. Where would you place yourself on this ladder?

Please select the rung where you think you stand at the time in your life, relative to other people in your community.

1▫ 2▫ 3▫ 4▫ 8▫ 9▫ 10▫

Now, think of the ladder as representing where people stand in Scotland.

At the top of the ladder are the people who are best off, those who have the most money, the most education and the most respected jobs. At the bottom are the people who are the worst off - who have the least money, least education and the least respected jobs or no job. The higher up you are on the ladder, the closer you are to the people at the very top.

17. Where would you place yourself on this ladder?

Please select the rung where you think you stand at the time in your life, relative to the other people in Scotland.

1▫ 2▫ 3▫ 4▫ 5▫ 6▫ 7▫ 8▫ 9▫ 10▫

18. Are you the primary earner in your house? ☐ Yes ☐ No

19. What range is your personal income?

▫ Less than £12,500

▫ £12,501 to £14,549

▫ £14,550 to £24,994

▫ £24,945 to £43,430

▫ £43,431 to £150,000

▫ More than £150,000

▫ Do not know

▫ Do not wish to answer

20. If you have any feedback, suggestions or improvements relating any of the sections of this questionnaire please leave a comment in the free text box:

End of Questionnaire

Appendix ii. Chapter 4 questionnaire invitation

Dear student,

We are writing to you on behalf of the 'Gender aspects of cardiovascular medicine' research group (gacm@glasgow.ac.uk) relating to a study being undertaken at the University of Glasgow.

If you have already completed this questionnaire, we would like to thank you for doing so. Please do not fill out the study form a second time.

Sex and gender are important concepts in clinical research, yet we often confuse the meanings of sex and gender. The term sex refers to the classification of a person as male or female, typically occurring at birth. Gender refers to a person's intrinsic sense of masculinity or femininity. A questionnaire that allows a standardised measurement of gender has been developed in Canada but has not yet been validated in the UK.

If you are interested in taking part in this study, over the age of 18 and a University of Glasgow student, please follow the link below. The questionnaire will take at most 15 minutes to complete. All data collected is anonymous.

Taking part in this study is not mandatory and you have no obligation to volunteer. Your decision to take part or not to take part in this research will have no implications to your related to study courses or degrees.

https://glasgow-research.onlinesurveys.ac.uk/gacm2020

If you have any questions about taking part in the study please do not hesitate to contact us: **gacm@glasgow.ac.uk**

Thank you for your time,

Appendix iii. Chapter 4 consent form

If you would like to ask any questions about this study before completing this consent form please contact the study team at the following email: GACM@glasgow.ac.uk

I confirm that I have read and understood the Participant Information Sheet version 1.1 dated 07/01/2020.

I confirm that I have read and understood the Privacy Notice version 1.1 dated 07/01/2020. I have had the opportunity to think about the information and ask questions, and understand the answers I have been given.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my legal rights being affected.

I confirm that I agree to the way my data will be collected and processed and that data will be stored for up to 10 years in University archiving facilities in accordance with relevant Data Protection policies and regulations.

I understand that all data and information I provide will be kept confidential and will be seen only by study researchers and regulators whose job it is to check the work of researchers.

I understand that if I withdraw from the study, my data collected up to that point will be retained and used for the remainder of the study.

I agree to take part in the study.

By clicking the 'Submit' button below, you are consenting to participate in this study, as it is described in the participant information sheet.

Appendix iv. Chapter 4 patient information sheet

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.

What is the purpose of the study?

Sex is recognized as an important demographic detail in the conduct of clinical research. Yet the terms sex and gender are often confused or miscategorised. Sex is assigned at birth and is based on biological factors. On the other hand gender is shaped by social context.

Consequently, our gender can lie along a spectrum of masculinity, femininity or both and this might not match with the sex we are given at birth.

A research study in Canada has demonstrated people with feminine genders, which were assessed using a gender questionnaire were more likely to have recurrent

heart attacks, regardless of what sex they were. The purpose of this study is to validate a modified version of this questionnaire in the UK so that it can be used in future cardiovascular research.

Why have I been invited to participate?

You have been invited to take part in this study because you are a student at University of Glasgow and over the age of 18 years.

Do I have to take part?

No, it is up to you to decide whether or not to take part. If you decide to take part, you are still free to withdraw at any time and without giving a reason. Taking part in this study is not mandatory and you have no obligation to volunteer. Your decision to take part or not to take part in this research will have no implications to your related to study courses or degrees.

