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A framework for developing sex-specific engineered heart models

Roberta Lock[1], Hadel Al Asafen[2,3], Sharon Fleischer[1], Manuel Tamargo[1], Yimu Zhao[1], Milica Radisic [2,3] and Gordana Vunjak-Novakovic [1,4 ✉]

Abstract | The convergence of tissue engineering and patient-specific stem cell biology has enabled the engineering of in vitro tissue models that allow the study of patient-tailored treatment modalities. However, sex-related disparities in health and disease, from systemic hormonal influences to cellular-level differences, are often overlooked in stem cell biology, tissue engineering and preclinical screening. The cardiovascular system, in particular, shows considerable sex-related differences, which need to be considered in cardiac tissue engineering. In this Review, we analyse sex-related properties of the heart muscle in the context of health and disease, and discuss a framework for including sex-based differences in human cardiac tissue engineering. We highlight how sex-based features can be implemented at the cellular and tissue levels, and how sex-specific cardiac models could advance the study of cardiovascular diseases. Finally, we define design criteria for sex-specific cardiac tissue engineering and provide an outlook to future research possibilities beyond the cardiovascular system.

Cardiovascular disease is a leading cause of death around the world[1]. Notably, major differences between male and female patients exist in nearly every aspect of cardiovascular disease, from epidemiology to outcome[2]. Investigating the influence of sex on cardiac (patho)physiology could lead to better mechanistic understanding of diseases, increase efficacy and safety of drug development, and allow the design of targeted treatments for both sexes. Unfortunately, although differences between the sexes have been acknowledged, sex is often overlooked as a variable in scientific research[3–5]. Historically, male patients have dominated clinical and preclinical trials, owing to the belief that sex-based differences are only relevant for the reproductive system and to reduce variability in experiments caused by hormone fluctuations in women. However, sex differences are increasingly considered in scientific studies[6,7].

The observed clinical disparities are a result of the amalgamation of differences owing to sex and gender, which are analytically distinct but inextricably connected. 'Sex' refers to biologically determined attributes (chromosomes, sex organs, endogenous hormone profiles), whereas 'gender' refers to characteristics that are socially constructed[7,8]. Neither sex nor gender are binary, and different sexes exist (for example, Turner syndrome (X), Klinefelter syndrome (XXY) and XYY or XXXY syndromes); however, in this Review, we compare only between male (XY) and female (XX) biological sexes, which are referred to here as men and women,

respectively. Although most differences are attributed to sex-specific hormones, they are only one of several contributing factors (FIG. 1). This is exemplified by gonadectomy studies in animal models, which have shown that female-specific, angiotensin-induced vasodilation is maintained regardless of hormone status[9,10]. In humans, the impact of societal stressors associated with gender are considerable. Recent studies on the association between cardiovascular risk factors and transgender status support the idea that, although important, sex hormone status is not entirely responsible for the differences between men and women. Interestingly, transgender women receiving exogenous oestrogen as part of their treatment experience increased risk of myocardial infarction and ischaemic stroke compared with cisgender women[11,12]. By contrast, there is no convincing evidence that treatment with testosterone in transgender men increases the risk of cardiovascular disease compared with cisgender men, but it leads to a higher rate of myocardial infarction compared with cisgender women. As multiple sex-related factors are involved in regulating cardiac function, it is important to understand their individual and synergistic roles. To this end, tissue engineering and stem cell biology provide a unique opportunity to parse out sex-based and gender-based differences and investigate the mechanisms contributing to sexual dimorphisms in humans. The incorporation of sex-specificity into tissue-engineered constructs will increase our understanding of the role of sex in health and disease.

[1]Department of Biomedical Engineering, Columbia University, New York, NY, USA.

[2]Institute of Biomedical Engineering, University of Toronto, Toronto, Ontario, Canada.

[3]Department of Chemical Engineering and Applied Chemistry, University of Toronto, Toronto, Ontario, Canada.

[4]Department of Medicine, Columbia University, New York, NY, USA.

✉*e-mail: gv2131@columbia.edu*

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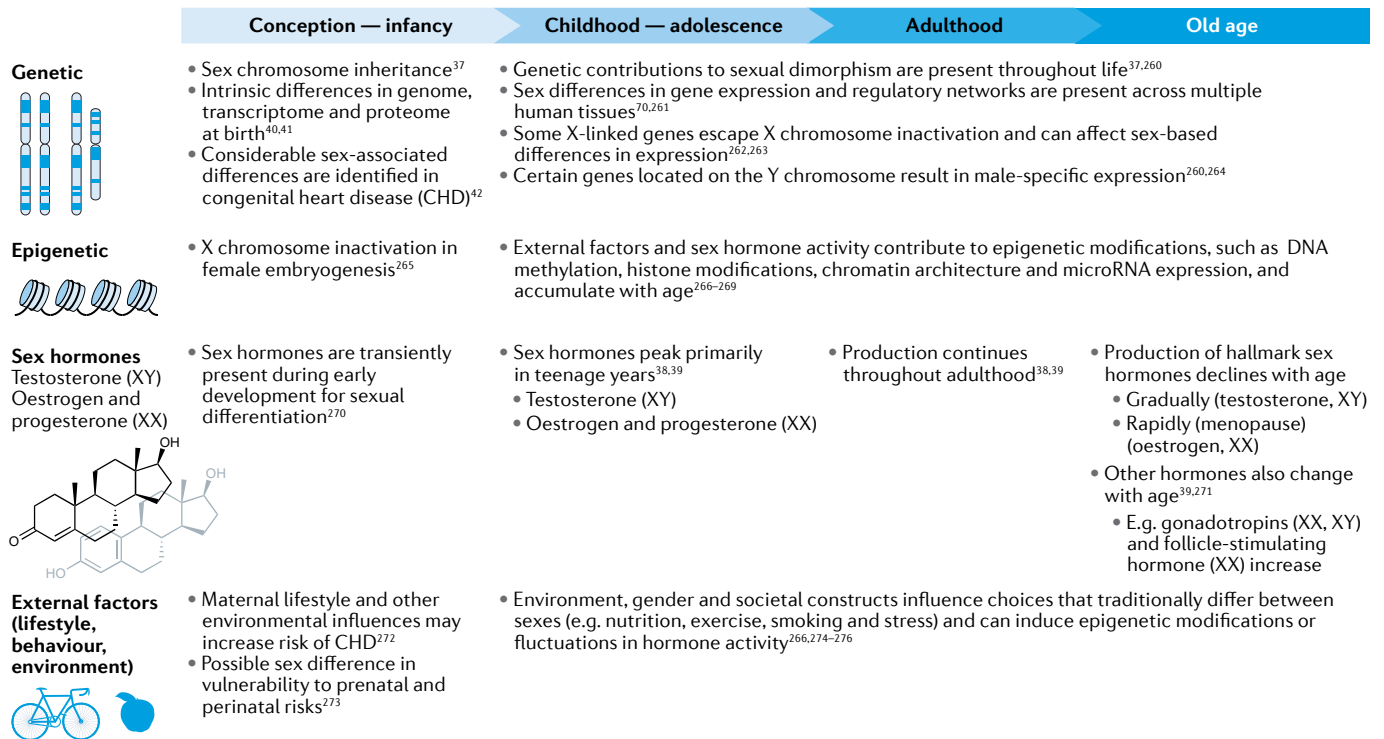|	Conception — infancy	Childhood — adolescence	Adulthood	Old age
Genetic	• Sex chromosome inheritance[37] • Intrinsic differences in genome, transcriptome and proteome at birth[40,41] • Considerable sex-associated differences are identified in congenital heart disease (CHD)[42]	• Genetic contributions to sexual dimorphism are present throughout life[37,260] • Sex differences in gene expression and regulatory networks are present across multiple human tissues[70,261] • Some X-linked genes escape X chromosome inactivation and can affect sex-based differences in expression[262,263] • Certain genes located on the Y chromosome result in male-specific expression[260,264]		
Epigenetic	• X chromosome inactivation in female embryogenesis[265]	• External factors and sex hormone activity contribute to epigenetic modifications, such as DNA methylation, histone modifications, chromatin architecture and microRNA expression, and accumulate with age[266–269]		

Sex hormones
Testosterone (XY)
Oestrogen and progesterone (XX)
	• Sex hormones are transiently present during early development for sexual differentiation[270]	• Sex hormones peak primarily in teenage years[38,39]
		  • Testosterone (XY)
		  • Oestrogen and progesterone (XX)
			• Production continues throughout adulthood[38,39]	• Production of hallmark sex hormones declines with age
				  • Gradually (testosterone, XY)
				  • Rapidly (menopause) (oestrogen, XX)
				• Other hormones also change with age[39,271]
				  • E.g. gonadotropins (XX, XY) and follicle-stimulating hormone (XX) increase

External factors (lifestyle, behaviour, environment)	• Maternal lifestyle and other environmental influences may increase risk of CHD[272] • Possible sex difference in vulnerability to prenatal and perinatal risks[273]	• Environment, gender and societal constructs influence choices that traditionally differ between sexes (e.g. nutrition, exercise, smoking and stress) and can induce epigenetic modifications or fluctuations in hormone activity[266,274–276]		

Fig. 1 | **Factors contributing to sex-based cardiac differences.** Differences between male and female cardiac physiology in humans arise from genetic, epigenetic, sex hormone, environmental, behavioural and lifestyle factors, which interact and change throughout life and are not easy to delineate[260–276].

Engineered cardiac models

In traditional cardiac tissue engineering approaches (FIG. 2), cardiomyocytes are cultured into large and elongated constructs expressing cardiac genes associated with contraction and calcium handling, displaying orderly ultrastructure and producing forces characteristic of a mature phenotype[13–16]. The addition of supporting stromal cells, such as cardiac fibroblasts and endothelial cells, can further increase the structural and functional properties of the tissue[17]. Subjecting engineered cardiac tissue to mechanical stress by stretching tissue anchored to pillars or wires improves cellular alignment and sarcomeric organization, and increases contractile force and expression of cardiac markers, as compared with statically grown tissue[18–20]. Further maturation can be achieved by subjecting the tissue to electrical pacing[19,21–25]. Electrical stimulation increases maturation efficacy if the stimulation starts at a low frequency and is gradually increased over time by applying an 'intensity training' regimen[26,27], resulting in tissues with adult-like gene expression, sarcomeric organization with networks of t-tubules, high mitochondrial density and more physiological contractile behaviour and drug responses[26], as compared with non-stimulated tissue.

