

## Targeting mitochondria for cardiovascular disorders: therapeutic potential and obstacles

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**Abstract** | A large body of evidence indicates that mitochondrial dysfunction has a major role in the pathogenesis of multiple cardiovascular disorders. Over the past 2 decades, extraordinary efforts have been focused on the development of agents that specifically target mitochondria for the treatment of cardiovascular disease. Despite such an intensive wave of investigation, no drugs specifically conceived to modulate mitochondrial functions are currently available for the clinical management of cardiovascular disease. In this Review, we discuss the therapeutic potential of targeting mitochondria in patients with cardiovascular disease, examine the obstacles that have restrained the development of mitochondria-targeting agents thus far, and identify strategies that might empower the full clinical potential of this approach.

### Regulated cell death

(RCD). A form of cell death that relies on the activation of a genetically encoded machinery and which, therefore, can be retarded or accelerated with specific pharmacological or genetic interventions.

### Autophagy

Evolutionarily conserved cellular process that culminates with the lysosomal degradation of ectopic, supernumerary, dysfunctional, or potentially dangerous cytoplasmic entities (of endogenous or exogenous derivation).

Mitochondria occupy a central position in the biology of most eukaryotic cells, including all the cells of the cardiovascular system, because mitochondria have a major role in catabolic and anabolic metabolism, regulation of intracellular Ca<sup>2+</sup> homeostasis, initiation of inflammatory reactions, and control of multiple pathways culminating in regulated cell death (RCD)<sup>1–4</sup>. In line with this notion, the mitochondrial network is constantly subjected to a tight quality-control system that segregates dysfunctional mitochondria and delivers them to lysosomes for degradation<sup>5,6</sup>. Such a mechanism, commonly known as mitophagy, involves not only the core molecular machinery for autophagy<sup>7</sup> but also a set of dedicated proteins that are required for the optimal recognition of damaged mitochondria<sup>8–10</sup>.

A tight control on mitochondrial fitness is paramount for the preservation of cardiovascular homeostasis for at least four reasons<sup>11</sup>. First, cardiomyocytes heavily rely on fatty acid-driven oxidative phosphorylation for ATP production, at least in physiological settings<sup>12</sup>. Thus, a decrease in the bioenergetic efficiency of the mitochondrial network can have a direct detrimental effect on the contractile capacity of cardiomyocytes. Second, Ca<sup>2+</sup> fluxes are at the core of overall cardiac activity<sup>1</sup>. Therefore, defects in the capacity of the mitochondrial network (in conjunction with the endoplasmic reticulum) to regulate Ca<sup>2+</sup> homeostasis can alter cardiac functions such as electrical conduction. Third, physiological inflammatory homeostasis is particularly important not only for normal cardiac functions<sup>13</sup> but also for the preservation of vascular

compartments<sup>14</sup>. Thus, damaged mitochondria accumulating in the cytosol of cardiomyocytes or endothelial cells can drive pathogenic inflammatory responses. Finally, the integrity of the cardiovascular system is crucial for optimal contractile and circulatory functions<sup>15</sup>. Severe mitochondrial dysfunction and/or the accumulation of permeabilized mitochondria (beyond a threshold that depends on multiple parameters) can initiate several variants of RCD that culminate in pathological tissue loss (FIG. 1).

In line with these observations, mitochondrial defects have been involved, at least to some extent, in the pathogenesis of a variety of cardiovascular disorders, including (but not limited to) myocardial infarction (MI), cardiomyopathies of different aetiology, some forms of arrhythmia, hypertension, atherosclerosis, and other vascular conditions<sup>16,17</sup>. Starting in the late 1990s, the identification of mitochondrial dysfunction as a central aetiological determinant of cardiovascular disease (CVD) drove an intensive wave of preclinical and clinical investigation aimed at the development of novel targeted therapies<sup>18</sup>. Thus far, the results of such an effort have been disappointing, as no molecules specifically conceived to target mitochondria are currently available for use against CVD in clinical settings<sup>19</sup>. In this Review, we discuss the rationale for using mitochondria-targeting agents (MTAs) in the treatment of CVD, dissect the obstacles that have limited their development over the past 2 decades, and put forward strategies that might unleash the full potential of these promising — but hitherto unrealized — therapeutic tools.