What will happen to me if I take part?

You will be asked to complete an online questionnaire (approximately 15 minutes). This questionnaire will ask you about your age, ethnicity, sex assigned at birth, gender which you identify as, if you are an undergraduate or postgraduate student and what college you attend. We will also ask you questions related to gender-based characteristics and how masculine and feminine you feel. The data collected from you will be anonymised and stored securely. The data may be used in future research.

What do I have to do?

Fully complete the questionnaire provided.

What are the possible disadvantages and risks of taking part?

We appreciate that this is a time inconvenience, and the questionnaire will take 15 minutes. We are very grateful for your time.

What are the possible benefits of taking part?

You will receive no direct benefit from taking part in this study. The information that is collected during this study will give us a better understanding of cardiovascular risk and gender links.

Will my taking part in this study be kept confidential?

All information which is collected about you, or responses that you provide, during the course of the research will be kept strictly confidential. You will be identified by an ID number. Please note that assurances on confidentiality will be strictly adhered. Any data in paper form will be stored in locked cabinets in rooms with restricted access at the University of Glasgow. All data in electronic format will be stored on secure password–protected computers. No one outside of the research team or appropriate governance staff will be able to find out your name, or any other information which could identify you.

What will happen to my data?

We may be collecting and storing information from you in order to undertake this study. This means that the University is responsible for looking after your information and using it properly. We may keep identifiable information about you until the end of the study and will not pass this information to a third party without your express permission.

Your rights to access, change or move the information we store may be limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights,

we will use the minimum personally identifiable information possible. You can find out more about how we use your information from the research team (GCAM@glasgow.ac.uk).

Researchers from the University of Glasgow collect, store and process all personal information in accordance with the General Data Protection Regulation (2018). All study data will be held in accordance with The General Data Protection Regulation (2018) .The data will be stored in archiving facilities in line with the University of Glasgow retention policy of up to 10 years. After this period, further retention may be agreed or your data will be securely destroyed in accordance with the relevant standard procedures.

Your identifiable information might be shared with people who check that the study is done properly and, if you agree, in coded form with other organisations or universities to carry out research to improve scientific understanding. Your data will form part of the study result that will be published in expert journals, presentations, student dissertations/theses (if applicable) and on the internet for other researchers to use. Your name will not appear in any publication.

What will happen to the results of the research study?

All results will be anonymous. The results may be published in the wider scientific community.

Who has reviewed the study?

The project has been reviewed by the College of Medical, Veterinary & Life Sciences Ethics Committee.

Contact for Further Information:

Dr Paul Connelly, Clinical Research Fellow

Email: GACM@glasgow.ac.uk

Appendix v. Chapter 4 privacy notice

Your Personal Data

The University of Glasgow will be what's known as the 'Data Controller' of your personal data processed in relation to the completion of the gender questionnaire. This privacy notice will explain how The University of Glasgow will process your personal data.

Why we need it

We are collecting your basic personal data such as age, ethnicity, sex assigned at birth, gender which you identify as, if you are an undergraduate or postgraduate student and what college you attend. We will also ask you questions related to gender-based characteristics and how masculine and feminine you feel in order to validate a gender questionnaire that can be used in future clinical research. We will also ask you to consider taking part in the second stage of this study where we will measure your blood pressure, BMI and waist to hip ratio. To do this we will ask you to provide your email address. We will only collect data that we need in order to provide and oversee this service to you.

Legal basis for processing your data

We must have a legal basis for processing all personal data. In this instance, the legal basis is consent of the data subject and processing is necessary for the performance of a task carried out in the public interest.

What we do with it and who we share it with

All the personal data you submit is processed by staff at the University of Glasgow in the United Kingdom.

How long do we keep it for

Your data will be retained by the University for 10 years. After this time, data will be securely deleted.

What are your rights?

You can request access to the information we process about you at any time. If at any point you believe that the information we process relating to you is incorrect, you can request to see this information and may in some instances request to have it restricted, corrected or, erased. You may also have the right to object to the processing of data and the right to data portability.

Where we have relied upon your consent to process your data, you also have the right to withdraw your consent at any time.

If you wish to exercise any of these rights, please contact dp@gla.ac.uk.

*Please note that the ability to exercise these rights will vary and depend on the legal basis on which the processing is being carried out.