Advances in cardiomyocyte differentiation protocols have enabled differentiation into atrial and ventricular cardiomyocytes for the fabrication of chamber-specific tissue, which produces distinct electrophysiological and drug responses between the atrial-specific versus ventricular-specific tissues[27–29]. Moreover, 3D bioprinting allows fabrication of scaled models of the heart ventricles, incorporation of vasculature and

even reproduction of patient-specific anatomical structures[30–33]. Furthermore, organ–organ interactions are being explored; for example, cardiac tissue models can be cultured with other tissues (such as liver, kidneys and lung)[34–36]. Tissue engineering approaches have more physiological relevance than traditional monolayer cultures, and mature engineered cardiac tissues provide versatile platforms for cardiac research (FIG. 3), which can be tailored to study specific biological questions.

Although much progress has been made in cardiac tissue engineering, the complete recapitulation of the physiology of the adult human heart has yet to be achieved. The physiological relevance of cardiac models can be further improved by tailoring their design to represent specific populations, such as men and women. Cardiac tissues are often modified to model specific diseases; however, to the best of our knowledge, a systematic investigation, comparison and modelling of male and female engineered cardiac tissues remains elusive thus far.

Modelling sex-based differences
Cardiac physiology

Here, we briefly review factors contributing to sexual dimorphism in cardiac muscle, identify quantifiable differences that can be recapitulated in cardiac tissue models and discuss engineering tools. Differences in male and female cardiac physiology in humans arise from combined effects of genetics, epigenetics, sex hormones, environmental, behavioural and lifestyle factors (FIG. 1). Genetics contribute to sex differences largely via the sex chromosomes, which are determined
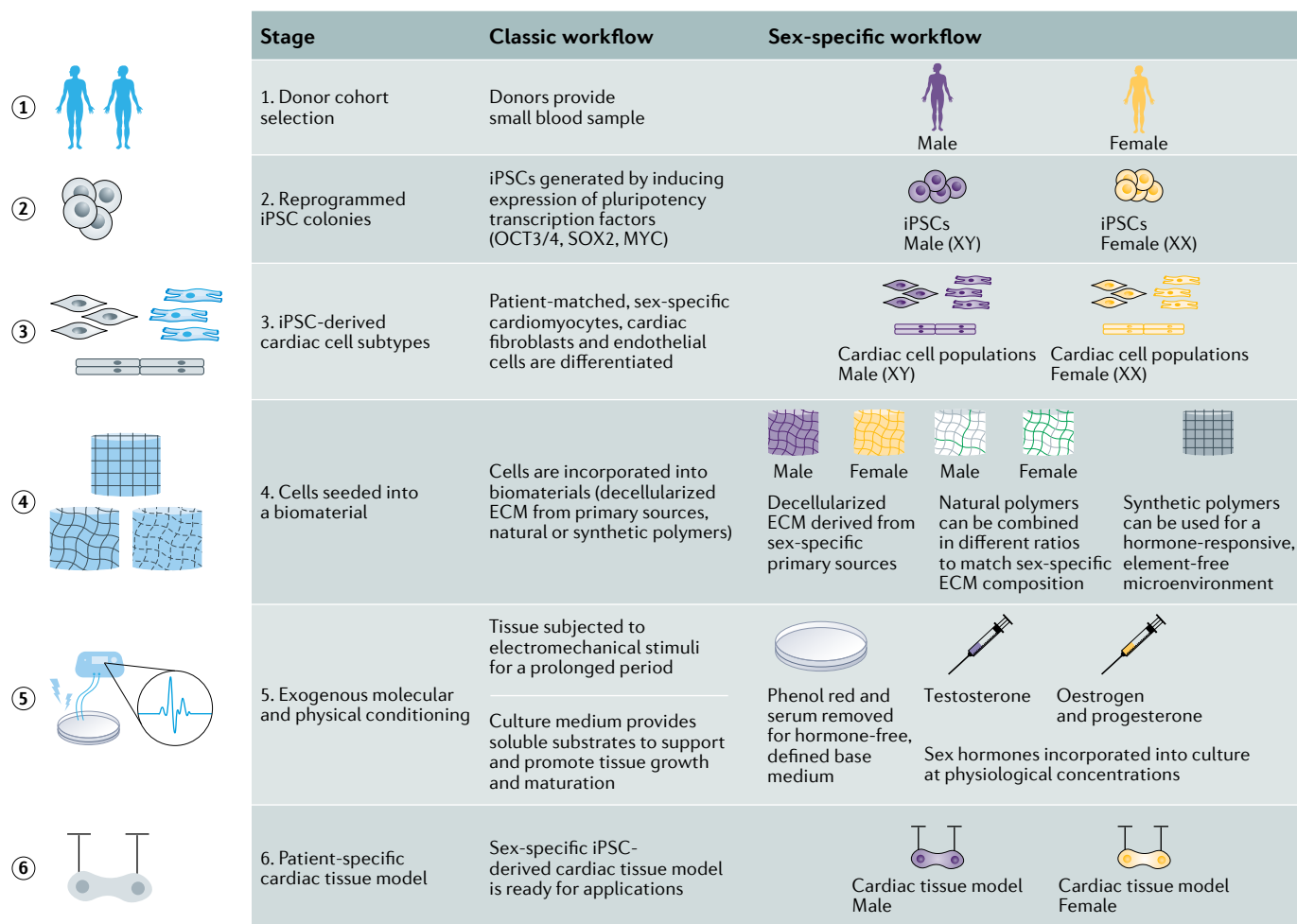
Fig. 2 | **Workflow for creating engineered cardiac models.** Engineered cardiac tissue models are 3D constructs that are grown using cells, biomaterials and exogenous factors to recapitulate patient-specific cardiac phenotypes in vitro. Regardless of the specific tissue design, a common workflow can be outlined and each step can be designed in a sex-specific way. Human induced pluripotent stem cells (iPSCs) are first derived from donors, differentiated into cardiomyocytes and supporting cells, and seeded into a biomaterial. The resulting engineered tissue is cultured in an appropriate culture medium and subjected to electromechanical conditioning. Finally, the cardiac tissue is matured to recapitulate the phenotype of the donor. ECM, extracellular matrix.

at conception[37]. In general, sex-specific differences can be related to the imbalance in gene dosage caused by inefficient X chromosome inactivation in women and the genes located in the male-specific region of the Y chromosome. Epigenetic modifications, such as DNA methylation, histone modification and chromatin remodelling, begin during development and accumulate with age. Production of sex hormones increases and peaks during adolescence[38], continues throughout adulthood and begins to decline around the age of 50 years. In men, testosterone levels decrease gradually, whereas in women, oestrogen and progesterone levels fall rapidly during menopause[39]. Environmental, social and behavioural influences include exercise and diet, which indirectly impact sex differences by modifying sex hormone activity and epigenetics. For the heart, the resulting sex differences are evident at the molecular level in the transcriptome and proteome at birth, and are pronounced during congenital heart disease[40-42]. After the onset of puberty, sex differences become more evident and

change with age throughout life. The vast majority of studies investigating effects of sex on cardiac physiology are conducted within the context of disease. However, to truly understand the differences in abnormal conditions, the differences in normal physiology need to be properly defined (TABLE 1).

Cells. In a healthy heart, the cellular distribution is generally similar in women and men, with a slightly higher percentage of ventricular cardiomyocytes in women ($56\% \pm 9\%$) compared with men ($47\% \pm 11\%$)[43]. In mice, amongst the non-myocytes, more endothelial cells were reported in male than in female cardiac tissue, whereas the proportion of resident stromal cells (fibroblasts, smooth muscle cells, Schwann cells and pericytes) is higher in female tissue[44]. Interestingly, the study also suggests that cardiac cellularity is at least partially regulated by sex hormones, because after gonadectomy, previously distinct cellular profiles of male and female hearts become similar.

In tissue engineering, the consistency of cell sources is vital for the creation of a robust tissue model. Primary animal cells (primary human cells are highly limited in supply) or cells differentiated from human stem cells are most commonly used. Induced pluripotent stem cells (iPSCs) can be derived from a small blood sample and differentiated into the types of cell comprising the heart muscle (ventricular, atrial and sinoatrial node cardiomyocytes, cardiac fibroblasts, endothelial cells, immune cells)[45–48]. Therefore, cell lines can be generated from male and female patients, allowing the creation of sex-specific cells. Sex-based differences have been studied in monolayers of iPSC-derived cardiomyocytes[49–52]; however, the immature state of these cells limits their capability to recapitulate human (patho)physiology and

sex-based differences in tissue function[53,54], which can be addressed by developing mature cardiac tissues, adding stromal cells and optimizing media formulations.

