From novel discovery tools and biomarkers to precision medicine-basic cardiovascular science highlights of 2021/22

Basic Cardiovascular Science Highlights of 2021/2022 – from novel discovery tools and biomarkers to precision medicine.

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Here we review the highlights of cardiovascular basic science in published in 2021 and early 2022 on behalf of the European Society of Cardiology Council for Basic Cardiovascular Science. We begin with non-coding RNAs which have emerged as central regulators cardiovascular biology, and then discuss how technological developments in single-cell 'omics are providing new insights in cardiovascular development, inflammation and disease. We also review recent discoveries on the biology of extracellular vesicles in driving either protective or pathogenic responses. The Nobel Prize in Physiology or Medicine 2021 recognised the importance of the molecular basis of mechanosensing and here we review breakthroughs in cardiovascular sensing of mechanical force. We also summarise discoveries in the field of atherosclerosis including the role of clonal haematopoiesis of indeterminate potential, and new mechanisms of cross-talk between hyperglycemia, lipid mediators and inflammation. The past 12 months also witnessed major advances in the field of cardiac arrhythmia including new mechanisms of fibrillation. We also focus on inducible pluripotent stem cell (iPSC) technology which has demonstrated disease causality for several genetic polymorphisms in long QT syndrome and aortic valve disease, paving the way for personalized medicine approaches. Finally, the cardiovascular community has continued to better understand COVID-19 with significant advancement in our knowledge of cardiovascular tropism, molecular markers, the mechanism of vaccine-induced thrombotic complications and new anti-viral therapies that protect the cardiovascular system.

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ABSTRACT

Here we review the highlights of cardiovascular basic science in published in 2021 and early 2022 on behalf of the European Society of Cardiology Council for Basic Cardiovascular Science. We begin with non-coding RNAs which have emerged as central regulators cardiovascular biology, and then discuss how technological developments in single-cell 'omics are providing new insights in cardiovascular development, inflammation and disease. We also review recent discoveries on the biology of extracellular vesicles in driving either protective or pathogenic responses. The Nobel Prize in Physiology or Medicine 2021 recognised the importance of the molecular basis of mechanosensing and here we review breakthroughs in cardiovascular sensing of mechanical force. We also summarise discoveries in the field of atherosclerosis including the role of clonal haematopoiesis of indeterminate potential, and new mechanisms of cross-talk between hyperglycemia, lipid mediators and inflammation. The past 12 months also witnessed major advances in the field of cardiac arrhythmia including new mechanisms of fibrillation. We also focus on inducible pluripotent stem cell (iPSC) technology which has demonstrated disease causality for several genetic polymorphisms in long QT syndrome and aortic valve disease, paving the way for personalized medicine approaches. Finally, the cardiovascular community has continued to better understand COVID-19 with significant advancement in our knowledge of cardiovascular tropism, molecular markers, the mechanism of vaccine-induced thrombotic complications and new anti-viral therapies that protect the cardiovascular system.

1. INTRODUCTION

The aim of this review from the European Society of Cardiology (ESC) Council for Basic Cardiovascular Science is to highlight the most noteworthy developments over the past year, in the field of cardiovascular basic science. The cited reports were selected as representative examples of studies which provided robust evidence for particularly novel insights. *Cardiovascular Research* previously reviewed the highlights of 2020 divided into vascular and cardiac topics,[1 2] but here we integrate both areas to generate the Basic Cardiovascular Science Highlights of 2021/2022.

2. CARDIOVASCULAR RNA UNIVERSE

2.1 Non-coding RNAs (nc RNAs)

In addition to the role of messenger RNA (mRNAs) in the 'central dogma' of molecular biology as a template for protein synthesis, the RNA universe also contains multiple constellations of microRNAs (miRNAs; miRs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) that control fundamental processes of life. These RNA species adopt complex structures and interact with nucleotides, proteins and lipids to control multiple functions including chromatin structure, transcription, RNA splicing and stability, intracellular signalling and organelle dynamics. Research reported in 2021 has provided further insight into the role of miRNAs, lncRNAs, and circRNAs in the regulation of vascular remodeling and cardiac disease. Using both single-cell (sc) and bulk RNA-sequencing to investigate transcriptional changes associated with endothelial-to-mesenchymal transition (EndMT), *Monteiro et al* identified for the first time the genomic locus hosting the lncRNA MIR503HG as necessary to maintain endothelial cell (EC) identity and function[3]. In a series of our loss- and gain-of-function experiments the group demonstrated that loss of lncRNA is a causal event in EndMT observed in pulmonary arterial hypertension (PAH) in association with vascular remodelling (Figure 1). Further, located upstream from the vascular smooth muscle cell (vSMC)-associated miR-143 and -145 cluster, the lncRNA CARMN (Cardiac Mesoderm Enhancer-associated Noncoding RNA) was recently identified as key regulator of vSMC function and the pathophysiology of atherosclerotic disease[4]. Crucially, while crosstalk between lncRNA host genes and coupled miRNAs is often seen, CARMN was found to function independently from miR-143/-145 in regulating vSMC and activating a pro-atherogenic proliferative state (Figure 1).

Gong et al identified in atherosclerotic mouse models a novel circRNA, circEsyt2, involved in vascular remodeling through the targeted inhibition of alternative mRNA splicing. By performing loss- and gain-of-function mutation analyses in vascular smooth muscle cells, circEsyt2 was shown to enhance cell proliferation and migration and blunt apoptosis and differentiation. Furthermore, silencing of circEsyt2

prevented neointima formation while circEsyt2 overexpression enhanced neointimal hyperplasia in an *in vivo* model of carotid artery injury.[5] The role of miRNAs in atherosclerosis progression was examined by Liu *et al* by describing the role of the Nuclear Factor of Activated T-cell isoform c3 (NFATc3)/miR-204 axis in the regulation of foam cell formation in atherosclerosis. Using genetically modified mice, they showed that NFATc3 prevents macrophage foam cell formation and limits the expression of scavenger receptors SR-A and CD36 by inducing expression of the microRNA miR-204,[6] suggesting the NFATc3/miR-204 axis as a potential therapeutic target to reduce plaque formation. In a separate study involving macrophages, *Schober et al* illuminated the circadian patterns of myocardial infarction (MI) by evaluating macrophage-related miRNAs. They evidence, in a murine model of atherosclerosis, that macrophage miR-21 drives circadian regulation of macrophage apoptosis by targeting proapoptotic Xaf1 (XIAP-associated factor 1), thereby regulating plaque composition and susceptibility to rupture.[7] Further studies in a murine model of pressure-overload heart failure have also found a key role for macrophage miR-21 in modulating cardiac fibrosis by regulating macrophage polarization towards a pro-inflammatory (M1) phenotype.[8] In addition, *Hinkel et al* identified a pivotal role of miR-132 in the mediation of pathologic cardiac hypertrophy in a novel porcine model of percutaneous aortic constriction by stent implantation.[9]

ncRNAs have also continued to attract attention as biomarkers with prognostic and diagnostic potential. A landmark study from *Blanco-Domínguez et al.* identified a novel miRNA with potential diagnostic value in acute myocarditis. The authors performed miRNA microarray analyses in sorted CD4+ T cells and type 17 helper T (Th17) cells after inducing experimental autoimmune myocarditis or MI in mice and identified mmu-miR-72 as a differentially expressed miRNA. They further identified the human homologue hsa-miR-Chr8:96 and demonstrated its potential to distinguish patients with myocarditis from those with MI and healthy controls.[10] Thus, miR-Chr8:96 has translational potential as a novel biomarker to diagnose myocarditis. miR-133a is a well-established, diagnostic circulating biomarker in patients with heart failure.[11] *Escate et al.* expanded on the diagnostic potential of this miRNA by demonstrating that elevated plasma levels of miR-133a predict the future occurrence of major adverse cardiovascular events (MACE) in patients with familial hypercholesterolaemia (FH).[12] This observation supports the potential utility of miR-133a in improving risk stratification and prognosis in high-risk patients. More broadly, an international consortium supporting collaboration and research on ncRNAs in cardiovascular disease (CardioRNA Cost Action CA17129) published a Position Paper on the pathophysiologic role of ncRNAs, and to provide recommendations to translate this into clinical practice.[13]