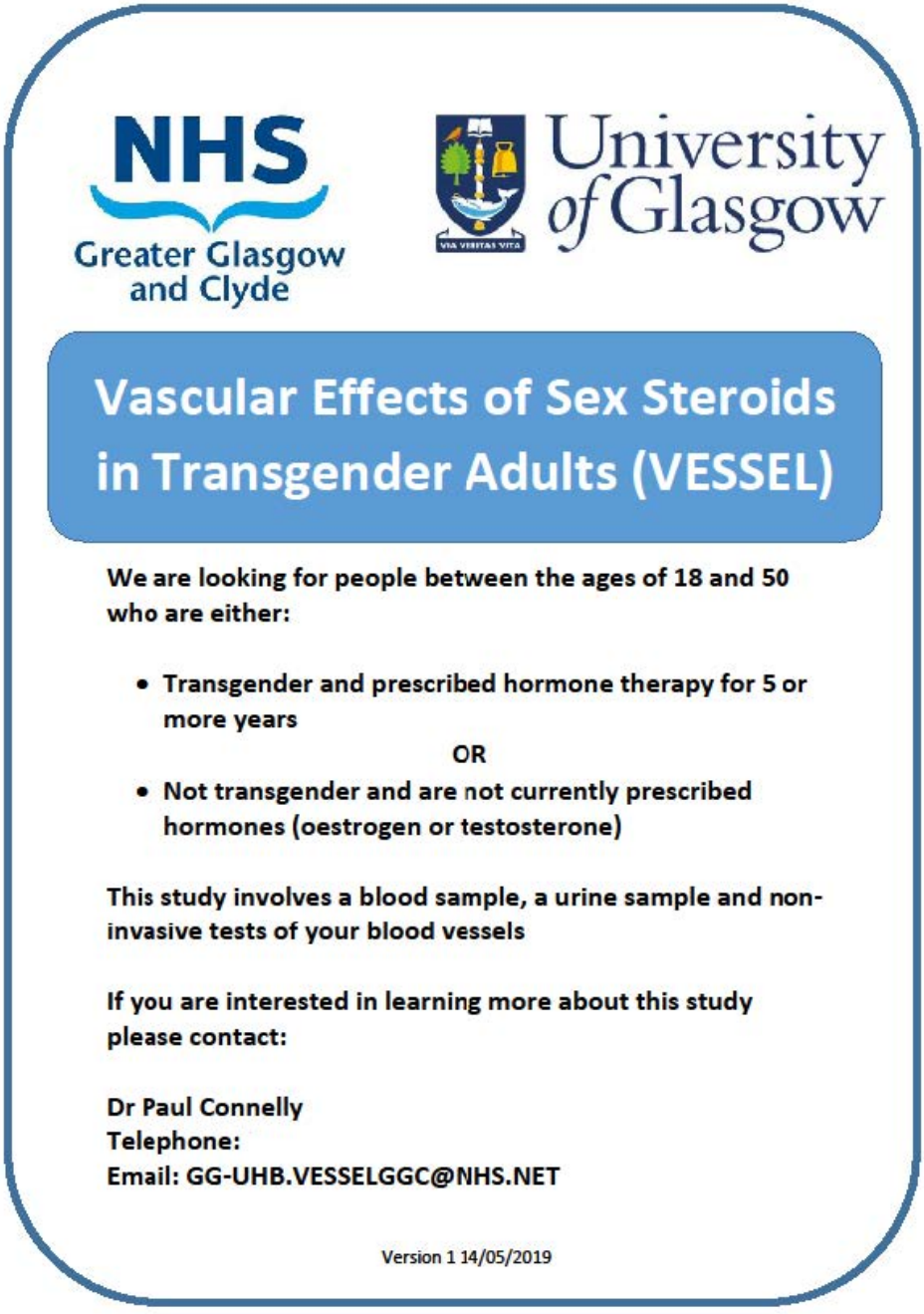
Complaints

If you wish to raise a complaint on how we have handled your personal data, you can contact the University Data Protection Officer who will investigate the matter.