Extracellular matrix. Studies of sex differences in extracellular matrix (ECM) composition in myocardial tissue remain limited. Analysis of healthy human left ventricular tissue revealed sex-specific regulation of several ECM proteins[55]. Tissues from young women contain less collagen type I, III and IV in comparison with men, whereas this is reversed with age. The expression of transforming growth factor-β (TGFβ) and tissue inhibitors of metalloproteinases (TIMPs), both involved in cardiac remodelling processes, also differs. At young age, women have less SMAD2 and SMAD3, which are proteins that
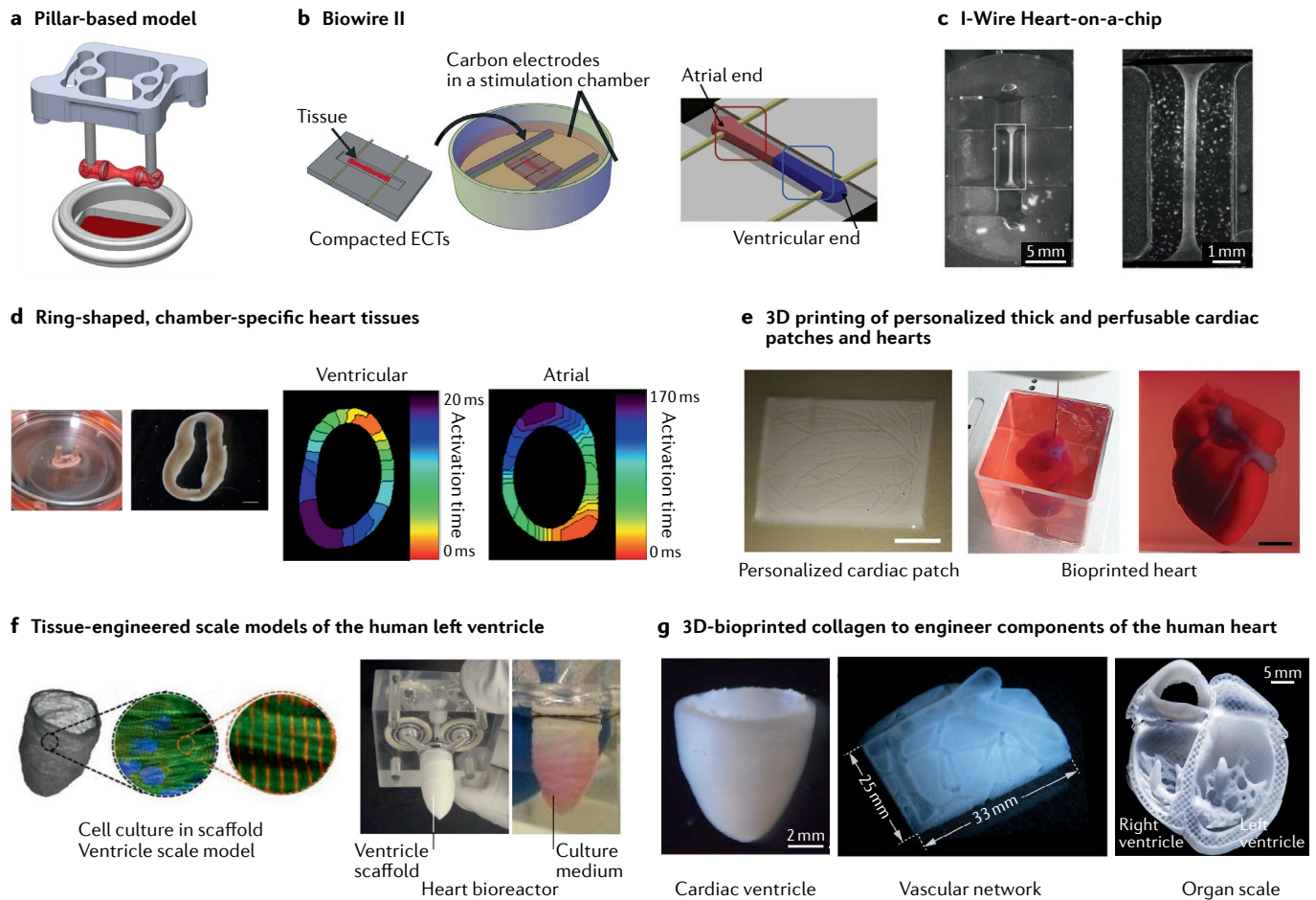

a Pillar-based model

b Biowire II

Tissue

Carbon electrodes in a stimulation chamber

Atrial end

Compacted ECTs

Ventricular end

c I-Wire Heart-on-a-chip

5 mm 1 mm

d Ring-shaped, chamber-specific heart tissues

Ventricular Atrial

20 ms 170 ms

Activation time Activation time

0 ms 0 ms

e 3D printing of personalized thick and perfusable cardiac patches and hearts

Personalized cardiac patch Bioprinted heart

f Tissue-engineered scale models of the human left ventricle

Cell culture in scaffold
Ventricle scale model

Ventricle scaffold Culture medium

Heart bioreactor

g 3D-bioprinted collagen to engineer components of the human heart

5 mm

25 mm 33 mm

Right ventricle Left ventricle

Cardiac ventricle Vascular network Organ scale

2 mm

Fig. 3 | **Engineered cardiac tissue models. a** | Human induced pluripotent stem cell-derived cardiomyocytes and supporting cardiac fibroblasts encapsulated in a fibrin hydrogel can be subjected to tension between two elastic pillars[26]. **b** | The Biowire II platform allows the creation of electrophysiologically distinct atrial and ventricular tissues attached to two polymer wires. **c** | The I-Wire platform combines neonatal ventricular rat cells in a fibrinogen–Matrigel–thrombin hydrogel into a polydimethylsiloxane mould with a channel for the 3D tissue and titanium wires on either side. **d** | Chamber-specific heart tissues can be created from ventricular and atrial human pluripotent stem cell-derived cardiomyocytes embedded in a collagen hydrogel. **e** | Using a hydrogel derived from human omentum and patient-specific cardiomyocytes and endothelial cells, personalized bioinks can be created to bioprint vascularized patches. **f** | Tissue-engineered ventricles are prepared from seeding ellipsoidal ventricle scaffolds with

cardiomyocytes. Their contractile properties are evaluated by pressure–volume catheterization in the heart bioreactor. **g** | Collagen can be 3D-printed using freeform reversible embedding of suspended hydrogels to generate parts of the human heart at different scales. The selection of the most appropriate model depends on the specific question and the functional outcomes being measured (for example, tissues anchored to pillars are especially useful for studies requiring measurements of force generation). Ultimately, the choice should be made at the user's discretion. ECT, engineered cardiac tissue. Panel **b** reprinted with permission from REF.[27], Elsevier. Panel **c** reprinted with permission from REF.[21], Elsevier. Panel **d** reprinted from REF.[28], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/). Panel **e** reprinted from REF.[30], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/). Panel **f** reprinted from REF.[31], Springer Nature Limited. Panel **g** reprinted with permission from REF.[32], AAAS.

Table 1 | **Differences between male and female hearts**

Parameters		Females	Males	Model	Refs
Cells	Proportion of ventricular CMs	56 ± 9%	47 ± 11%	Human heart transmural sample	43
	Proportion of non-myocyte cells	Lower proportion of endothelial cells	Higher proportion of endothelial cells	Mouse	44
		Higher proportion of resident mesenchymal cells	Lower proportion of resident mesenchymal cells		
	Ventricular myocyte loss through apoptosis	Lower expression of apoptotic genes	Higher expression of apoptotic genes	Rat and monkey hearts	108,229
		No ventricular myocyte loss with ageing	Ventricular myocyte loss with ageing		
Extracellular matrix	Collagens	Lower collagen I, collagen III and collagen IV at young age (higher at old age)	Higher collagen I, collagen III and collagen IV at young age (lower at old age)	Healthy human left ventricular myocardial samples	55
	Cytoskeletal proteins	Higher vimentin and vinculin at young age (lower at old age)	Lower vimentin and vinculin at young age (higher at old age)		
	TIMPs	Lower TIMP1 and TIMP3 at young age (higher at old age)	Higher TIMP1 and TIMP3 at young age (lower at old age)		
	TGFβ-associated factors	Lower SMAD2 and SMAD3 at young age (higher at old age)	Higher SMAD2 and SMAD3 at young age (lower at old age)		
		Increased expression of TGFβ receptor 1 with age	No change in expression of TGFβ receptor 1 with age	Mouse	56
	Collagen metabolism-associated factors	Higher periostin at young and old age	Lower periostin at young and old age		
		Lower lysyl oxidase at young age (higher at old age)	Higher lysyl oxidase at young age (lower at old age)		
		Lower MRC2 at old age	Higher MRC2 at old age		
Gene expression	*MYL4* (important for contractile functions by increasing force production)	Lower levels of *MYL4*	Higher levels of *MYL4*	Human ventricular myocardial samples	73
	SCN10A (associated with hypertrophic cardiomyopathy)	Higher expression	Lower expression	Human heart tissue	71
	KCNE1 (associated with long QT syndrome)				
	NPPB (associated with cardiomyopathy)	Higher expression	Lower expression	Human heart tissue	69
Contractility	Contraction frequency	78–82 beats min⁻¹	70–72 beats min⁻¹	Healthy human subjects	230
	Ejection fraction	Higher LVEF (median (25th, 75th percentile)) 75% (70%, 79%)	Lower LVEF (median (25th, 75th percentile)) 70% (65%, 75%)	Healthy human subjects	77
	Fractional cell shortening	Lower	Higher	Isolated rat myocytes	78,79
		No change with ageing	Decreases with ageing		
Electrophysiology	QT interval	470 ms	450 ms	Human patients	86
	APD90	870 ms	670 ms	Human ventricular myocytes isolated from failing human hearts	90
	Potassium repolarizing currents	Smaller	Larger	Isolated rabbit myocytes	82

Table 1 (cont.) | **Differences between male and female hearts**

Parameters		Females	Males	Model	Refs
Calcium handling	Ion channels (Cav1.2a, NCX1)	Higher expression	Lower expression	Cardiomyocytes derived from male and female human iPSCs	49
	Calcium transients	Smaller	Larger	Rat myocytes, mouse myocytes	79,94–96,231
	β-Adrenergic stimulation response	Reduced response (compared with males)	–	Rat myocytes, mouse myocytes	95–97
		Decreased changes to APD	Increased changes to APD		
Energy metabolism	Cardiac mitochondria morphology	Greater number	Fewer number	Mouse	100
		Larger area	Smaller area		
		Elongated morphology	Circular, fragmented morphology		
	Gene expression (genes associated with fatty acid metabolism, oxidative phosphorylation capacity, mitochondrial biogenesis)	Higher expression	Lower expression	Mouse, rat	108,109
	Mitochondrial transition pore opening	Lower calcium sensitivity	Higher calcium sensitivity	Rat	105–107
		Higher myocyte calcium retention capacity	Lower myocyte calcium retention capacity		

APD, action potential duration; APD90, action potential duration at 90% repolarization; Cav1.2a, L-type voltage-dependent calcium channel α1C subunit; CM, cardiomyocyte; iPSC, induced pluripotent stem cell; LVEF, left ventricular ejection fraction; MRC2, mannose receptor C type 2; NCX1, sodium–calcium exchanger; SMAD, fusion of *Caenorhabditis elegans* 'small' worm phenotype (SMA) and *Drosophila* mothers against decapentaplegic (MAD) genes; TGFβ, transforming growth factor-β; TIMPs, tissue inhibitors of metalloproteinases.

function as the main signal transducers for TGFβ receptor signalling, as well as less TIMP1 and TIMP3 than men. In mice, age-dependent increases in TGFβ receptor 1 (TGFβR1) and SMAD2/3 have been reported in females but not in males[56]. Thus, there is evidence for sex-based differences in cardiac ECM composition and remodelling throughout life; however, underlying regulatory mechanisms and impact on disease pathology remain to be explored.