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## Key points

- Mitochondrial dysfunction is involved in the pathogenesis of multiple cardiovascular disorders, including myocardial infarction, cardiomyopathies of various aetiologies, arrhythmias, hypertension, and atherosclerosis.
- Mitochondria are essential for the physiological activity of the cardiovascular system owing to their crucial role in bioenergetic and anabolic metabolism and their central position in intracellular  $\text{Ca}^{2+}$  fluxes.
- In addition to losing their physiological functions, damaged mitochondria actively drive inflammatory responses and waves of regulated cell death that contribute to the pathogenesis of cardiovascular disease.
- An intensive wave of investigation attempted to develop mitochondria-targeting agents for preventing or treating cardiovascular disorders in patients, with rather dismal results.
- Molecules with improved pharmacological features, precise mechanistic insights into mitochondrial processes, and reconsidering the pathogenesis of some cardiovascular disorders are instrumental for the development of mitochondria-targeting agents with clinical use.

### $\beta$ -Oxidation

Biochemical pathway whereby fatty acids are converted into acetyl-CoA, which enters the TCA cycle, and NADH and  $\text{FADH}_2$ , which fuel oxidative phosphorylation.

### Ketolysis

Biochemical pathway whereby ketone bodies are converted into acetyl-CoA, which enters the TCA cycle, and NADH, which fuels oxidative phosphorylation.

## Therapeutic potential of MTAs

Targeting mitochondria from multiple angles has been associated with beneficial effects in a variety of experimental CVD models (TABLES 1,2). However, limited benefits have been documented in clinical trials investigating the safety and efficacy of MTAs for the treatment of CVD, as discussed below.

**Mitochondrial metabolism.** Healthy cardiomyocytes satisfy their elevated energy needs by catabolizing fatty acids (via  $\beta$ -oxidation), branched-chain amino acids, and, to a lesser extent, ketone bodies (via ketolysis) to fuel the tricarboxylic acid (TCA) cycle and drive ATP production via the mitochondrial respiratory chain (BOX 1). By comparison, pyruvate derived from glycolysis contributes minimally to ATP synthesis in the healthy heart<sup>11</sup>. Such a predominantly mitochondrial metabolic profile

shifts in the course of numerous cardiac pathologies. Heart failure (HF) is accompanied by a gradual decline in the bioenergetic reserve capacity of the myocardium, which — beyond a specific threshold — can no longer be compensated for by endogenous mechanisms<sup>20</sup>. In multiple variants of cardiomyopathy culminating with HF, cardiomyocytes undergo metabolic reprogramming involving decreased  $\beta$ -oxidation and branched-chain amino acid metabolism coupled with intracellular lipid deposition and increased glucose utilization<sup>21–24</sup>. The TCA cycle intermediate succinate accumulates in the ischaemic myocardium, and such an accumulation is mechanistically linked to oxidative damage at reperfusion<sup>25</sup> (see below). Along similar lines, TCA cycle activity is impaired 6 weeks after MI<sup>26</sup>, potentially representing an early maladaptive phase of the surviving tissue.

The molecular mechanisms underlying metabolic reprogramming in the diseased myocardium remain to be fully elucidated, although a role for specific transcription factors has been postulated. For instance, nuclear receptor subfamily 2, group F, member 2 (NR2F2; also known as COUP-TF2) is upregulated in patients with HF, and transgene-driven *Nr2f2* overexpression in mice favours dilated cardiomyopathy accompanied by pathological metabolic remodelling<sup>27</sup>. Similarly, hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) initiates a transcriptional programme involving peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) that leads to increased glucose uptake and consequent lipid accumulation, apoptotic cell death, and contractile dysfunction<sup>21</sup>. Corroborating an aetiological role for this transcriptional module, ventricular-specific deletion of *Hif1a* prevents pressure-overload-induced cardiomyopathy in mice<sup>21</sup>.

Additional metabolic functions ensured (at least in part) by mitochondria are relevant for CVD, including the folate cycle. An efficient folate cycle is indeed required for the optimal conversion of homocysteine into methionine, and defects in this pathway, including genetic variants in *MTHFR* (which encodes methyl-entetrahydrofolate reductase) are associated with an increased incidence of vascular disorders (such as thrombosis and atherosclerosis) secondary to, or at least paralleled by, homocysteine accumulation<sup>28</sup>. Of note, several mutations in mitochondrial or nuclear genes coding for components of the mitochondrial respiratory chain have been associated with familial cardiomyopathies in humans<sup>29</sup>. Moreover, experimental interventions inducing respiratory defects in myocardial cells, such as the tissue-specific deletion of *Aifm1* (which encodes apoptosis inducing factor mitochondria associated 1)<sup>30</sup> or *Tfam* (which encodes mitochondrial transcription factor A; TFAM)<sup>31</sup>, result in spontaneous, early-onset cardiomyopathy. Taken together, these observations exemplify the involvement of mitochondrial metabolic dysfunction in CVD.