Our Data Protection Officer can be contacted at dataprotectionofficer@glasgow.ac.uk

If you are not satisfied with our response or believe we are not processing your personal data in accordance with the law, you can complain to the Information Commissioner's Office (ICO) https://ico.org.uk/

Appendix vi. Chapter 6 VESSEL poster



Appendix vii. Chapter 6 VESSEL patient information sheet

Dear recipient,

I am writing to invite you to participate in a research study taking place at the University of Glasgow, which aims to investigate the effects of hormone therapy upon cardiovascular health.

Sex hormones (oestrogen & testosterone) have been suggested to play a role in the development of cardiovascular disease by influencing blood vessels. However, the effect of hormone therapy on the risk of developing heart attacks, strokes, high blood pressure or blood clots is not well understood.

We would like to invite you to take part in a study that will help us better understand the effects of hormones upon your blood vessels. This study would involve you attending the Clinical Research Facility, Queen Elizabeth University Hospital, where we would take blood and urine samples and undertake other tests that are non-invasive (e.g. do not cause discomfort or break the skin) that will tell us about the health of your blood vessels. We have provided an information sheet explaining this study further.

If you are interested in finding out more about this study, please read the information sheet and contact one of our researchers (Dr. Paul Connelly) on the phone number or email provided below.

Phone Number: xxxxxxxx

Email address: GG-UHB.VESSELGGC@NHS.NET

If you are unsure whether you would like to take part in this study, we would be happy to discuss it further.

Thank you for taking the time to read this letter.

Yours faithfully,

Professor Christian Delles

Institute of Cardiovascular and Medical Sciences

BHF Glasgow Cardiovascular Research Centre

University of Glasgow

The Vascular Effects of Sex Steroids in Transgender Adults (VESSEL) Study

Introduction

You are being invited to take part in a research study. Before you decide to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Please take the time to decide whether or not you wish to take part.

What is the purpose of this study?

Sex hormones (oestrogen & testosterone) have been suggested to play a role in the development of cardiovascular disease by influencing blood vessels. The purpose of this study is to test if people who are transgender and prescribed hormone therapy are at higher risk of cardiovascular disease. We aim to assess the health of blood vessels in people who are transgender and have been taking hormone therapy for **5 years or more,** and compare that to the vascular health of people who have not received this hormone therapy. This research will contribute to the PhD course of one of the research team, Dr Paul Connelly.

Why have I been invited to participate?

You have been invited to participate because you are currently prescribed hormone therapy (oestrogen or testosterone).

You can only take part in this study if you are:

- Between the ages of 18 and 50 years
- Have been using hormone therapy for 5 or more years

You cannot take part in this study if:

- You have a health condition that may affect tests of your blood vessels (Raynaud's disease, atrial fibrillation or cardiac arrhythmia)
- You are a transgender man currently prescribed the following: Goserelin, Leuprorelin or Triptorelin
- You are using anticoagulation therapy (warfarin, apixaban, rivaroxaban, dabigatran, edoxaban or heparin)

The research team would be more than happy to discuss whether you are suitable to be included in this study if you are not sure. In total we aim to invite one hundred transgender men and women who are prescribed hormone therapy and compare the results of our tests to one hundred men and women who are not prescribed hormone therapy.

Do I have to take part?

No. Taking part in this research is completely voluntary. It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason. This will in no way impact on your clinical care if you decide not to take part or withdraw from this study.

What will happen to me if I take part?

Approximately 200 people will be invited to take part in this study. When we have enough people taking part in this study, we will not include or invite any more.

This study will consist of one visit to the Glasgow Clinical Research Facility, Queen Elizabeth University Hospital. Reasonable travel expenses will be reimbursed. In total this study visit would be expected to last around 3 - 3.5 hours. We will ask you not to have anything to eat and to avoid caffeine (tea, coffee or energy drinks), cigarettes, E-cigarettes and alcohol from the midnight the night before. Our study appointments will be held in the morning. If you are using oestrogen tablets we will ask you to not take that on the morning of the study until the blood samples have been taken. All other medications should be taken as they normally are and you can drink normally except for the drinks mentioned above.

When you arrive we will go over what the study involves with you and ask if you have any questions you would like to ask us about this study. We will then ask you to sign a consent form. You will be given a copy of this to keep.

If you consent to being included in this study we will ask you some questions about any medical conditions you have, any medical conditions that run in your family that you are aware of, your current medications, how often you exercise and if you smoke. If you consent to it, we will access your secondary care (not GP) medical records to ensure that information is accurate.

The following study procedures will then be performed. A diagram of these can be found on page 5 of this Participant Information Sheet.