Cardiac tissue engineering requires the incorporation of cells into a biomaterial, which acts as the ECM and provides cells with physical and biochemical cues that affect cellular signalling, organogenesis, tissue homeostasis and disease processes. Initially, cardiomyocytes seeded within biomaterials are round and lack cell–cell interaction. Biomaterials, such as decellularized ECM from primary sources, natural polymers, such as fibrin and collagen, and synthetic polymers, such as polyethylene glycol[57], can guide cardiac cell spreading and elongation, and enable the establishment of cell–cell interactions to form an electrically coupled tissue that generates strong contraction forces. These biomaterials can be fabricated into hydrogels, macroporous and electrospun scaffolds. Biomaterials can also be designed to mimic the cardiac ECM; for example, electrospun fibre scaffolds with patterned biochemical cues and microchannels and nanochannels can control cell directionality and elongation[58,59], enabling the formation of aligned tissue that supports anisotropic electrical propagation. Cardiomyocytes are also highly sensitive to matrix stiffness[60]. The mechanical properties of biomaterials can be adjusted by varying polymer and crosslinker concentrations, and the mechanical anisotropy of the heart can be recapitulated by controlling macroscopic biomaterial geometry[61,62]. As the cardiac ECM also serves as a depot for regulatory molecules, biomaterials can be functionalized with these molecules to support cardiomyocyte survival and maturation, as well as vascularization[63]. Finally, the incorporation of conductive materials, such as gold nanoparticles, graphene and carbon nanotubes, can improve the electrical properties of cardiac tissues[64–66].

Overall, the type of biomaterial needs to be chosen and designed based on the specific biological question. Although sex-specificity of ECM in cardiac engineering has not yet been studied, it has been investigated in other fields, such as breast cancer tissue models, in which, for example, oestrogen receptor activity must be considered.

Gene expression. Genome-wide studies suggest sex-specific genetic architectures of human tissues. RNA sequencing of rodent hearts revealed substantial sexual dimorphism of cardiac tissue, identifying approximately 600 genes with sexually dimorphic expression[67,68]. RNA sequencing and microarray datasets of human tissues revealed slightly fewer sexually differentiated genes (average of 300–400 genes), with the heart being one of the most sexually dimorphic organs in the body, following the reproductive organs, breast tissue and the brain[69–71]. Single-cell RNA sequencing further showed sexual dimorphism in gene expression in all cell populations within the heart, with the most sexually dimorphic genes in cardiac fibroblasts[72]. In particular, pathways involved in glucose and lipid metabolism, as well as contraction, have been implicated in differential gene expression in the heart. For example, expression

of myosin light chain 4 (MYL4), which increases force production in cardiomyocytes, was found to be higher in men than in women, correlating to stronger cellular contraction in men[73]. Several genes linked to cardiomyopathy, most notably sodium voltage-gated channel α-subunit 10 (SCN10A), natriuretic peptide B (NPPB) and potassium voltage-gated channel subfamily E regulatory subunit 1 (KCNE1), are more highly expressed in women than in men[69,71]. Unsurprisingly, the genes with the highest sex-based differential expression in human cardiac tissue are located on the sex chromosomes. However, about two-thirds of sex-biased genes are not directly influenced by sex hormones or under the control of sex hormones or sex chromosomes[71]. Although the mechanisms that underpin these differences are not yet fully understood, the identification of sex-biased genes provides a foundation for investigating sexually dimorphic tissue, as well as a tool and resource for benchmarking sex-specificity of engineered cardiac tissues.

Various tools are available to study differences in gene expression in engineered cardiac tissues. Sexually dimorphic gene expression has mainly been investigated by high-throughput sequencing technologies, such as RNA sequencing, enabling transcriptome-wide analysis of differential gene expression[74]. This technology can also be used with in vitro tissue models, in which gene expression can be further interrogated using gene editing techniques[75]. For example, CRISPR technology can be used to modulate the expression of a specific gene or pathway of interest to evaluate its impact on tissue function. Additionally, reporter lines can be generated to investigate the activity of specific genes in response to different conditions. Many reporter lines have been used in cardiac tissue engineering studies, such as ventricular-specific and atrial-specific gene expression reporters[76]. In sex-specific studies, reporter lines could be used to evaluate changes in cardiac tissue function in response to hormone treatment or activity of particular hormone receptors. Fluorescent reporters can further provide functional readouts; for example, genetically encoded calcium indicators can be used for evaluation of calcium handling, and markers can be generated for apoptosis, reactive oxygen species (ROS) generation and mitochondrial function[76].

Contractility. The main function of cardiomyocytes is coordinated contraction to allow the heart muscle to pump blood. Macroscopically, there are very few differences in contractility between healthy men and women. For example, the slower beat rate of male hearts (70–72 beats per minute) as compared with female hearts (78–82 beats per minute) can be accounted for by the overall size of the heart, which is proportional to body size and typically larger in men. At rest, women have higher left ventricular ejection fraction (LVEF) than men, independent of the left ventricular volume, reflecting a higher stroke volume[77]. In animal models, however, contractions in isolated rat cardiomyocytes are weaker in female cells than in male cells[78]. This difference changes with age, as male rats show decreased fractional cell shortening with ageing, whereas female rats show no such decrease[79]. A trend in both animals and humans is the lower ability of female hearts to respond to stress than male hearts. During exercise, men have a greater increase in ejection fraction than women[77].

In vivo, the contractile function of the heart is evaluated by the ejection fraction, measured by echocardiography. In vitro, measurement of contractility depends on the model design. For isolated cardiomyocytes and cell monolayers, contractile function can be quantified through visual assessment of cellular fractional shortening[80]. Traction force microscopy can also be used to measure the forces a cardiomyocyte transmits to an underlying substrate during contraction. Contraction–relaxation kinetics can be further evaluated by high-speed video microscopy and motion vector analysis[81]. In tissues anchored by pillars (FIG. 3a), the amplitude and frequency of contraction can be measured by image analysis of pillar deflection[26]. For ring-shaped models (FIG. 3d), forces can be measured by progressively stretching tissues placed on hooks that are attached to a force transducer and a length controller[28]. In a 3D ventricle model (FIG. 3f), contractile force can be analysed by catheterizing the ventricle and by taking pressure and volume recordings[31]. The selection of the tissue model for studying sex-specific differences will depend on the physiological property that is investigated. For example, contractile behaviour can be studied using tissues on anchors (FIG. 3a–d), whereas pumping capacity may be studied using the model of ventricles (FIG. 3f).

Electrophysiology. It has long been known that women have longer QT intervals — measurements taken from an electrocardiogram representing the time from ventricular depolarization to the end of ventricular repolarization — and smaller potassium-repolarizing currents[82] than men, leading to less stable human ether-à-go-go-related gene (hERG) potassium channels that contribute to the greater prevalence of drug-induced QT prolongation[83]. These differences emerge after the onset of puberty, when QT interval duration in men begins to shorten[84,85]. Electrocardiogram recordings show that QT values corrected for heart rate (QTc values) post-puberty are less than 450 ms for men and less than 470 ms for women[86]. Indeed, in clinical studies, more cardiac arrhythmias were observed in women than in men in the 15–50-year age group, when the sex difference in QT interval is greatest[87]. In an orchiectomized male rabbit model as well as in isolated guinea pig cardiomyocytes, testosterone shortens the QT interval[88,89], which has also been shown in a clinical study, demonstrating the association between physiological levels of sex hormones and QT interval duration in humans[85]. The same study found that testosterone levels are inversely correlated with the QT interval in men but not in postmenopausal women, suggesting that testosterone levels may explain the differences of QT intervals between men and women[85]. In human ventricular myocytes isolated from the failing hearts of female and male patients at the time of cardiac transplantation, the action potential duration at 90% repolarization (APD90) was 670 ms for male and 870 ms for female cells[90]. Although these APD90 values are much higher than the APD90 values in a healthy adult heart (200–400 ms), these measurements prove

that men exhibit shorter APD90s than women, that APD90 values of female ventricular cardiomyocytes are 29% longer than those of men and, although QTc duration is age-dependent, that APD90 has little or no age-dependence[86,90].

The patch clamp assay is the gold standard for analysing the electrophysiology of cardiomyocytes, allowing the study of ion currents directly from the cell[81]. To perform this assay, tissues must be dissociated into single cells by enzymes, followed by plating at low cell density. Dissociation removes the cells from their native environment and affects membrane proteins, thereby changing cell behaviour. Electrophysiological assessment of intact tissues would provide more accurate functional data. Electrophysiology can be studied in tissues using a calcium indicator or a voltage-sensitive dye, enabling evaluation of real-time changes in calcium flux and voltage by fluorescence microscopy. Hybrid tissues with built-in electronics further allow non-invasive monitoring of electrophysiological tissue properties in real time[91]. Moreover, external electrical stimulation can be used to interrogate tissue function. For example, electrical impulse propagation rates can be determined using point stimulation and optical mapping techniques[92].