Other studies have progressed ncRNA candidates with therapeutic potential towards clinical translation.[9, 14, 15] *Kay et al* examined the potential of targeting ncRNAs to promote cell-based regenerative strategies for heart disease. Using an integrated approach, they identified CARMA (CARdiomyocyte Maturation-Associated lncRNA), a conserved lncRNA controlling cardiomyocyte differentiation and maturation in human embryonic stem cells. CARMA knockdown promoted cardiogenic commitment and cardiomyocyte differentiation in embryonic stem cells, and is therefore a novel target for improving human ESC-derived cardiomyocyte production in regenerative cardiovascular medicine.[14] On the other hand, *Modica et al* provided evidence for the effectiveness of a novel nanotechnology-based approach for delivering exogenous synthetic miR-133a. The authors demonstrated that intra-tracheal nebulization of miR-133a-nanoconstruct once-a-day on alternate days for 4 consecutive weeks protects against heart failure progression (improved cardiac function parameters and lower fibrosis) in a murine model. This improvement was associated with the restoration of physiological levels of miR-133a in cardiomyocytes without significant accumulation in other myocardial cells or organs.[15]

2.2 Single cell approaches

Single-cell RNA sequencing (scRNAseq) has emerged as a powerful tool to dissect transcriptional profiles of the complex cardiovascular system at single-cell resolution. scRNAseq has been insightful in our understanding of the earliest stages of cardiac development by identifying the epicardial progenitor field, which is anatomically and transcriptionally distinct from the currently known first and second heart fields.[16] In the formed heart, scRNAseq and spatial transcriptomics were used to show that dysregulation of TBX5, the mutated gene causing septal and conduction defects in patients with Holt-Oram syndrome, leads to transcriptional consequences in specific cardiomyocyte subtypes.[17] The study went on to show using cell-based analyses and mice that the stability of many gene regulatory networks, including those that have been shown to be relevant to congenital heart disease, are sensitive to TBX5 dosage.

At the level of the vasculature, the number of publications of atlas-type human or primate scRNAseq, or Assay for Transposase-Accessible Chromatin (ATAC) datasets has steadily increased, which provides a valuable, yet often descriptive resource.[18-21] scRNA-seq has been used to identify transcriptional changes upon conditional cell type-specific genetic deletion, otherwise obscured in bulk tissue RNA sequencing.[22] As for immune cells in atherosclerosis, the detection of different subsets has culminated in a consensus on cell type markers,[23] yet to be achieved for the many varieties of vSMCs identified using scRNAseq in recent years, i.e. fibromyocytes, proinflammatory or

modified vSMCs, SMC-derived intermediate cells.[21, 24-26] scRNAseq has also progressed our understanding of EC,[27 28] with *Rodor et al* identifying CD74 as potential target in PAH and showing its capacity to regulate barrier integrity.[28]

At a cardiac level, the implementation of scRNAseq allowed the impact of heart failure on circulating immune cells to be determined.[29] Furthermore, it demonstrated an exacerbated inflamed transcriptome in circulating monocytes and a signature of T-cell activation in heart failure patients harbouring clonal haematopoiesis-driver mutations in DNA methyltransferase DNMT3A, thereby providing further insights into the potential effect of DNMT3A mutations in heart failure progression.[30] On the other hand, *Hesse et al.* have defined a high level of heterogeneity of epicardial stromal cells following MI, similar to cardiac fibroblast heterogeneity, with evidence of regenerative capacity and hypoxic signalling.[31] *Tombor et al* used scRNAseq of endothelial-lineage traced mice to change the dogma on EndMT in MI, showing this is a transient affair, often without a definite mesenchymal endstage.[32]

Moving forward, cardiovascular scientists will benefit greatly from the generation of multi-omics reference atlases, including different layers of information on RNA, protein, spatial anatomy, interactome and cell ontology.[33-35] Overall, scientific progress can be expedited by open-access science and data sharing. Thus, the integration of available datasets for mesenchymal cells,[36] as previously carried out for immune cells in atherosclerosis,[37] and a web-based application by the *Miller* lab (plaqview.com),[38] pave the way for new, meaningful discoveries in cardiovascular biology.

3. CARDIOVASCULAR DEVELOPMENT

2021 witnessed progress in several important aspects of heart development with implications for our understanding of both congenital and acquired heart conditions. Genomic studies of congenital heart malformations now allow the analysis of variants within the context of gene networks. A good example of this is the recent genomic study on hypoplastic left heart syndrome (HLHS),[39] where whole-exome sequencing, coupled to nuclear transcriptomics and scRNAseq identified genetic heterogeneity in HLHS that converges to alter fundamental processes (e.g. autophagy, apoptosis, proliferation) in myogenesis.

Despite the relative ease in differentiating functional, if immature, cardiomyocytes from iPSC, it has proven remarkably difficult to create organoids resembling the cellular and structural complexity of the vertebrate heart *in vitro*. However, *Lewis-Israeli et al*[40] described a robust protocol for producing

cardiac organoids from iPSC using a three-step Wnt signaling modulation strategy. These organoids develop a broad range of cardiac cell types, including those that are induced through interactions between distinct primary cardiac cell types, and develop cavities that superficially resemble the lumen of the chambers. Moreover, they are vascularised and display regular beating. Importantly, the transcriptome of the organoids more closely resembles foetal hearts than monolayer cardiomyocytes. This method is an important step on the path to developing a robust human-based *in vitro* model of the heart.

It is increasingly apparent that the majority of valve malformations and dysfunction arise from abnormal development, and yet the mechanisms of valve development are incompletely understood. The study by *Fukui* et al focussed on the role of mechanical factors using zebrafish embryos.[41] They identified a critical role for shear stress by showing that ectopic activation of wall shear stress, using agarose beads implanted into the atrium of the early zebrafish heart, resulted in the formation of valve-like structures that expressed the characteristic molecular signature of primitive valves, including the activation of NFATc and klf2a. Downstream of this, they ruled out a number of well-known mechanosensitive pathways, and instead identified adenosine tri-phosphate (ATP) signalling as a mediator of Ca^{2+} oscillations that were essential for specifying valve cell identity. Overall, the convergence of large-scale genomic network analyses, scRNAseq and spatial transcriptomics and experimental developmental biology is coming close to explaining the mechanisms underlying heart malformations presenting at birth and in adulthood.

4. VASCULAR DISEASE AND REPAIR

4.1 Mechanosensing

The Nobel Prize in Physiology or Medicine 2021 was awarded to *David Julius* from the University of California San Francisco and *Ardem Patapoutian* from The Scripps Research Institute La Jolla for explaining the molecular basis for sensing heat, cold and mechanical force.[42] *Ardem Patapoutian* identified PIEZO 1 and 2 as ion channels activated by mechanical force,[43] and they are central responders of arterial responses to flow.[44] Recently, the protein kinase N2 (PKN2) has been shown to be activated by flow through the mechanosensitive ion channel PIEZO1 and mediate flow-induced endothelial NO synthase activation and vascular tone regulation[45] (Figure 2). As another important mechanosensor, the glycocalyx modulates the endothelial redox state in response to shear stress and could mediate an atheroprotective synergism between glycocalyx sialic acids and nuclear factor erythroid 2-related factor (NRF2) antioxidant signaling.[46] The regulation of NRF2 plays also a major role in the reduced endothelial cell viability and wound healing in response to cigarette smoke

extracts under atherogenic low flow conditions.[47] The concept of disturbed flow as an initial stimulus for the development of atherosclerotic plaques has led to exciting new therapies to target mechanosensitive genes like *TWIST1*, *GATA4*, and bone morphogenic proteins (*BMPs*) using siRNA-based technologies in an attempt to slow down the progression of atherosclerosis.[48, 49]

4.2. Atherosclerosis risk factors

The metabolic syndrome – in concert with inflammation - plays a central role in atherosclerosis. In particular, the causal role low-density lipoprotein (LDL) in atherosclerosis is indisputably supported by multiple lines of evidence such as epidemiological studies, Mendelian randomization and genetic analyses, as well as randomized clinical trials and animal model experimentation.