Height and weight:

Your height and weight will be measured and recorded

Electrocardiogram (ECG):

An ECG is a simple test that can be used to check your heart's rhythm and electrical activity. Sensors attached to the skin are used to detect the electrical signals produced by your heart each time it beats.

Urine Sample:

You will be asked to provide a urine sample.

Blood Sample:

Blood will be taken at your study visit. In total we will take 52.5 ml (10 and a half teaspoons) of blood. Bruising may occur at puncture site and some people may

feel faint. After the blood is taken you will be offered a snack and if you take oestrogen tablets these can now be taken.

Measurements of blood pressure and blood flow

These are non-invasive tests and by that we mean that they do not involve breaking of the skin. Most of these tests require you to lie on an examination bed for up to 30 minutes that can sometimes cause muscle aches. Similarly many of these tests involve inflating a blood pressure cuff on your arm or leg for up to 5 minutes. Some people may experience discomfort from wearing a blood pressure cuff for several minutes at a time. When the blood pressure cuff is inflated, blood flow to the hand and fingers will be temporarily stopped. When the blood pressure cuff is released, the strong flow of blood will stimulate the arteries to dilate and deliver more blood to the hand and fingers. Some people report the temporary sensation of 'pins and needles' within their hands when the blood flow is returning to their hands and fingers, however, this will resolve over a few minutes. We would advise that you wear comfortable clothing that would allow you to roll up your sleeves. The blood pressure cuff over the leg can be done over most clothes. However, stiffer material such as jeans might prevent this measurement and we may ask you to remove this piece of clothing for this measurement. We would provide a blanket and make sure you are covered up during this procedure.

You will be asked to lie down on a bed and we will perform a test that give us a measurement of how stiff the blood vessels are **(pulse wave velocity and analysis)**. A blood pressure cuff will be applied to your thigh. We will then measure the distance between the pulse in your neck (carotid artery), your breast bone and thigh. We will then place a pencil sized probe against the skin in your neck. We will then wrap a blood pressure cuff around your arm that will inflate.

This will assess the pulse of the artery in your arm (brachial artery). This will take up to 30 minutes.

We will then perform a test called **flow mediated dilatation**. This test tells us how well your blood vessels respond to stress. A blood pressure cuff will be placed around the lower part of your arm and will be inflated for 5 minutes. We will use an ultrasound machine to take pictures of the main artery in your arm (brachial artery) before the cuff is inflated and after it has been removed. This will last up to 20 minutes.

Another similar test will then be performed to test **endothelial function**. Once again a blood pressure cuff wrapped around your arm. However, this time fingertip sensors will be placed on your index fingers. The blood pressure cuff will inflate for 5 minutes and then deflate. Blood flow will be measured by the finger sensor to assess how well the arteries dilate. This will last up to 20 minutes. After these tests are finished you will be offered a break.

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On the night before you come for your study visit we would ask that you refrain from eating anything from midnight until your blood samples are taken the next morning. We would also ask that your refrain from using caffeine, cigarettes, Ecigarettes or alcohol. You should continue to take you regular medications with the exception of oestrogen tablets (if you are taking them), which should not be taken until the blood samples have been taken. You can drink normally except for caffeine and alcohol.

What are the possible disadvantages and risks of taking part? There are no major disadvantages in taking part of this study. You will be giving up your valuable time to take part in this study. You may experience minor bruising or bleeding from the blood samples we take or some discomfort from the tests we perform.

What are the possible benefits of taking part?

You will receive no direct benefit from taking part in this study. The information that is collected during this study will give us a better understanding of the role of hormone therapy in the development of cardiovascular disease. This will allow doctors prescribing these treatments to provide more detailed information to people who are commencing hormone therapy in the future.

What will happen to the data I provide?

NHS Greater Glasgow and Clyde is the sponsor for this study based in the United Kingdom. We will be using information from you and your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. NHS Greater Glasgow and Clyde will keep identifiable information about you for 10 years after the study has finished.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information http://www.nhsggc.org.uk/patients-and-visitors/faqs/data-protection-privacy/#.