Calcium handling. Male and female cardiac tissues show differences in ion channel composition, with higher expression of L-type voltage-dependent calcium channel α1C subunit (Cav1.2a) and sodium–calcium exchanger (NCX1) in women[49,93]. Upon oestrogen conditioning, L-type Ca^{2+} and sodium–calcium exchange currents are upregulated and their respective mRNA levels increase in female but not in male cardiomyocytes, suggesting a role for oestrogen in modulating the expression of these ion channels. In murine models, calcium transients, calcium sparks and excitation–contraction coupling gain differ between males and females[79,94,95]. Although data vary between different rodent studies, females are considered to have lower calcium operational status than males[95,96]. Differences in cyclic adenosine monophosphate (cAMP)–protein kinase A (PKA) signalling, which is responsible for sarcoplasmic reticulum calcium release, is at least partly responsible for these disparities between sexes. Oestrogen regulation of cAMP–PKA signalling is supported by an ovariectomy (OVX) study in mice, showing significant differences in intracellular cAMP and calcium transients in females and oestrogen-treated OVX females compared with males and OVX females[94]. Oestrogen regulation of cAMP–PKA activation may also be responsible for differences in the response to β-adrenergic stimulation. Female hearts have a reduced response to isoprenaline, with smaller changes in action potential duration compared with males in small animal models[95–97]. β-Adrenergic signalling is involved in heart failure and ageing, and, thus, a better understanding of the mechanisms underlying the differential responses between the sexes to stress stimuli is needed.

Analysis and characterization of calcium handling can be monitored in real time using calcium indicator dyes or reporter lines and optical mapping[98]. Optical mapping can visualize the propagation of calcium waves in tissues, allowing the calculation of calcium transient kinetics (amplitude, duration, decay time and conduction velocity). Optical mapping can also be used to identify functional abnormalities, such as spiral waves, which indicate arrhythmia[99]. Agents, such as caffeine, can be applied to induce Ca^{2+} pulses to measure particular parameters, including sarcoplasmic reticulum calcium content and release. Finally, as calcium homeostasis is maintained by coordinated function of numerous proteins, such as ion channels and transporters, mechanisms underlying changes in calcium handling can also be evaluated through interrogation of individual proteins, by introducing calcium channel blockers into the culture media or by editing the cell genome to knock out particular proteins.

Energy metabolism. Cardiac mitochondria in murine female cardiac tissue are greater in number, larger, more elongated and less fragmented than those in male tissue[100]. Regulated by oestrogen, mitochondrial biogenesis is disrupted by OVX, resulting in downregulation of the expression of associated genes, such as peroxisome proliferator-activated receptor-γ co-activator (PGC1α) and nuclear respiratory factor 1 (NRF1)[101]. Loss of oestrogen also causes substantial mitochondrial swelling, which can be counteracted by supplementation of oestrogen[102]. OVX is further accompanied by functional changes in the mitochondria, providing evidence that loss of oestrogen leads to mitochondrial dysfunction. OVX causes a reduction in oxidative capacity, a decrease in ATP synthesis and decline in antioxidants, with a significant increase in stress-induced ROS production in the OVX group compared with the control group[101,103,104].

Compared with females, males have a lower capacity for calcium retention and greater sensitivity to Ca^{2+}-induced mitochondrial permeability transition pore opening, further supporting the cardioprotective role of oestrogen in the heart[105–107]. Gene expression analyses in mice and rats support these findings, revealing considerable sexual dimorphism in cardiac mitochondria at the transcriptional level for genes related to fatty acid metabolism, oxidative phosphorylation and apoptosis[108,109]. Interestingly, these differences in expression are age-dependent. At a young age, genes associated with fatty acid metabolism are upregulated in females compared with males, whereas differences in oxidative phosphorylation mainly appear in old rats, with gene expression indicating a higher oxidative capacity in females than in age-matched males. Although these differences may vary by age and species, sexual dimorphism in cardiac metabolism is evident, owing to the many differences in mitochondrial properties. Oestrogen, in particular, plays an important role, influencing regulation of mitochondrial structure and biogenesis, apoptosis, ATP production, ROS generation and calcium kinetics.

Energy metabolism can be evaluated by longitudinal studies that assess functional changes over time without interrupting cell culture, by monitoring labelled substrates collected from media samples. Metabolite profiles of cell-free supernatants can indicate metabolic shifts between glycolysis and fatty acid metabolism, which is an important determinant of cell maturity[110].

Table 2 | **Sex-based differences in cardiac disease conditions**

Condition	Prevalence	Differences in phenotype (relative to opposite sex)		Differences in outcome (relative to opposite sex)		Refs
		Women	Men	Women	Men	
Ischaemic heart disease	M > F	Increased plaque erosion Increased prevalence of spontaneous coronary artery dissection	Increased plaque rupture	Worse outcome overall Increased incidence of cardiogenic shock Increased incidence of heart failure as a result of ischaemic heart disease Higher mortality within 1 year and 5 years	–	232,233
Heart failure (HF)	M > F	Increased incidence of HFpEF and diastolic heart failure	Increased incidence of HFrEF and systolic heart failure	Worse outcome overall Increased mortality	–	234–237
Hypertrophic cardiomyopathy (HCM)	M > F	Increased interventricular septum thickness Higher prevalence of obstructive phenotype Worse diastolic function at presentation More severe heart failure symptoms	–	Worse outcome overall Increased incidence of HF symptoms with obstructive HCM Increased incidence of symptoms of refractory HF with nonobstructive HCM	–	129,238–240
Myocarditis/dilated cardiomyopathy	M > F	More moderate–severe LV dilation Increased incidence of left bundle branch block	–	Better long-term outcome overall	–	241–246
Takotsubo cardiomyopathy	F > M	Increased prevalence of emotional or no stress trigger	More severe systolic dysfunction Increased prevalence of physical stress trigger	–	Higher incidence of cardiac complications Higher mortality	247–249
Long QT syndrome/ torsades de pointes	F > M	Longer QT interval (M: QTc > 440 ms; F: QTc > 460 ms) Increased propensity towards torsades de pointes	–	Increased risk of adverse cardiac events at older age (>15 years)	Increased risk of adverse cardiac events at young age (<15 years)	176,250,251

F, female; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; LV, left ventricle; M, male; QTc, corrected QT interval.

Supernatant can also be tested for changes in cell function by measuring ROS, troponin I and lactate dehydrogenase, indicating oxidative stress levels, cardiomyocyte injury and cell damage, respectively[111,112]. Imaging can reveal mitochondrial density, morphology and subcellular organization in cell populations[113]. Tissues can also be analysed by high-throughput liquid chromatography mass spectrometry, allowing comprehensive profiling of energy metabolism. Biosensors that non-invasively measure glucose and lactate[114] could also be applied to monitor sex-specific energy metabolism in tissues and assess tissue responses to different factors, such as hormonal changes, in real time and over long culture periods of weeks to months.

Overall, sex-based differences in cardiac physiology are complex, arise from multiple sources and change throughout life, making it challenging to investigate those differences. Although these differences affect nearly every aspect of cardiac function, from

contractility to metabolism, they are largely overlooked under homeostatic conditions, because they do not disrupt normal heart function for either sex. However, sex-based disparities at baseline likely contribute to and exacerbate clinically observed differences in disease pathology and drug response.

Cardiac pathologies

In disease, sex disparities become even more apparent. Owing to the cardioprotective effect of sex hormones and genetic differences, women have considerably less cardiovascular-related diseases than men, which is also reflected in less cardiovascular-disease-related deaths in women[115]. These differences gradually disappear after menopause[116]. The sex disparity is relevant in several prevalent conditions (TABLE 2) and for drug efficacy and safety of cardiac therapies, and, thus, should be incorporated in tissue-engineered models of cardiac disease (TABLE 3).

Table 3 | **Tissue-engineered disease models**

Cardiac pathological conditions		Tissue-engineered disease model	Cells	Biomaterials	Readouts	Refs
Ischaemic cardiac diseases	Ischaemia–reperfusion injury	Cardiac engineered tissue attached around flexible pillars in small volume of ischaemic media to promote metabolic waste combined with hypoxic environment (ischaemia), followed by sudden return to normal conditions (reperfusion)	hiPSC-CMs	Collagen–fibrinogen hydrogel	Cell death, contractile force, conduction velocity, pH change, ROS generation	125
Cardiomyopathy	Hypertrophy	Biowire II platform, in which cardiac tissues are suspended between two POMaC wires and electrically conditioned for weeks	A ratio of 10:1 hiPSC-CMs to human cardiac fibroblasts	Collagen hydrogel	Contractile dynamics, conduction velocity	27
		Cell–matrix mixture seeded on PDMS mould, leading to compaction and self-assembly of cylindrical engineered cardiac tissues anchored by end posts	hiPSC-CMs derived from patients with cardio-facio-cutaneous syndrome due to an activating BRAF gene mutation	Collagen–Matrigel mix	Tissue size, twitch force	252
		Engineered heart tissue cast between two hollow elastic silicone posts, reinforced with metal braces	Rat ventricular cardiomyocytes	Fibrin	Contractile dynamics, collagen I deposition, cardiomyocyte size	254
	Mitochondrial cardiomyopathy of Barth syndrome	Cardiac microtissues seeded on cantilevers to form muscular thin films	iPSC-CMs derived from patients with Barth syndrome	Fibronectin	Mitochondria respiratory capacity reserve, sarcomere morphology, contractile dynamics	253
	Dilated and acquired cardiomyopathies	Human engineered cardiac tissues on single-tissue and multi-tissue bioreactors to model hereditary phospholamban-R14 deletion-dilated cardiomyopathy; and cryo-injury and doxorubicin-induced hECT models of acquired cardiomyopathy	hiPSC-CMs	Collagen–Matrigel mix	Contractile dynamics	255
Cardiac fibrosis		Human cardiac fibrosis-on-a-chip model with two-material microwell chip consisting of a cell culture compartment and two parallel flexible horizontal rods	A ratio of 3:1 hiPSC-CMs to human cardiac fibroblasts	Fibrin gels	Contractile dynamics, collagen deposition, BNP secretion, tissue stiffness	256
		Biowire II model of interstitial and focal cardiac fibrosis	hiPSC-CMs co-cultured with 75% ventricular cardiac fibroblasts	Fibrin-based hydrogel	Contractile dynamics, electrophysiological function	257
Torsades de pointes		3D cardiac tissue sheets on a culture surface grafted with a temperature-responsive polymer	hiPSC-CMs and non-myocytes	Collagen	Contractile dynamics, extracellular field potential	258
Drug-induced cardiomyopathy		Biowire platform that combines 3D cell cultivation around a suture with electrical stimulation to investigate the effect of chronic drug exposure to isoprenaline, angiotensin II and endothelin 1	hESC-CMs and hiPSC-CMs	Collagen matrix	Cell size, contractile dynamics, cardiac troponin secretion	259
		Cardiac tissues stretched between two flexible pillars providing mechanical forces, subjected to electrical stimulation to induce contractions	hiPSC-CMs	Fibrin hydrogel	Beat frequency	26

BNP, brain natriuretic peptide; hECT, human engineered cardiac tissue; hESC, human embryonic stem cell; hESC-CMs, hESC-derived cardiomyocytes; hiPSC, human induced pluripotent stem cell; hiPSC-CMs, hiPSC-derived cardiomyocytes; PDMS, polydimethylsiloxane; POMaC, poly(octamethylene maleate (anhydride) citrate); ROS, reactive oxygen species.