Traditional lipid-lowering drugs such as statins aim to reduce lipid uptake and/or cholesterol synthesis and are still widely used. However, the availability of genetic data and the identification of the genetic cause for rare diseases linked to dyslipidaemias has prompted spectacular advances in the identification of pharmacological targets for the treatment of dyslipidaemias (Figure 3). The most recent advances in lipid-lowering relate to the inhibition of proprotein convertase subtilisin kexin 9 (PCSK9), angiopoietin-like 3 (ANGPTL3) and lipoprotein (a) (Lp(a)). Besides monoclonal antibodies, additional options to inhibit PCSK9 are emerging, including gene silencing with an siRNA or gene editing employing the CRISPR/Cas system. Inclisiran, a siRNA conjugated with *N*-acetylgalactosamine residues ensuring hepatic selectivity, decreases PCSK9 production by promoting the degradation of its mRNA. This approach allows for twice-yearly dosing, with long-term lowering of LDL-C (~50%), potentially enhancing patient compliance compared with other cholesterol-lowering drugs.[50, 51] Along the same line of RNA interference, Lp(a)-reducing drugs are being investigated in phase 2-3 trials.[52] At earlier stages of development are gene-editing technologies, which introduce permanent genomic changes to alter gene function. A single treatment with *PCSK9* gene or base editors has been shown to confer durable LDL-C reduction in primates[53]. Evinacumab is a monoclonal antibody targeting ANGPTL3. It reduces significantly triglycerides (TG) by up to 80% in hypertriglyceridaemic subjects[54] and it is highly effective in reducing LDL-C levels in patients with homozygous FH carrying null *LDLR* mutations[55] providing a new pharmacological tool. In a recent study, membrane type 1 matrix metalloproteinase (MT1-MMP), in addition to activating MMP-2, was shown to regulate LDL-receptor (LDLR) shedding, affecting circulating lipid concentrations and atherosclerosis.[56]

The past year has further blurred the borders between traditional risk factors and the role of inflammation in atherosclerosis as their connections and interplay become more evident. Diabetes mellitus elevates cardiovascular risk, and hyperglycaemia contributes strongly to metabolic syndrome. Besides these known effects, *Edgar et al* elucidated a pro-inflammatory and pro-atherogenic switch in macrophages from diabetic mice persisting even when cultured under normoglycaemic conditions.[58] This persevering effect of earlier hyperglycaemia may explain the relatively low degree of risk reduction upon glucose level normalisation in diabetics. The inseparable connection between cholesterol and inflammation and atherosclerosis is further supported by a recent study that showed how sensing of cholesterol crystals by macrophages induces complement component C5aR1 signaling on mitochondrial membranes and results in interleukin (IL)-1β production and sterile inflammation.[59] Hence, intracellular C5aR1 targeting may be used to normalize mitochondrial function and reduce IL-1β release. This has translational relevance since inhibition of IL-1β production through targeting the inflammasome has been identified as a target in cardiovascular disease previously. Another old acquaintance in cardiovascular disease therapy, rivaroxaban, a direct oral anticoagulant, not only targets factor Xa activity, but may also reduce inflammasome formation. In mice treated with rivaroxaban, macrophage autophagocytic activity increased significantly, which the authors were able to trace back to the Xa-PAR2 axis.[57]

Recent studies show the complex intertwinement between traditional risk factors, vascular biology and immunology. Cardiovascular risk factors can affect haematopoiesis through defective angiogenesis in the bone marrow towards generation of inflammatory leukocytes, thereby creating a self-energizing circle of cardiovascular risk factors – defective angiogenesis – release of inflammatory cells – cardiovascular disease exacerbation.[60] Sakic et al emphasised crosstalk between vSMCs and vascular inflammation by demonstrating that S100A4 induces vSMC change towards a proinflammatory phenotype to drive features of plaque instability[61]. Together, these studies call for an integrated and unprejudiced approach in atherosclerosis research to link traditional risk factors with novel molecular mechanisms.

4.3 Inflammation in Atherosclerosis

The immune response is critical throughout the development of atherosclerotic lesions, during disease initiation, as a trigger for episodic plaque progression, and a contributor to thrombotic complications.[62] A failure in the resolution of inflammation can prevent healing and repair of the vascular wall. [62-64] This concept was advanced by *Arnardottir et al* who found that lipid-specialized, pro-resolving mediators (SPM) signalling through G-protein coupled receptor (GPR)-32,

Is critical for inflammatory resolution and atheroprotection.[64]

The proposal that macrophage uptake mechanisms are decisive for the turning point that leads either to inflammation resolution or to chronic inflammation and plaque progression has received further support from analysis pro-resolving pathways[64] or phagocytic immune checkpoints in murine models.[65] Focussing on the CD47- signal-regulatory protein (SIRP)α immune checkpoint, loss of SIRPα in macrophages stimulated efferocytosis, attenuated oxidized LDL-induced inflammation and induced an M2 macrophage phenotype.[65] These findings may pave the way for novel interventions to promote inflammatory resolution through macrophage uptake mechanisms and phenotypic transitions to protect the vasculature.

Adaptive immune responses are critical regulators of atherosclerosis. On a systemic level, pro-inflammatory and cytotoxic T-lymphocytes prevail in atherosclerosis, as demonstrated by a preferential expansion and function of $CD28^{null}$ T lymphocytes after *ex vivo* IL-7 and IL-15 stimulation of high-purity sorted CD4+ cells isolated from patients with acute coronary syndrome.[66] The local recruitment of regulatory T lymphocytes (T_{reg}) is critical for the control of atherosclerotic lesion inflammation and is, in part, regulated by cellular metabolism.[67] As an approach to use T_{reg} recruitment as a therapeutic strategy to selectively target adaptive immune regulation in the atherosclerotic plaque, adoptive transfer of the fractalkine receptor CX3CR1 overexpressing T_{reg} was shown to increase their recruitment to atherosclerotic lesions and decreased atherosclerosis burden.[68]

However, inhibition of some immune checkpoints can lead to enhanced atherosclerosis. This isi exemplified by *Poels et al.* who found that short-term immune checkpoint inhibitors (ICIs) therapy aggravates T cell-mediated plaque inflammation and drives plaque progression in mice.[69] Also, ICIs used to treat cancer, such as monoclonal antibodies targeting CTLA-4, PD-1, and PD-L1, have been associated with adverse cardiovascular events.[70] For example, *Michel et al.* discovered that anti-PD1 therapy in a mouse model of melanoma led to impaired left ventricular function and promoted myocardial infiltration with CD4+ and CD8+ T cells via a TNF-dependent mechanism.[71, 72] Therefore, the use of ICIs in the treatment of cancer provides exciting new opportunities for therapies but should be pursued with caution.

4.4 Haematopoiesis of Indeterminate Potential

Clonal haematopoiesis of indeterminate potential (CHIP) has recently emerged as an exciting topic in cardiovascular medicine and biology. CHIP is defined as positive selection of specific somatic mutations in haematopoietic stem cells that provide a proliferative advantage and finally result in a clonal population carrying the mutation. Besides being associated with a 0.5 to 1% risk per year to develop leukaemia, CHIP is also associated with ageing, smoking, obesity and type 2 diabetes mellitus, chronic inflammation, infections, sleep deprivation, stress, hyperlipidaemia and atherosclerosis. Most mutations identified in CHIP affect the epigenetic regulators DNA (cytosine-5)-methyltransferase 3A (*DNMT3A)*, tet methylcytosine dioxygenase 2 (*TET2*) and ASXL transcriptional regulator 1 (*ASXL1*) and the tyrosine kinase janus kinase 2 (*JAK2*) which result in a pro-inflammatory state that offers a possible explanation for the association of CHIP with a two-fold increase in risk to develop cardiovascular disease.[73, 74] Using mice that express the $JAK2^{V617F}$ variant exclusively in macrophages, *Fidler et al* reported increased proliferation of macrophages in atherosclerotic lesions and greater necrotic cores. These effects were ameliorated when caspase 1 and 11, which are key components of the inflammasome or gasdermin D, which plays a major role in pyroptosis, were deleted. The authors also noted increased lesional expression of absent in melanoma 2 (AIM2) and found that atherosclerosis was reduced in mice deficient in *Aim2*. The authors concluded that enhanced proliferative stress caused by $JAK2^{V617F}$ leads to DNA damage and to activation of the AIM2 inflammasome resulting in IL-1β activation, which then in turn starts a feed forward loop resulting in even more macrophage proliferation thereby aggravating atherosclerosis.[75]