The University of Glasgow will collect information from you for this research study in accordance with instructions from the sponsor. The University of Glasgow will use your name, NHS number and contact details to contact you about the research study, and make sure the relevant information about this study is recorded for your care, and oversee the quality of the study. Individuals from NHS Greater Glasgow and Clyde and regulatory organisations may look at your medical and research records to check the accuracy of the research study. The only people in NHS Greater Glasgow and Clyde who will have access to information that identifies you will be people who need to contact you to follow-up a concern, audit the data collection process. However, if you consent to it you may be contacted in this future with regards to further research studies. The people who analyse the information will not be able to identify you and will not be able to find out your name, NHS number or contact details.

The University of Glasgow will keep identifiable information about you from this study for 10 years after the study has finished.

The University of Glasgow will collect information about you for this research study from your routine records. The University of Glasgow will not provide any identifying information about you to NHS Greater Glasgow and Clyde. We will use this information to look at your demographic information, medical prescriptions and medical conditions affecting your health.

When you agree to take part in a research study, the information about your health and care may be provided to researchers running other research studies in this organisation and in other organisations. These organisations may be universities, NHS organisations or companies involved in health and care research in this

country or abroad. Your information will only be used by organisations and researchers to conduct research in accordance with the UK Policy Framework for Health and Social Care Research. This information will not identify you and will not be combined with other information in a way that could identify you. The information will only be used for the purpose of health and care research, and cannot be used to contact you or to affect your care. It will not be used to make decisions about future services available to you, such as insurance.

What will happen to the samples I give?

You will donate blood and urine samples for research purposes. Some examinations will be done straight away whilst others will be done at a later stage when we collect more samples from other participants. RNA (ribonucleic acid) will be measured in your blood. Genes are made out of DNA (deoxyribonucleic acid), which will not be measured. RNA is the version of the DNA code that the body uses to direct how proteins are made. We will also store some samples for up to 10 years at the BHF Glasgow Cardiovascular Research Centre accessible to the research team to perform additional tests if required and for use in future research. Further tests on stored samples will again require review and approval by the ethics committee.

What will happen to the results of the research study?

Your data will form part of the study result that will be published in expert journals, presentations, student theses and on the internet for other researchers to use. Your name will not appear in any publication. Most of the results of the research study will not have any impact on your medical care. Any abnormal blood tests would need to be repeated in NHS laboratories. However, if we identify a medical concern (e.g. very high blood pressure) we would ask for your explicit consent before contacting your health care provider.

Who is organising and funding this research?

This research will be completed by research staff at the University of Glasgow. This study is funded by the British Heart Foundation.

Who has reviewed this study?

This project has been reviewed by the West of Scotland Research Ethics Committee.

Contacts for further information:

Professor Christian Delles & Dr Paul Connelly

Institute of Cardiovascular and Medical Sciences

BHF Glasgow Cardiovascular Research Centre

University of Glasgow

Tel: xxxxxxxxx

Email: **GG-UHB.VESSELGGC@NHS.NET**

Appendix viii. Chapter 6 VESSEL consent form

Study ID:

Participant Identification Number:

Title of Project: Vascular Effects of Sex Steroids in Transgender Adults (VESSEL)

Name of Researcher(s): Professor Delles, Dr Gemma Currie, Dr Paul Connelly

CONSENT FORM

I confirm that I have read and understood the Participant Information Sheet (A/B) version 1.1 dated 17/06/2019.

I have had the opportunity to think about the information and ask questions, and understand the answers I have been given.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

I confirm that I agree to the way my data will be collected and processed and that data will be stored for up to 10 years in University archiving facilities in accordance with relevant Data Protection policies and regulations.

I understand that all data and information I provide will be kept confidential and will be shared and seen only by study researchers and regulators whose job it is to check the work of researchers.

I agree that my name, contact details and data described in the information sheet will be kept for the purposes of this research project.

I agree that my medical records can be accessed to gain information relevant to this study

I understand that if I withdraw from the study, my data collected up to that point will be retained and used for the remainder of the study.

I agree that researchers can contact my GP if this study identifies any significant medical findings that may affect my health

I agree to blood and urine samples being stored for a period of 10 years at University facilities for further analysis within this study or use in future research studies

I give consent for the research team of this study to contact me in future with regards to future research projects

I understand that my gender history will be disclosed to the members of the research team and that this information will be held confidentially

I agree to take part in the study.

_____ _____ _____

Name of participant Date Signature

_____ _____ _____

Name of Person taking consent Date Signature

(if different from researcher)

Researcher	Date	Signature

(1 copy for participant; 1 added to clinical notes)