Ischaemic cardiac diseases. Sex-based disparities in ischaemic cardiac disease are evident in humans and animals, with females more inclined to have vascular damage during myocardial infarction[117]. Female rats are more tolerant to oxygen deprivation than males[118], potentially owing to better preserved metabolic capacity and energy production under stress[119]. These protective effects have been attributed to oestrogen. Cardiomyocyte-specific overexpression of oestrogen receptor-β improves survival after induced myocardial infarction in both male and female rats[120]. Activation of oestrogen receptor-α in the heart endothelium by an oestrogen–dendrimer conjugate reduces ischaemia–reperfusion injury in mice[121]. In vitro, functional responses of hypertrophic rat cardiac myocytes to ischaemic stress show increased ischaemic resilience, with attenuated reduction of cell shortening and fewer changes in Ca^{2+} handling in females compared with males[96]. Oestrogen, in the form of 17β-oestradiol, helped restore mitochondrial activity[122], and reduce cardiomyocyte apoptosis[123] and infarct size[124], consistent with clinical outcomes comparing women and men following myocardial infarction[124].

Engineered human cardiac tissues can be used to model acute injury; for example, a model of ischaemia–reperfusion injury has been established by encapsulating human iPSC-derived cardiomyocytes in a collagen hydrogel (FIG. 3a). These engineered cardiac tissues were exposed to anoxic conditions and allowed to accumulate metabolic waste to mimic nutrient deprivation, hyperkalaemia, high lactate concentration and low extracellular pH in ischaemia. To model reperfusion, cardiac tissues were switched back to normoxic conditions and media was replenished to re-establish normal pH and ion balance[125].

Cardiomyopathy. In cardiomyopathy, women develop more severe concentric hypertrophy with smaller ventricular diameter, greater wall thickness and a better systolic function, whereas men demonstrate more pronounced chamber dilation[126]. Genetic profiling revealed distinct molecular processes involved in men and women during maladaptive left ventricular remodelling. Genes and pathways involved in fibrosis were highly upregulated in overloaded male ventricles, whereas genes and pathways related to the ECM and inflammation were upregulated in overloaded female ventricles[127]. In addition to the effects of oestrogen, intrinsic sex differences in myocardial calcium handling and energy metabolism may also contribute to differential remodelling mechanisms[128].

Hypertrophic cardiomyopathy is characterized by decreased diastolic function and increased interstitial fibrosis, caused by genetic mutations of sarcomere proteins, such as cardiac myosin-binding protein C (MYPBC3) and myosin heavy chain 7 (MYH7)[129,130]. Female patients tend to have a higher degree of interstitial fibrosis, a lower passive stiffness of cardiomyocytes owing to higher expression of a more compliant isoform of titin and reduced capillary density[129,130] compared with male patients. In rat models, males with hypertrophic cardiomyopathies are more responsive to β-adrenoceptor stimulation than females[96]. Two

polymorphisms in oestrogen receptors have been associated with increased left ventricular mass and wall thickness in female patients, indicating a role for oestrogen[131].

Myocarditis, or myocardial inflammation, and resulting dilated cardiomyopathy are 50% more common in men than in women[132]. COVID-19-related studies suggest a higher rate of acute myocarditis in men than in women, with cardiac injury markers (troponin, myoglobin, brain natriuretic peptide, creatine kinase–myocardial band fraction test) expressed at higher levels in male patients[133,134]. This disparity may result from sex-specific immune responses to virus infection and subsequent injury[135]. Across species, females commonly develop more sensitive innate and adaptive immune responses than males, facilitating faster progression of inflammation towards tissue remodelling and restoration of homeostasis. Accelerated immune responses in females favour the resolving of inflammation caused by cardiac injury, suggesting protective effects of oestrogen[136,137]. In addition, in a clinical study, genes associated with energy transduction and antioxidant activity were upregulated in female patients, suggesting higher metabolic capacity and energy production under stress[119] than in male patients. These results may inform the design of sex-specific therapeutic strategies.

Takotsubo (stress) cardiomyopathy primarily occurs in postmenopausal women, comprising close to 90% of all reported cases, with symptoms similar to acute coronary syndrome[138,139]. The root cause of the disease is unclear, and its onset is commonly related to emotional and physical triggers. Clinical evidence suggests that high levels of stress hormones (noradrenaline, adrenaline, dopamine) cause negative inotropy and left ventricular contractile dysfunction through β2-adrenoceptors[139]. The cardioprotective effects of oestrogen showed high correlation to the aetiology of the syndrome in rodent models[138,140].

Torsades de pointes. Longer systole duration and longer QT intervals in women lead to a higher risk of torsades de points[141] compared with men. By contrast, men are at a higher risk of short QT syndrome[142]. Human iPSC-derived cardiomyocytes from male and female donors can be used to recapitulate these sex-related differences in vitro. Female cardiomyocytes show significantly steeper slopes of field potential durations (FPDs) to interspike interval ratios and a higher sensitivity to cardiac delayed rectifier (IKr) blocker-induced FPD prolongation. Sex hormones also affect FPD; 17β-oestradiol increases FPD and 5α-dihydrotestosterone shortens FPD. However, the addition of sex hormones has a limited effect on the responses of human iPSC-derived cardiomyocytes to IKr blockade and cannot explain the higher proarrhythmic tendency in women than in men[50]. A ring-shaped engineered heart tissue model (FIG. 3d) can be used to establish an atrial arrhythmia model, controlled by an electrical field potential. Application of the electrical field potential terminates the arrhythmic activity of the atrial engineered heart tissue, converting the arrhythmia into normal activity with synchronous AP propagation[28].

Drug efficacy and toxicity. Owing to the sex-specificity of cardiac pathophysiology, drug efficacy and toxicity have been reported to differ in men and women[143,144]. Women are at a nearly twofold higher risk for adverse drug reaction than men across all drug classes[145]. Drug regimens also differ between the sexes. For example, in the case of hypertrophic cardiomyopathies, men are more commonly prescribed beta blockers (80% for men versus 58% for women), whereas women are more frequently prescribed calcium channel blockers (58% versus 27%) and dual therapy (38% versus 20%)[130]. Owing to differential metabolic enzyme secretion, cardiovascular drugs can have different pharmacokinetics and pharmacodynamics in men and women. Many cardiovascular drugs are metabolized by enzymes of the cytochrome P450 (CYP) monooxygenase system[146]. Sex differences in the expression of CYP1A2, CYP2D6, CYP2E1 and CYP3A4 are particularly relevant in cardiovascular medication; for example, verapamil has a faster clearance rate in women than in men, owing to higher CYP expression[147].

Despite the complex, long and expensive process of drug discovery, many non-cardiovascular medicines had to be withdrawn from the market, because they caused risks to patients, mainly cardiotoxicity. Although sex differences are implemented in the experimental design of clinical trials, sex-specific regulations for in vitro or preclinical studies remain elusive. Stratifying by sex leads to more informative results in clinical trials. Notably, the oestrogen-dependent efficacy of the phosphodiesterase type 5 (PDE5) inhibitor sildenafil was only revealed in clinical trials, and not in preclinical studies[148]. The higher cardiotoxicity of sunitinib in women, caused by reduced expression of drug efflux transporters and suppressed receptor tyrosine kinases, was demonstrated in in vitro cultures and animal models[149]. Engineered cardiac tissues could be used in parallel with animal studies to better validate drug safety before clinical studies. Moreover, refining engineered cardiac tissues by including both sexes would remove biased results, expediting drug development. In addition, gene editing and stem cell technologies enable the generation of customized, genetically modified disease cell lines for studying sex-specific diseases (TABLE 3).

Design criteria for sex-specific models
Cardiac models based on engineered human cardiac tissues would have several advantages for the study of sex-specific differences in cardiac (patho)physiology. First, human-based models are not limited by species-specific effects inherent to animal studies. iPSC technology[150] allows implementation of patient specificity[30,151–153], providing an essentially unlimited source of patient-specific cells, which can be expanded and differentiated into almost any cell type, including cardiac lineages[45,46]. Second, understanding the roles of sex-related factors in clinical and preclinical studies is challenging, owing to confounding variables. Sex-specific tissues enable control of such environmental and behavioural factors to study genetic, epigenetic and hormonal variables. Third, experimental variables can be optimized, for example, the timing and dosage

of hormone conditioning. The cellular microenvironment can be manipulated by modulating the stiffness, degradation and composition of biomaterials to mimic properties of the heart matrix in health or disease. Gene editing can be used to knock out particular genes to create or correct disease models. These advantages allow quantitative, mechanistic studies of sex-specific differences in cardiac physiology in vitro (FIG. 3).