A new perspective to the field added *Heyde et al* who recently showed by mathematical modeling and murine models that increased proliferation of haematopoietic stem cells occurs in individuals suffering from atherosclerosis thereby increasing the risk to develop clonal haematopoiesis by the age of 70 3.5-fold. Based on their findings the authors propose a vicious cycle in which atherosclerosis leads to clonal haematopoiesis, which in turn aggravates atherosclerosis.[76]

5. CARDIAC DISEASE AND REPAIR

5.1 Extracellular vesicles and nanoparticles

2021 was another exciting year in the field of extracellular vesicle (EV) biology for regenerative medicine, including cardiac repair and regeneration (Figure 4). There was increasing interest in understanding the mechanism of EV-based intercellular communication within the myocardium during ventricular remodeling after acute MI. In terms of the role of EVs in cardiac fibrosis after MI, however, findings differ. For example, *Li et al* showed that miR-30d is mainly secreted in EVs by

cardiomyocytes and inhibits fibroblast proliferation by acting on integrin α5 via paracrine signaling.[77] Counterbalancing this view, *Wang et al* evidenced, in a mouse model of MI, that EVs released by myocardial M2 macrophages exacerbate migration, proliferation and myofibroblastic transformation of cardiofibroblasts.[78] By performing mechanistic studies in cocultured primary cardiofibroblasts and M2 macrophages, the authors linked these effects to activation of miR-138-5p/RhoC signaling after delivery of the M2 macrophage-derived EVs containing circular RNAcirCUbe3a into the cardiofibroblasts.[78] These findings may offer an additional therapeutic target to optimize the endogenous mechanism of cardiac repair but suggest that EV function may depend on cell of origin.

There is great interest in the potential for EVs prepared from stem or progenitor cells to enhance cardiac repair. Increasing evidence suggests the mechanism may involve the resolution of inflammation. For example, *Correa et al* reported that EVs secreted from human iPSC-derived cardiovascular progenitor cells (CPC) can trigger a pro-resolving immune response in preclinical murine models of either chronic or acute heart failure. Similar results were confirmed *in vitro* on human inflammatory cells, suggesting that this EV formulation can instruct the immune cell response towards a pro-resolving phenotype.[79] *Patil et al* showed a similar pro-resolving effect of mesenchymal stem cell (MSC)-derived small EVs, which they attributed to the EVs both enhancing opsonisation of dead cells and activating phagocytic signaling, thereby augmenting removal of apoptotic cells, resolution of inflammation, and improving cardiac recovery after injury.[80]

In order to investigate a clinically feasible translational approach, *Katsur et al* assessed whether cardioprotection could be achieved using a reproducible, clinical-grade preparation of small EVs obtained from the CTX0E03 human neural stem cell line. Systemic administration of small EVs from differentiating CTX0E03 reduced infarct size in mice and prevented *in vitro* cardiomyocyte mitochondrial permeability transition pore opening, which is responsible for cardiomyocyte death during reperfusion injury. These findings provide evidence for considering non-cardiovascular, yet stabilised, cell lines as additional candidate source of therapeutic EVs.[81] Interestingly, however, EVs from proliferating CTX0E03 cells were not cardioprotective, which suggests that the status of cells of origin can impact their secreted EV activity.[81] Further evidence of this is provided by a study showing that systemic administration of serum small EVs from young rats into aged ischaemic rats improved functional outcomes after ischemic stroke, in contrast to small EVs from aged rats that worsened outcome.[82] This provides further evidence that EV function is altered in disease, and further suggests that EV-mIR-mediated vascular intercellular communication is altered in patients with chronic kidney disease and coronary artery disease.

A major goal in cardiac regenerative medicine is to identify novel methods to reinstate cardiomyocyte renewal. In such a scenario, EVs released from cardiac progenitors have been widely investigated, given the role of cardiac stromal cells such as the epicardium-derived progenitor cells play in cardiac muscle growth during embryonic development, and in heart regeneration in zebrafish and in neonatal mice. *Villa del Campo et al* reported that epicardial EVs isolated from the secretome of both mouse and human progenitors enhanced the proliferative activity of neonatal murine cardiomyocytes *in vitro* and promoted cell cycle re-entry when injected into the injured area of infarcted neonatal hearts. These EVs also enhanced regeneration in cryoinjured engineered human myocardium constructs, as a novel model of human myocardial injury. Notably, the epicardial EV cargo was found enriched with specific miRNAs, including miR-30a, miR-100, miR-27a, and miR-30e, which recapitulated the EV regenerative influence on human stem cell-derived cardiomyocytes and cryoinjured cardiac constructs *in vitro*.[83]

The relevance of the content of cardiovascular cell-derived EVs was highlighted by publications showing that miRNAs of the miR-106a-363 cluster,[84] periostin[85] and mitochondrial cargoes[86] can act as effectors of cardiac repair. While such encouraging evidence supports the exploitation of stem/progenitor cell-EVs as candidate therapeutics to promote adult cardiomyocyte proliferation, a general consensus has not been reached yet on their mechanism of action. In fact, *Lima Correa et al* recently showed that EVs obtained from human iPSC-derived cardiac progenitor cells failed to trigger the generation of new cardiomyocytes in chronically infarcted hearts in mouse models. Despite this negative result, the authors confirmed that EVs from cardiac progenitor cells remained capable of significantly improving cardiac function by non-regenerative mechanisms.[87]

These findings suggest that further analyses and accurate lineage tracing are required to better understand the regenerative potential of cardiac EVs. At present, the rapid clearance of EVs from circulation is a limitation for their clinical application. During 2021, a number of studies aimed to overcome this barrier by constructing specific nanoparticles and genetically modifying cells to improve retention time of the cell-derived EVs. Thus, *Wei et al* demonstrated that intravenously-injected EV derived from modified mouse bone marrow MSC overexpressing CD47, a transmembrane protein known to elicit blockade of the mononuclear cell phagocytosis, have prolonged retention in the circulation and accumulate at greater levels in the ischemic heart.[88]

5.2 Cardiotoxicity and regeneration

A wide range of drugs, including but not limited to antineoplastic chemotherapeutic agents, can cause heart electrophysiology dysfunction, muscle damage and other cardiovascular pathologies. For example, anthracyclines such as doxorubicin (DOX) are a cornerstone for the treatment of many cancers, but their use is complicated by cardiotoxicity, especially left ventricular dysfunction.

An interesting 2021 paper reported that transcutaneous vagal nerve stimulation prevented DOX-induced cardiotoxicity in rats by rebalancing autonomic tone, ameliorating cardiac dysfunction and remodelling. It was hypothesized that the mechanism involved crosstalk between autonomic neuromodulation, innate immune cells such as macrophages and chemokines.[89] Indeed, there are multiple mechanisms responsible for anthracycline cardiotoxicity.[70, 90, 91] *Chan et al.* found that two orally available MMP inhibitors ameliorated DOX cardiotoxicity by attenuating intracellular and extracellular matrix remodelling, suggesting that they may be a potential prophylactic strategy to prevent heart injury during chemotherapy.[90] Remote ischaemic preconditioning can ameliorate DOX-induced cardiotoxicity by preserving mitochondrial integrity[92] and this is currently the subject of the RESILIENCE clinical trial.[93]

Other recent studies (discussed in [94]) have identified harmful effects of anticancer therapies on the ability of stem/progenitor cells to repair cardiac damage, through a reduction of stem cell viability and paracrine activity. Thus numerous animal and clinical studies have demonstrated that local or systemic administration of mesenchymal stem cells significantly improve cardiac function, through a reduction in inflammatory responses and myocardial fibrosis.[95] Antivirals can also induce cardiotoxicity, including the only FDA-approved treatment for hospitalized COVID-19 patients, remdesivir which can induce toxicity in human iPSC-derived cardiomyocytes through mitochondrial fragmentation, electrophysiological alterations and sarcomere disarray.[96]