Cells
iPSCs can be derived from male and female patients and differentiated into the different types of cell comprising the heart muscle[45–48]. X chromosome dosage and sex affect iPSC differentiation and cardiac fate outcomes[51], and, therefore, iPSC-based engineered tissues might differ on the molecular, ultrastructural and functional levels, even without the addition of sex hormones. Moreover, as the cellular landscape in cardiac tissues differs between men and women, adequate ratios of cardiomyocytes and supporting cells should be considered to recapitulate in vivo cell composition[43].

Soluble factors
Sex-based differences in cellular responses to soluble factors have been observed in several diseases[154,155]. Notably, sex-specific differences have been reported in valvular interstitial cell (VIC) responses to conditioning with serum derived from male versus female patients both pre and post transcatheter aortic valve replacement (TAVR). VICs pretreated with male pre-TAVR serum showed reduced ability to deactivate when in male post-TAVR serum relative to VICs treated with female serum[156]. Therefore, growth factors, cytokines and exosomes in the cell environment should be included in sex-specific studies[157]. The source of sera is often not reported for cell culture experiments and, thus, chemically defined supplements should be used. Importantly, phenol red, a commonly used pH indicator dye, should be removed from basal media because of its oestrogen-like bioactivity[158].

In addition to controlling the base media and identifying sex-specific soluble factors, the timing of their administration and dosage should be considered. For healthy adult tissues, physiological hormone levels should be mimicked, matching clinical data. In healthy adult men, serum concentrations of testosterone range between 300 and 1,000 ng dl^{-1}, and remain at a constant level throughout adulthood[39,159]. Hormone conditioning is more complicated in women; ideally, the temporal variations in hormone levels throughout the menstrual cycle should be recapitulated, with β-oestradiol and progesterone levels fluctuating between 30 and 800 pg ml^{-1} and 1 and 20 ng ml^{-1}, respectively[160,161].

Biomaterials can be functionalized with bioactive molecules to increase cell attachment and growth[162,163]. Similarly, instead of supplementing media with hormones or other sex-specific soluble factors, biomaterials could be modified to deliver hormones by physical absorption, covalent binding or microparticle delivery[164,165]. For example, sequential release of oestrogen and progesterone from a biomaterial could mimic temporal variations in hormone levels in women.

Alternatively, on-demand release of sex-specific soluble factors from biomaterials could be achieved by external stimuli, such as a magnetic field or light[166,167]. Alternatively, flexible electronics could be applied to tissues for triggered release of sex-specific factors at a desired time and location[168]. Microfluidic devices could also be harnessed to administer hormones to engineered tissues in a controlled manner[169]. For example, female organs have been engineered to study women's health (BOX 1), featuring integrated platforms with tissues capable of physiologically relevant hormone secretion[170–174]. Notably, a microfluidic culture model can reproduce the hormone profile of the human 28-day menstrual cycle[171]. Integrated with engineered cardiac tissues by perfusion, this platform could provide sustained hormone signalling and induce sex-specific effects without the need for external supplementation. Of note, many microfluidic devices are made of poly-dimethylsiloxane (PDMS)[175], which is biocompatible, transparent and easy to use; however, PDMS absorbs hydrophobic molecules and may, thus, change available concentrations of soluble factors. Therefore, more inert elastomers should be used for studies involving sex hormones.

Box 1 | Bioengineered models to study women's health and disease

Bioengineered organs-on-chips have been developed to address the many unmet needs in the area of women's health. For example, a 3D tissue model has been designed, termed EVATAR, which imitates the human female 28-day menstrual cycle hormone profile and allows organ–organ communication through hormonal signals. The system includes engineered decellularized endometrial scaffolds to provide the structure of the native tissue-like environment and promote cellular interactions leading to tissue formation. In addition, female mouse ovaries and human reproductive tissues are used to design ovarian, fallopian, uterine, cervical and hepatic microtissue modules. These modules are then connected by microfluidic channels to provide the required nutrients and to remove the metabolic waste. Addition of follicle-stimulating hormone causes the ovary module to produce oestrogen. On day 14, luteinizing hormone is provided to cause the ovary module to start producing progesterone and stop producing oestrogen. This dynamic microfluidic device provides female steroid and peptide hormones to other tissue modules, which respond as they normally would in the female body[171].

Decellularized endometrial scaffolds can also be used in a 3D endometrial model recapitulating the crucial hallmarks of the menstrual cycle changes of a human endometrium. This model provides a more physiologically relevant setting to study the physiology and pathophysiology of the innermost lining layer of the woman's uterus compared with cell monolayers. The 3D endometrium is fabricated from decellularized endometrial extracellular matrix (ECM), which is then recellularized by endometrial cells and treated with oestrogen and progesterone to induce the secretion of the decidual markers insulin-like growth factor-binding protein 1 (IGFBP1) and prolactin, in response to addition of dibutyryl cyclic adenosine monophosphate (cAMP)[277].

A mucosal barrier tissue model allows investigation of the mucosal barrier function and of diseases, such as HIV and infertility. The model is based on a hybrid ECM hydrogel consisting of poly(ethylene glycol) macromers, adhesion peptides that connect the epithelial and stromal cells, peptides that bind the ECM of each cell type and crosslinkers. Endometrial epithelial and stromal cells can be co-cultured in the mucosal barrier model and the ECM hydrogel is easy to fabricate and can be applied in other applications in mucosal barrier tissue engineering[278].

A multilayer engineered ovarian tissue secreting sex steroids and peptide hormones in response to gonadotropins was designed as hormone replacement therapy. The native follicular structure can be recapitulated by encapsulating theca and granulosa cells, isolated from rat ovaries, in multilayer alginate microcapsules, with granulosa cells in the inner layer and theca cells in the outer layer, separated by a layer of poly-L-ornithine. The 3D arrangement allows nutrient and waste exchange, crosstalk between cell types and sustained release of key sex hormones for 30 days[173].

External stimuli

Electrical stimulation is well established in cardiac tissue engineering. Extended regimens of electrical pacing are used to mature cardiac tissues towards a more adult-like phenotype[26,27]. Electrical stimulation can also help elucidate sex-specific cardiac phenotypes. In rats, sex differences in contractility are more pronounced at rapid pacing rates[78]. However, a study in rabbits showed that in the context of long QT syndrome, pacing at rapid rates eliminates the sex differences seen in the electrocardiogram[176]. For example, exercise-induced cardiac hypertrophy is more predominant in female rodents than in males, although this hypertrophic response is different from that observed in pathological hypertrophy[177]. Whether electrical pacing differentially affects male and female tissues remains to be explored; of note, the frequency of electrical stimulation of cardiac tissue may be important, because it could either reveal or obscure sex-based differences.

Biomaterials

In the heart, cells are surrounded by the cardiac ECM, which is a hierarchically organized network of fibres. The ECM plays a crucial role in controlling cell migration, proliferation, differentiation and assembly into cardiac tissues[13,59,178,179]. A myriad of physical and biochemical signals are transmitted by the cardiac ECM and sensed by receptors on cardiac cells, in which they activate signalling pathways to regulate cellular behaviours[179,180]. The molecular, structural and mechanical properties of the cardiac ECM play important roles in promoting cell assembly into an aligned syncytium, enabling anisotropic electrical signal propagation and orchestrating cardiac tissue contractions[181]. The cardiac ECM can be harnessed or mimicked to generate functional cardiac tissues[59,62,182–184]; for example, cardiac cells are able to repopulate a decellularized whole heart and restore some pump function, indicating the capability of the native ECM to guide tissue assembly[185]. Biochemical cues on the native ECM further provide cells with a protective microenvironment, support their growth and function, and promote heart regeneration following myocardial infarction[178,186,187]. Moreover, recapitulating the mechanically anisotropic, honeycomb-like structure of the endomysial fibres or the spring-like perimysial fibres improves cardiac tissue functionality[188,189].

Material-based scaffolds can be customized to design biomaterials recapitulating the male and female cardiac microenvironment[190–192]. For cardiac tissue engineering, biomaterials need to be mechanically stable while allowing tissue contractions, promoting cell spreading and elongation, and supporting the formation of a tissue with electrical signal propagation. Synthetic materials, natural materials and decellularized hearts have been explored for cardiac tissue engineering, each with advantages and disadvantages, depending on the biological question and goal of the sex-specific study.

Synthetic polymers, such as poly(DL-lactic-co-glycolic acid), poly(glycerol sebacate), polycaprolactone and poly(ethylene glycol), can be fabricated into hydrogels or electrospun into fibrous scaffolds for cardiac tissue engineering[193]. Synthetic materials are readily available

and they can be easily manipulated to tune their mechanical properties. In addition, many synthetic polymers are biodegradable and biocompatible, and their fabrication is highly reproducible. However, they lack biochemical cues for cell attachment and functional maturation. Importantly, the low remodelling rates of synthetic materials would limit tissue remodelling studies of male and female tissues, an aspect of particular interest.

Natural polymers, such as collagen, gelatin, fibrin and Matrigel, are used to fabricate hydrogels, macroporous and fibrous scaffolds, and as biological inks to print tissues. Natural polymers are also readily available, support cell adhesion and spreading, and have high remodelling rates. Furthermore, the properties of natural materials could be tailored to mimic sex-specific features, such as stiffness as well as protein and glycoaminoglycan content. For example, collagens of the cardiac ECM vary by age and sex, with more collagen type I, III and IV expressed in male cardiac tissues than in female tissues at a young age[55]. Male and female tissues could be fabricated using different combinations of collagens to match the native ECM properties. Of note, the source and properties, especially the sex of the original biological source of commercially available collagens should be reported, because they play an important role in sex-specific research.

It is impossible to fully recapitulate the complexity of the cardiac ECM. Therefore, decellularized heart ECM is often used as scaffold to preserve the native biochemical and structural properties of the heart. The importance of organ-specific ECM has been demonstrated for heart, lung and liver tissue engineering[194–196], with differences in male and female ECM architecture, mechanical properties and composition, including different remodelling and regenerative capabilities upon injury or disease[55,56,127,197,198]. Decellularized heart ECM has great potential to study sex-specific microenvironments and their effects on cells; for example, to investigate whether sex-specific ECM has differential effects on male and female cells. Cardiac ECM for tissue engineering is mostly sourced from rodents and swine, where sex is often not disclosed, and species-specific differences may still be present[199,200]. Harvesting human cardiac tissues, for example, by endomyocardial biopsy or using tissue from hearts not suitable for transplantation that are otherwise healthy, would allow the generation of male and female ECM that preserves the distinct human ECM architecture and mechanics. Alternatively, ECM could be digested to form hydrogels, in which only the biochemical composition is preserved.

The specificity of the ECM is especially important for disease modelling, because many diseases show sex-based differences in cardiac remodelling and fibrosis[130,201,202]. For example, in hypertrophic cardiomyopathy, women show more ECM remodelling than men[130]. Sex-specific tissue models would, thus, markedly increase the mimicry of specific disease phenotypes, enabling the study and control of sex-related differences.

Study design
Designing sex-specific studies for cardiac disease investigations is challenging. Sample size is important and a large number of cell lines may be required, similar to clinical trials. In vitro studies of sex-based differences have to consider the variability between cell lines, necessitating a power analysis to ensure statistical significance. For example, high variability between baseline phenotypes of iPSC-derived cardiomyocytes has been reported[203]. The timescale also has to be considered, because time is a limiting factor in vitro. In vivo, sex differences may slowly accumulate over decades of hormone conditioning, whereas in vitro studies are typically conducted on the scale of weeks or sometimes months. Therefore, it needs to be explored how long engineered tissues must be cultured to achieve sex-specific phenotypes.

Sex-specificity can be implemented into studies of cardiac (patho)physiology with engineered cardiac tissues by modulation of cells, ECM, soluble factors and external stimuli. However, the numbers and combinations of sex-specific components required to adequately capture sex-dependent physiology and clinical outcomes remain to be explored. These studies will rely on knowledge acquired in functional and mechanistic studies, as well as on rigorous benchmarking to data gathered in clinical studies. Although in vitro tissue models are unable to capture complex organismal behaviours, they afford a high level of control, with the potential to provide new insights into sexual dimorphisms observed in the clinic.

Applications and outlook
Incorporating sex-specificity into engineered cardiac tissue models and investigating the influence of sex on disparities in cardiac (patho)physiology would serve many purposes. A thorough mechanistic understanding of cardiac diseases would allow the development of targeted treatments for both sexes. In addition, sex-specific platforms could be used for drug screening to identify sex differences in cardiac responses to drugs prior to clinical trials. Sex-specific engineered cardiac tissues could also potentially enable studies of the effects of exogenous supplementation of sex hormones on tissues fabricated from cells derived from the opposite sex. Disparities in cardiac-associated risks occurring in transgender populations may then be explained to make gender-affirming hormone therapy safer. Furthermore, effects of pregnancy (that is, changes in hormone status) and ageing on the heart could be investigated. In particular, the temporal control of in vitro platforms enables the study of the effects of timing and duration of hormone replacement therapy on cardiac function[204].

Translational impact of sex-specific research
Sex-specific research could also have an impact on translational research. In addition to tissue models, cardiac tissue engineering aims at regenerating or replacing injured or diseased heart tissue[205–207]. Interestingly, sex-specificity may play an important role in tissue engraftment and replacement. Reduced survival after heart transplantation has been associated with donor–recipient sex mismatch, especially for male recipients of female donor organs, with sex mismatch increasing mortality independently of recipient–donor heart weight match[208–212]. The impact of sex mismatching may also

apply to engineered tissue patches or grafts fabricated for implantation. Whether matching the sex of the tissue-engineered product to the recipient is required or whether further conditioning for sex-specificity would be beneficial remains to be explored.

Increasing model complexity for sex-specific applications

Cardiac tissue engineering has greatly advanced, from engineering myocardial tissue to engineering organ-level structures. Integration of sex-specificity into more complex models of the heart would increase the ability to recapitulate clinical phenotypes seen at the organ level. For example, magnetic resonance imaging and micro-computed tomography imaging of male and female patients could inform tissue models and facilitate reproduction of sex-specific differences in the cardiac anatomical structure.

Vasculature. Cardiac tissue models with biologically relevant and functionally matured vasculature remain elusive thus far. Vascular tone, regulated by the renin–angiotensin–aldosterone system (RAAS) and nitric oxide synthases, can be considerably influenced by sex hormones[213] and abundances of X-chromosome-located proteins[214–216]. Differences in vascular tone may affect local oxygen and nutrient delivery, vascular barrier function and stromal cell behaviours, resulting in sex-specific mechanisms of cardiovascular diseases. For example, coronary heart diseases in women are linked to microvascular spasms and vasoconstriction, whereas in men, they are linked to coronary occlusion and deposition of plaque[217]. Haemodynamic overload, such as hypertension, induces cardiac remodelling and triggers sex-specific adaptation, with women developing hypertrophic remodelling with systolic function and ejection fraction better preserved compared with men[218]. Therefore, incorporation of vascular networks into cardiac tissue models will increase the mimicry of the sex-specific tissue microenvironment. Vasculogenic factors of interest include hydrostatic pressure, vascular smooth muscle tone, solute transport and biological interactions between endothelial, stromal and immune cells. Control over these parameters would enable investigation of sex-specific mechanisms in cardiovascular diseases, hypertension, atherosclerosis, stroke and aneurysms[213].

Multi-organ systems. Beyond the heart, sex-specific differences have been reported for other organs. For example, tissue-specific, sex-biased gene expression and regulation have been reported across 44 different human tissues, with at least 37% of genes differentially expressed between sexes in at least one of the tissues[219]. Organ-on-a-chip technologies can model the functionality of organs, including liver, lung, kidney, vasculature, gut and brain, and iPSCs can be incorporated in these platforms to engineer patient-specific tissues to study disease mechanisms and for precision medicine[220–225]. However, sex differences are generally overlooked in these models. The workflow for sex-specific cardiac tissue engineering could also be applied to in vitro models of other organs. Such sex-specific tissue models would provide excellent tools to study sexual dimorphisms in organ pathophysiology, which is crucial for precision medicine and drug development.

Importantly, sex-specific organ models could be integrated into larger systems. Although mechanistic insights into sex-based dimorphism in individual organs will provide the basis for precision medicine and drug development, organs in the human body do not function independently, and an affected organ system can influence multiple other tissues. For example, the RAAS is a hormone system essential for the regulation of blood pressure and fluid balance in the body. RAAS dysfunction is closely related to hypertension, chronic cardiovascular and kidney diseases[226]. The RAAS involves multiple proteins that cycle through organs connected by a common vascular network, including the liver, white adipose tissue (angiotensinogen), kidney (renin), lung (angiotensin-converting enzyme (ACE)) and adrenal gland (aldosterone).

Sex dimorphisms manifest themselves in different plasma levels of these proteins and different receptor densities in multiple organs and in the vascular endothelium. Angiotensin II, converted from angiotensinogen by renin and ACE, can induce pathological hypertrophy of cardiomyocytes, acting as a vessel constrictor and inducing the secretion of aldosterone, causing water retention in the kidney distal tubules[216]. Oestrogen reduces plasma levels of angiotensin II by increasing the production of the N-terminal angiotensin II fragment (Ang-(1-7)), which leads to a decrease in the activity of ACE[214] and a change in extracellular volume, vascular filtration and blood pressure[217]. Consequently, risks of hypertension are lower in premenopausal women than in men, but are similar between postmenopausal women and men[227]. Therefore, modelling RAAS in vitro would require high-fidelity, multi-hierarchical vascular networks that link liver, kidney, lung, adrenal gland, heart and reproductive organs in a biologically relevant manner, in terms of sizes and order. Moreover, in sex-specific tissue models of RAAS, adequate tissue function needs to be established, such as renin secretion and salt regulation of kidney tissues, protein production and drug metabolism in liver tissues, ACE production and O_2/CO_2 exchanges in lung tissues, contractile function of the heart, as well as hormone production from female or male reproductive systems. These tissue-specific functions need to be benchmarked using sex-specific clinical results and drug responses. For example, blockage of the angiotensin II receptor may be considered a good choice in female patients, because ACE inhibitors used for blood pressure management in male patients are less likely to have similar therapeutic benefits in female patients, owing to lower ACE activity[228]. Thus, in vitro multi-organ models would be beneficial for developing sex-specific, personalized therapeutic strategies for RAAS diseases. Similarly, different organs could be combined for customized multi-organ systems for various biological questions. Multi-organ-systems-on-a-chip would be particularly important for modelling drug absorption, distribution, metabolism and excretion, and for studying diseases and drugs with systemic effects, such as chemotherapy.

REVIEWS

In summary, sex can influence nearly every aspect of the heart, from gene expression to cellular composition and function. By focusing on differences at the cell and tissue levels, we proposed a framework for recapitulating sex-specific traits in cardiac tissues. Sex as a biological variable has been largely overlooked in tissue engineering thus far, despite numerous differences observed in the clinic. Incorporating sex-specificity will allow the development of male and female cardiac tissue models, towards the goal of personalized medicine, based on innovations in human stem cell technology and tissue engineering, without ethical constraints.

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Author contributions
R.L., H.A.A., S.F., M.T., Y.Z., M.R. and G.V.-N. conceptualized the manuscript. R.L., H.A.A., S.F., M.T. and Y.Z. surveyed relevant literature and wrote the manuscript. M.R. and G.V.-N. edited and finalized the manuscript.

Competing interests
G.V.-N. and M.R. are co-founders and shareholders of TARA Biosystems Inc. They serve on the Board of Directors and receive compensation for this role. Y.Z. and M.R. are inventors of a patent application on the Biowire II platform related to this work (US patent US20160282338A1), which has been licensed to TARA Biosystems Inc. G.V.-N. is inventor on a patent application on engineering adult-like human heart tissue (application no. 15/151,751). All other authors declare no competing interests.

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