5.3 Cardiac arrhythmias

Several key insights into fibrillation and re-entrant arrhythmias were obtained in 2021 (Figure 5). *Handa et al* revealed that the degree of gap junction coupling as well as the pattern of fibrosis influences mechanisms sustaining ventricular fibrillation.[97] Differentiating between these underlying mechanisms of maintenance of fibrillation may help to guide therapy. Re-entrant arrhythmias may also initiate in the absence of structural abnormalities, shown recently in a study on the spatiotemporal interaction between trigger and electrical substrate in the context of unexplained sudden cardiac arrest (SCA).[98] Analysis of explanted hearts and observations in survivors of

unexplained SCA, identified key elements required for re-entry initiation including the occurrence of an early premature beat from an early repolarizing region of the ventricles, which may block against a steep repolarization time (RT) gradient to start re-entry. They also showed that detection of the origin of premature beats and their relation to RT gradients in patients is possible with non-invasive electrocardiographic imaging (ECGI) and may provide targets for therapy. ECGI was also employed by *Leong et al* in survivors of SCA to show that not only repolarization abnormalities, but also underlying conduction abnormalities play a role in the initiation of SCA.[99] A similar mechanistic reasoning extends to atrial arrhythmias.[100] Bringing these studies together highlights that any cause of steep excitability dispersion – whether resulting from local changes in gap junction coupling, fibrosis, local conduction slowing, or inherent repolarization duration heterogeneity – play a critical role in the initiation and maintenance of re-entry and fibrillation.

New tools are essential to obtain mechanistic insights and recent reports highlight how the field of atrial fibrillation research should transition from a translational approach to an integrative research approach[101] and how personalized computer models may provide more individualised insights in disease and guide therapy.[102] Application of novel therapeutic tools also brings new mechanistic insights. Non-invasive radiation therapy for cardiac arrhythmias was initially thought to induce fibrosis, similar to invasive catheter-based therapy.[103] However, *Zhang et al* found that transmural fibrosis does not develop in the hearts of patients receiving radiation therapy within the timeframe of its ventricular tachycardia-reducing effects.[104] Interestingly, they showed that irradiating murine hearts results in a persistent supraphysiologic electrical phenotype, mediated by increases in sodium channel function and gap junction function. This functional restoration was confirmed by a shortening of QRS duration in patients receiving radiation therapy, highlighting that radiation-induced reprogramming of cardiac conduction is the potential mechanism beyond the initial success of radiation therapy for refractory ventricular tachycardia. This holds promise for extending the use of non-invasive radiation therapy to other applications, as for example recently demonstrated in heart failure with reduced ejection fraction.[105]

6. CARDIOVASCULAR PRECISION MEDICINE AND iPSC

Precision medicine aims to improve risk stratification and customize the management and therapy of patients based on their clinical and genetic characteristics, on datasets of large populations and the use of advanced technologies.[106] Genome-wide association studies (GWAS) has progressed through advances in genome-wide genotyping technology and large population and patient datasets to explore the role of common variants on phenotypic traits and disease susceptibility. According to the

GWAS catalogue database, there are known to be 1329 polymorphism-cardiovascular trait associations. This growing catalogue of genome-wide and nominally significant variants has also opened the door to creating polygenic risk scores that could identify individuals at risk of developing specific cardiovascular diseases or sub-groups of patients with a more severe prognosis.[107] However, this approach must consider numerous confounding factors such as epigenetic and transcriptomic data that may correlate with genetic variants. *Boix et al* undertook a *tour de force* to create EpiMap, a compendium comprising 10,000 epigenomic maps across 800 samples, which were used to define chromatin states, high-resolution enhancers, enhancer modules, upstream regulators, and downstream target genes.[108] This resource allowed the annotation of 30,000 genetic loci associated with 540 traits, predicting trait-relevant tissues, putative causal nucleotide variants in enriched tissue enhancers and candidate tissue-specific target genes for each of them. These different data integration layers could be essential for understanding the genetic architecture underlying the broad phenotypic traits encountered in common and complex cardiovascular diseases such as coronary artery disease. For instance, while "only" 56 'unifactorial' traits were enriched in the case of long QT syndrome (LQTS), a total of 192 'multifactorial' traits were enriched in an average of five different tissues, and in the case of coronary artery disease, 26 'polyfactorial' traits were enriched in 14 tissues. The study by *Boix et al* is at the same time a rich scientific resource, but also a lesson regarding the profound and magnificent complexity of the human genome and the causal basis of common diseases like coronary artery disease.

The GENMED consortium conducted a large GWAS study focused on dilated cardiomyopathy (DCM), enrolling 2719 cases and 4440 controls.[109] They identified and replicated two new DCM-associated loci on chromosome 3p25.1 and chromosome 22q11.23. *In silico* annotation and functional 4C-sequencing analyses on cardiomyocytes derived from iPSC-derived cardiomyocytes identified SLC6A6, a gene encoding a taurine, as the most likely DCM candidate at the 3p25.1 locus, and SMARCB1 as the candidate culprit gene at the 22q11.23 locus. The consortium also constructed a genetic risk score for DCM.

In another important study, exome sequencing data from 811 probands with tetralogy of Fallot (TOF) were used to identify rare loss-of-function and other likely pathogenic variants in genes associated with congenital heart disease.[110] The role of some likely pathogenic variants was confirmed and multiple loss-of-function variants provided support for 3 emerging congenital heart disease/TOF candidate genes: *KDR*, *IQGAP1*, and *GDF1*. Moreover, using composite genes in a STRING protein

interaction enrichment analysis, a biologically relevant network was revealed, with vascular endothelial growth factor receptor 2 (VEGFR2) and NOTCH1 representing central nodes.

The use of iPSC technology for disease modelling and drug testing is increasingly used for cardiovascular precision medicine. Last year, for the first time, the combination of patient-specific iPSC-derived cardiomyocytes, genetics and genome editing unveiled the mechanisms of action of modifier genes in subsets of patients affected by long QT syndrome (LQTS).[111, 112] By comparing patient-specific iPSC-CMs derived from symptomatic and asymptomatic LQT1 carriers of the same mutation, it was shown that genetic variants of *MTMR4*, an upstream regulator of neural precursor cell expressed developmentally downregulated gene 4-like (NEDD4L), control potassium channel turnover, thus influencing the clinical manifestations of the disease. iPSC technology has also been used to gain insights into the molecular mechanisms of atrial septum defect (ASD), a form of congenital heart disease, by implicating a mutation in *GATA4* that modifies *FGF16* induction.[113]

Pioneering work from *Srivastava* and collaborators developed a machine-learning approach to identify small molecules that broadly correct gene networks dysregulated in an iPSC model of aortic valve (AV) disease.[114] Correction of the gene network by the most effective therapeutic candidate, XCT790, was sufficient to prevent and treat AV disease *in vivo* in a mouse model. This strategy, made possible by combining iPSC technology, network analytics and machine learning, may can represent an effective path to discovering new therapies.

7. COVID-19

7.1 Cardiovascular tropism and molecular markers

The aetiology of myocarditis caused by cardiotropic viruses has become a major topic of interest during the COVID-19 pandemic.[115, 116] A comparative study revealed that while myocardial injury occurred with a similar frequency in infection with influenza and SARS-CoV-2, the mortality was almost 4-fold higher in COVID-19 compared with influenza.[117] Evidence of viral infection was seen mainly in endothelium and rarely in cardiomyocytes,[118] however, evidence for stromal cells infection by SARS-CoV-2 has been found.[119] Endothelial-dependent dilation in human arterioles is impaired for months after SARS-CoV-2 exposure, and could contribute to long-lasting symptoms of post-COVID-19 infection.[120] Consistently, *Bräuninger et al* performed massive analysis of cDNA ends–RNAseq in myocardial tissue from fatal COVID-19 cases with and without cardiac infection to reveal potential SARS-CoV-2-related pro-inflammatory transcriptomic alterations in EC, while no differences were detected in immune cell infiltrations.[121] Interestingly, the levels of several known cardiometabolic

biomarkers are associated with COVID-19 severity and mortality, particularly myocyte-derived miR-133a and liver-derived miR-122.[122] The potential for the use of cardiovascular RNA markers and artificial intelligence in the setting of COVID-19 has been reviewed in.[123] In a study of 95 SARS-CoV-2-positive autopsy tissue, cardiac SARS-CoV-2 infection was shown to increase transcription of interferon pathways, originating predominantly from EC.[118] The ESC has provided guidance for the diagnosis and management of cardiovascular disease during the COVID-19 pandemic[124, 125] and recommendations for future research.[126]

7.2 Virus- and vaccine-induced thrombotic complications and COVID-19

Accumulating evidence suggests that patients suffering from COVID-19 have an increased risk to experience thrombotic events such as microthrombosis, venous thromboembolism and ischaemic stroke (for a review see[127]). Two recent studies have found microthrombi in the hearts of patients who succumbed to SARS-CoV-2 infections. *Pellegrini et al* identified microthrombi as a cause of myocyte necrosis. Interestingly these microthrombi contained more fibrin and more of the complement components C5b-9 than thrombi isolated from the myocardium of patients of COVID-19 negative patients and coronary thrombi aspirated from COVID-19 negative and positive patients with ST-e*levation* MI.[128] *Bois et al* found nonocclusive microthrombi in myocardial arterioles in 12 out of 15 patients who died from SARS-CoV-2 infections. However, no evidence of acute ischaemic injury of the heart was detected in this study.[129] When tissue factor (TF)-bearing microvesicles isolated from the plasma of 100 patients with moderate and severe COVID-19 and from the plasma of 28 healthy subjects were studied, the authors found that TF-activity on such microvesicles, which is indicative of a procoagulatory state, was increased in patients suffering from COVID-19 and is significantly linked to disease severity and mortality.[130]

Thrombotic complications have been reported in 1 per 100 000 adenoviral COVID-19 vaccinated irrespective of age, rising to 1 in 50 000 above 50 years vaccinated with ChAdOx1.[131] This is referred to as vaccine-induced immune thrombotic thrombocytopenia (VITT).[131, 132] *F*ibrinogen, *A*ge, *P*latelet count, and the presence of *I*ntracranial haemorrhage, and *C*erebral venous sinus thrombosis (the *FAPIC* score) are significantly associated with mortality in cases of VITT.[133] Increased levels of anti-PF4 antibodies post-vaccination unrelated to previous heparin exposure implicates an augmentation of the antibody response by unknown PF4 co-factors.[132] The antigenic component with PF4 may be vaccine constituents but remains an unsolved critical question in VITT pathophysiology.[132] The immune complexes transduce platelet activation through the Fcγ receptor IIA (FcγRIIA) resulting in thrombosis with concomitant thrombocytopenia accompanied by a fulminant immune activation.[134]

Among novel therapeutic options for VITT, inhibitors of Bruton tyrosine kinase (Btk), which is used for B-cell malignancies, have been explored for their ability to block FcyRIIA for preventing the downstream platelet activation and aggregation. The Btk inhibitors ibrutinib and fenebrutinib prevented platelet aggregation induced by serum obtained from patients with VITT.[135] Additional possibly favourable effects of Btk inhibition in VITT are blocking of neutrophil-platelet complexes and reduced NET release,[136] which are part of the massive immune activation during VITT.[134]

7.3 Cardiovascular drugs and COVID-19

In the beginning of the COVID-19 pandemic, the interactions with cardiovascular drugs were focused on ACE-inhibition and anti-thrombotic treatments[137] and more recently extended to lipid-modulating agents.[138] In the latter context, omega-3 fatty acids may provide beneficial cardiovascular effects through immunomodulation, anti-thrombosis and improved endothelial function.[139] Specific cytokine antibodies to dampen the inflammatory storm in COVID-19 exhibit anti-inflammatory strategies explored for cardiovascular prevention and have shown some success in improving survival and clinical outcomes.[140] The RECOVERY trial tested multiple different therapeutic approaches including antiviral, immunomodulatory and antithrombotic treatments, in a multi-arm factorial design inspired by the International Study of Infarct Survival (ISIS) trials of the 1980s, and demonstrated benefit with tocilizumab and dexamethasone, but not hydroxychloroquine, convalescent plasma or other tested approaches.[141] In a separate study, anticoagulation with low-molecular-weight heparin (LMWH) may curtail viral persistence and reduce mortality.[142]

Perspectives

The substantial progress of basic cardiovascular science during the past year has revealed a plethora of novel therapeutic and diagnostic possibilities. Non-codíng RNA, scRNAseq, and iPSC are examples of discovery tools to widen the understanding of cardiac and vascular pathophysiology. Through the integration cardiovascular risk factors, genetics, and biomarkers, the basic cardiovascular science field is expanding towards applications in precision medicine. The year was still marked by the COVID-19 pandemic and several important contributions have increased our knowledge of the cardiac and thrombotic effects of SARS-CoV-2, and the underlying pathways behind reported vaccinal complications. Finally, the mechanistic insights from *in vitro* and *in vivo* basic science models have deepened our understanding of inflammation, CHIP, EVs, regeneration, and mechanosensing in cardiovascular disease.

Conflicts of interest

CGT has received funding from Amgen, and personal fees from VivaLyfe, and is listed as an inventor on 2 heart failure patents.

FIGURE LEGENDS

Figure 1. Novel insights into the role of ncRNAs.

Several complex loci composed of lncRNA and miRNA clusters have been identified throughout the genome. Nonetheless, despite their genomic and often transcriptional overlap, they have been found to have distinct functional and regulatory targets. The X-linked lncRNA MIR503HG maintains endothelial cell (EC) identity by interacting with the RNA splicing regulatory protein PTBP1, with decreased expression leading to broad changes associated with EndMT. Importantly, these phenotypic changes seem to be independent of miR-424 and miR-503 expression, which overlap the lncRNA locus[3]. Similarly, loss of the Cardiac Mesoderm Enhancer-associated Non-coding RNA (CARMN) primes vascular smooth muscle cells (vSMCs) into a pro-atherogenic proliferative state, while migration or dedifferentiation are regulated through the modulation of the overlapping miR-143 and miR-145[4].

Figure 2. Recent findings on cardiovascular mechanosensing.

Newly discovered flow-stimulated mechanosensitive signalling pathways. Flow-activated PIEZO1 was shown to activate the protein kinase N2 (PKN2) via PKD1, resulting in phosphorylation of Akt and eNOS, with subsequent vascular tone regulation via NO.[45] The glycocalyx component sialic acid, was shown to activate NRF2 antioxidant signalling, via phosphorylation of AKT[46], whereby modulating the endothelial redox state in response to shear stress. The pathways are likely to be interconnected as both result in phosphorylation of AKT and eNOS and as NRF2-induced antioxidant signalling is likely to affect NO bioavailability.

Figure 3. New insights and interventions in lipid biology.

Gene silencing with small interfering RNA (siRNA) like inclisiran or gene editing are becoming additional options to monoclonal antibodies for the inhibition of proprotein convertase subtilisin kexin 9 (PCSK9) leading to long-lasting circulating LDL-Cholesterol (LDL-C) decrease. Lipoprotein(a)

(Lp(a))-reducing drugs by RNA interference, via antisense oligonucleotide (ASO), like Pelacarsen or siRNA, like Olpasiran are holding promising results in clinical trials. The inhibition of angiopoietin-like 3 (ANGPTL3) via evinacumab, a monoclonal antibody or Vupanorsen, a GalNAc-conjugated ASO markedly reduces circulating triglyceride-rich lipoprotein (TGRL) levels. N-acetylgalactosamine (GalNAc) ligands conjugated with siRNAs or ASOs allow its hepatocyte-targeted delivery lowering incidence and severity of off-target effects, commonly observed with the first generation RNA interference.

Figure 4. Source of EVs affects their function

Several thought-provoking studies published in 2021/2022 demonstrated that the cardiovascular effects of extracellular vesicles (EVs) can depend upon their origin. For example, EVs originating from different cell types (cardiomyocytes vs M2 macrophages), different cellular states (proliferating vs differentiated), different ages (young vs old serum) or different health states (chronic kidney disease and coronary artery disease [CKD+CAD] vs healthy) can have opposite effects.

Figure 5. Novel mechanisms of arrhythmia.

Recent publications (top left) and accepted concepts (top right) on the mechanisms leading to re-entry may be combined to arrive at a generalized theory of the spatiotemporal interaction between triggers and substrate leading to re-entry arrhythmias (bottom). The generalized hypothesis highlights that re-entry can only initiate when there is a local dispersion of excitability, with some tissue excitable whereas other tissue is (still, or always) refractory at the time when the trigger occurs. The trigger should originate from the excitable tissue, may block and travel around (relatively large) refractory tissue before it arrives at the previously excited tissue again.

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Figure 1. Novel insights into the role of ncRNAs.

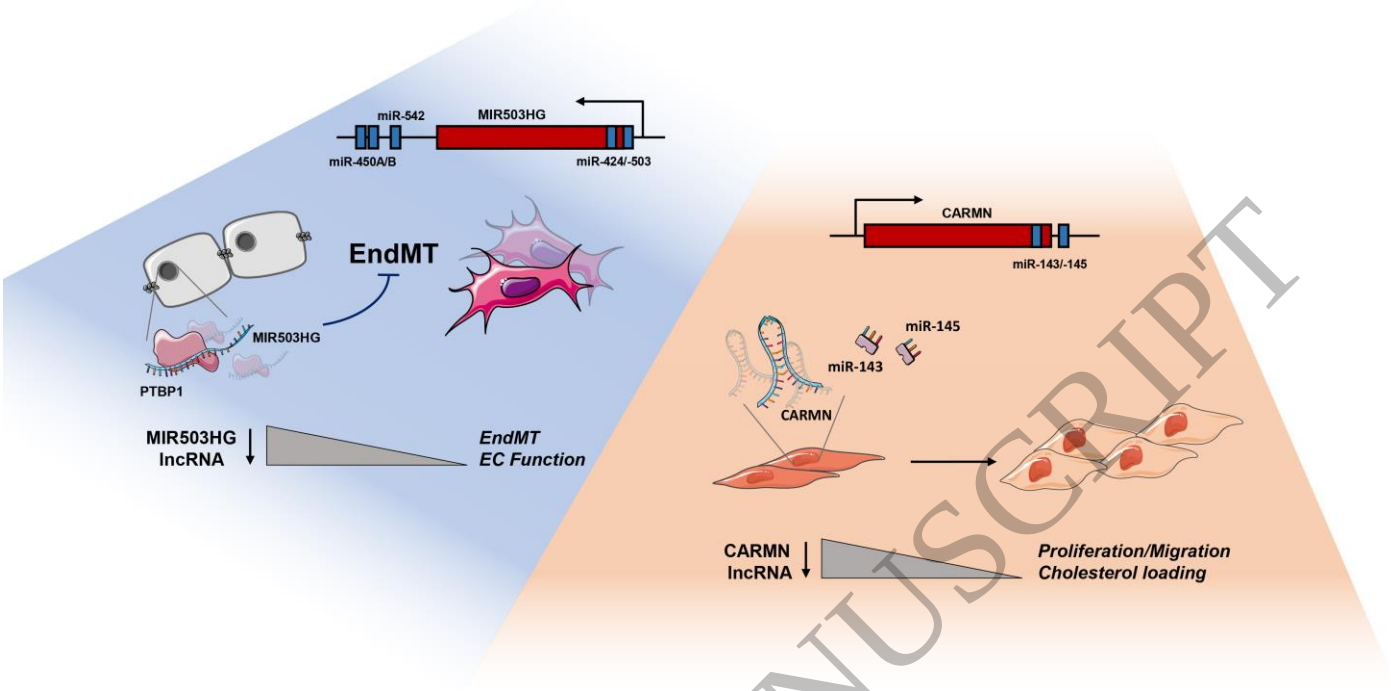

Figure 1
185x103 mm (x DPI)

Figure 2. Progress in mechanosensing.

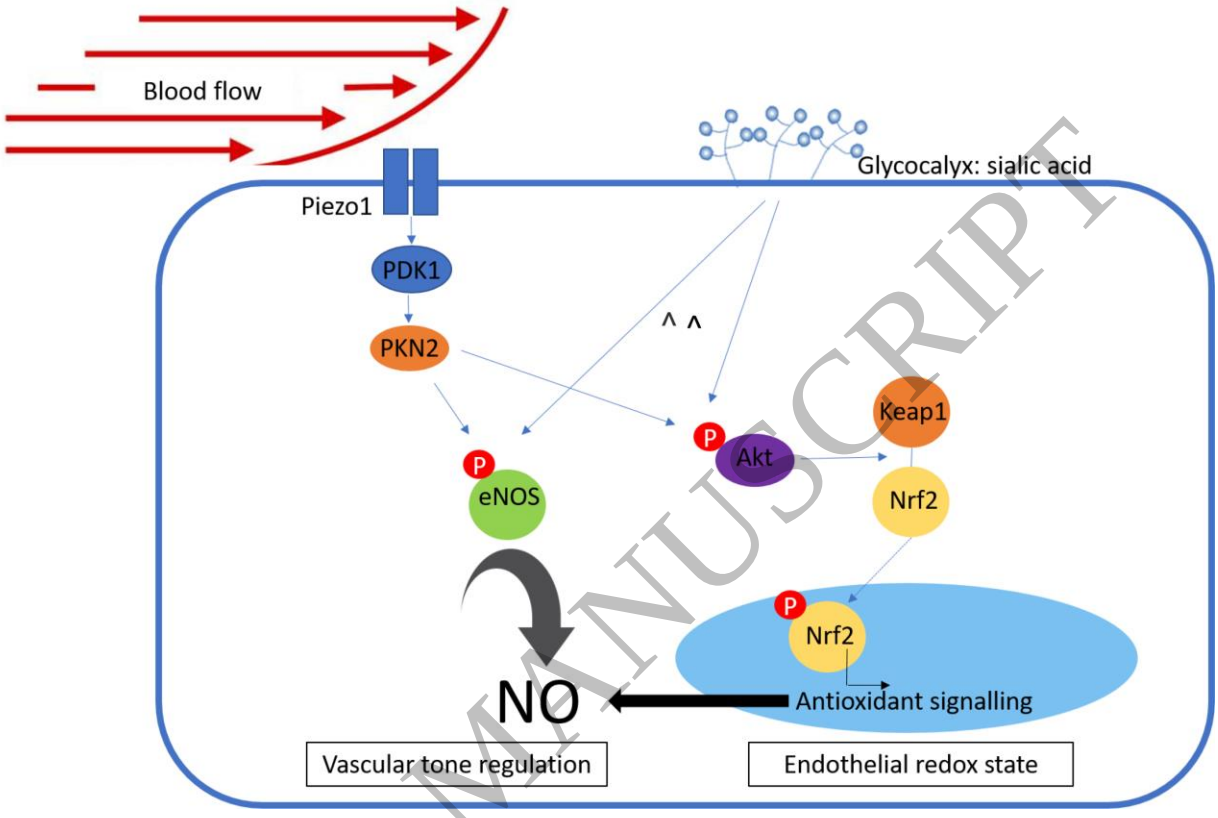

Figure 2
185x129 mm (x DPI)

Figure 3. New insights in lipid biology and cross-talk with inflammation

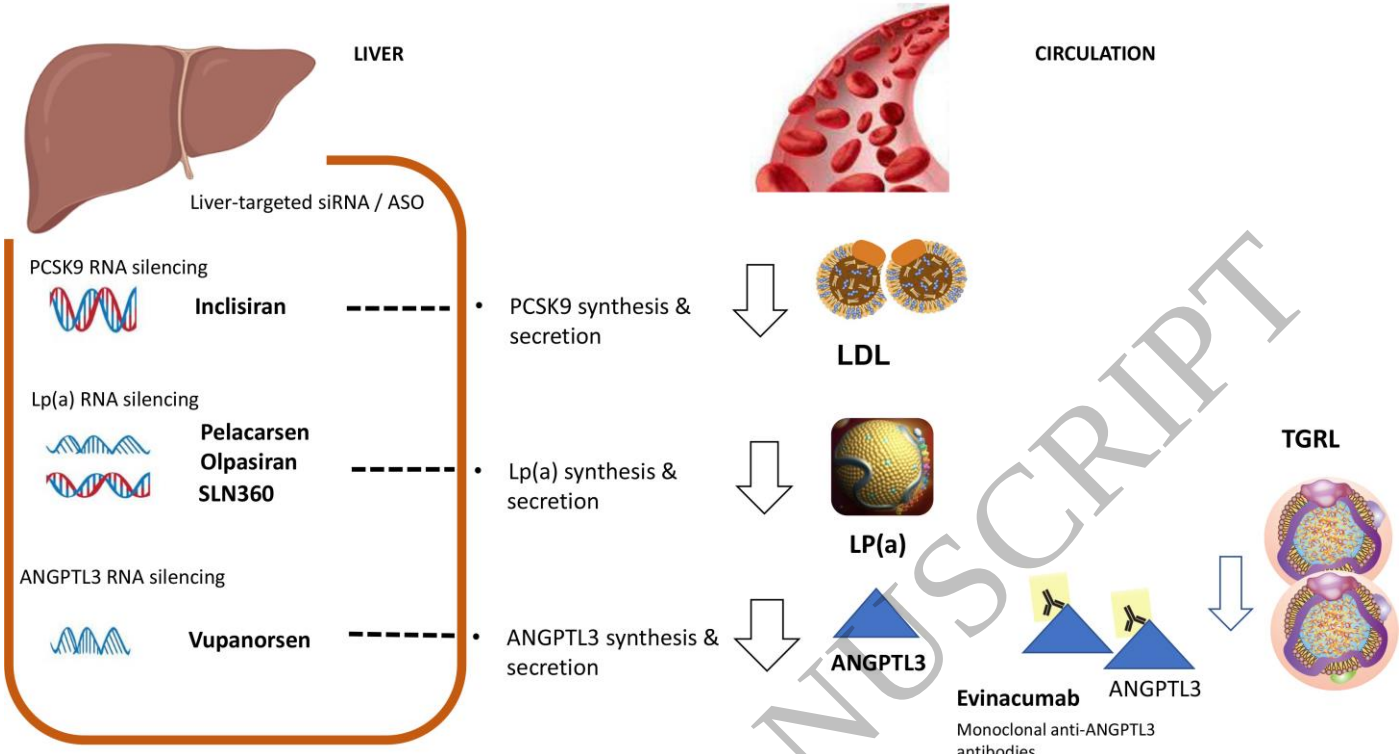

Figure 3
185x107 mm (x DPI)

Figure 4. Source of EVs affects their function.

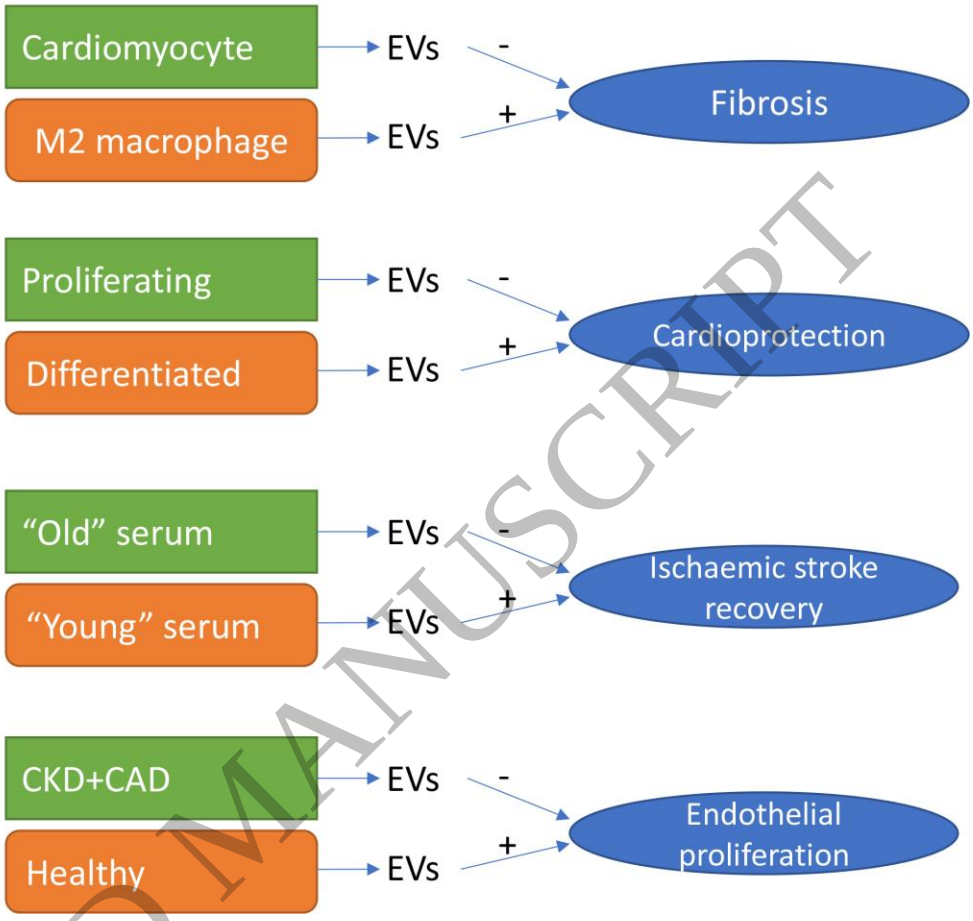

Figure 4
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Figure 5. Novel mechanisms of arrhythmia.

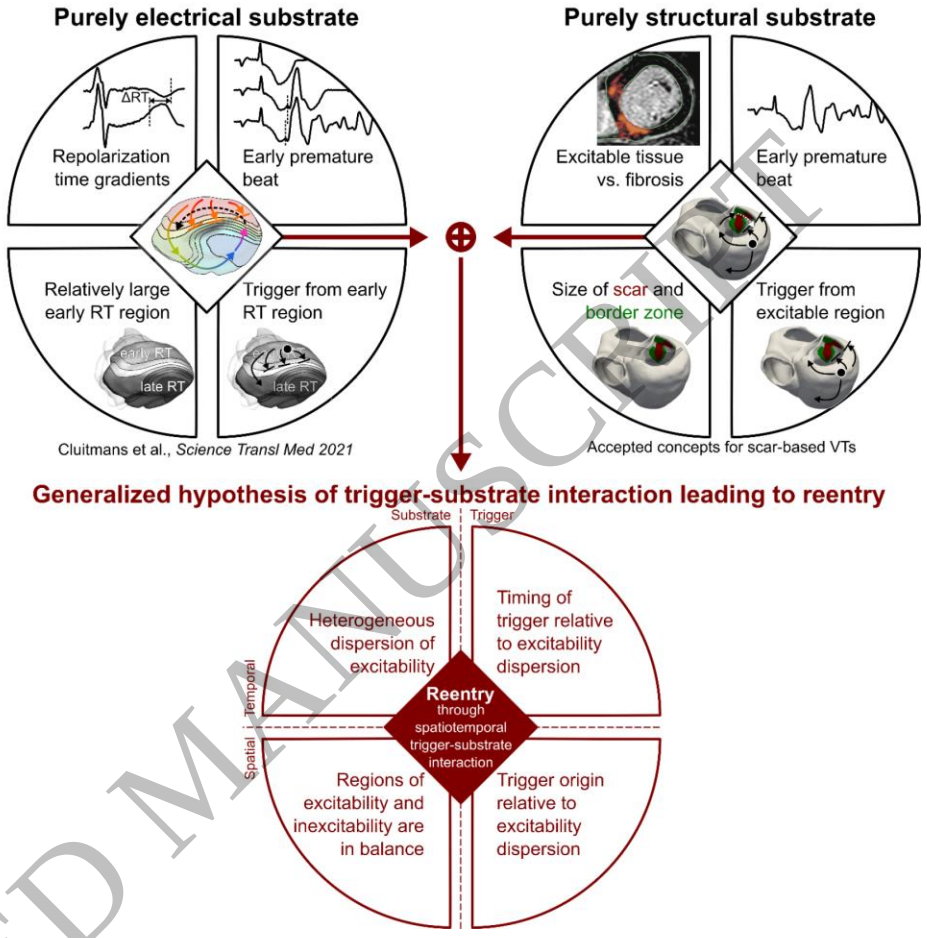

Figure 5
185x143 mm (x DPI)