Early clinical trials testing L-carnitine supplementation, which (among other effects) favours the mitochondrial uptake of cytosolic fatty acids, in patients recovering from acute MI documented some degree of efficacy in reducing the incidence or severity of HF, left ventricular enlargement, arrhythmias, and cardiac death<sup>32,33</sup>. However, subsequent studies did not

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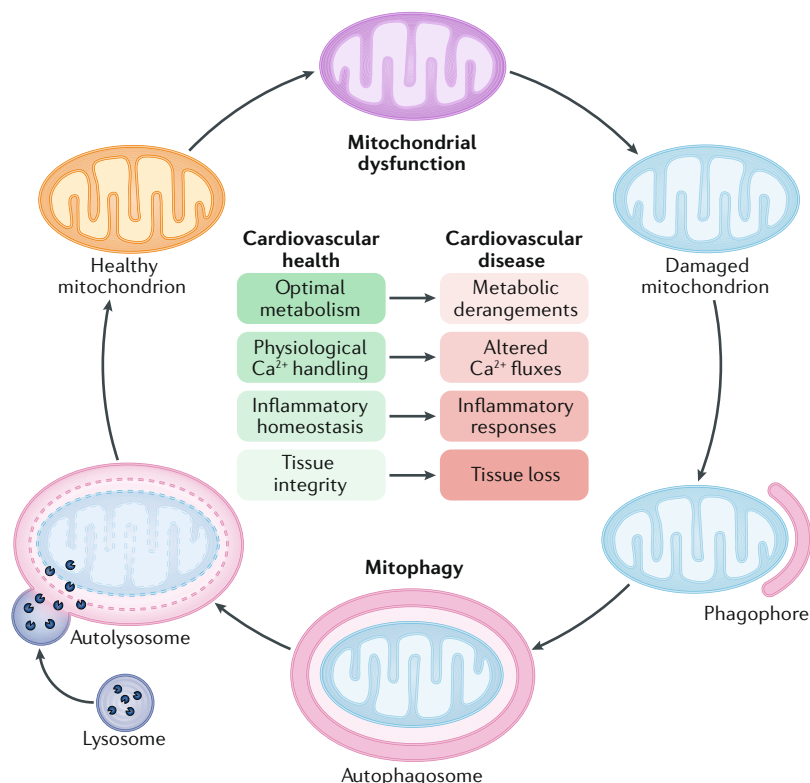
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**Fig. 1 | Contribution of mitochondrial dysfunction to cardiovascular disease.** In physiological conditions, healthy mitochondria support the functions of virtually all cells from the cardiovascular system by ensuring optimal catabolic and anabolic metabolism and regulating the intracellular trafficking of Ca<sup>2+</sup>. Additionally, an intact mitochondrial network promotes the preservation of inflammatory homeostasis and tissue integrity by preventing the activation of signal transduction cascades that lead to the release of pro-inflammatory factors and regulated cell death. In addition to being accompanied by metabolic derangements and alterations in intracellular Ca<sup>2+</sup> fluxes, mitochondrial dysfunction favours the establishment of an inflammatory milieu and facilitates regulated cell death, which culminates with tissue loss. By efficiently eliminating dysfunctional mitochondria that originate as a consequence of physiological cellular functions or accumulate in the context of pathological cues, mitophagy has a major role in the preservation of cardiovascular homeostasis.

**Folate cycle**  
Biochemical pathway catalysing the cyclic conversion of tetrahydrofolate, 10-formyl-tetrahydrofolate (which feeds into purine synthesis), 5,10-methylenetetrahydrofolate, and 5-methyl-tetrahydrofolate (which feeds into methionine metabolism).

**Mitochondrial permeability transition (MPT).** Rapid loss of the ionic barrier function of the inner mitochondrial membrane, culminating in mitochondrial breakdown and regulated necrosis.

conclusively confirm these observations<sup>34,35</sup>. Moreover, oral L-carnitine can be metabolized by the gut microbiota into trimethylamine N-oxide (TMAO), a pro-atherogenic molecule<sup>36</sup>. Accordingly, individuals with high L-carnitine levels and concurrently high TMAO levels in the blood are at increased risk of CVD and major adverse cardiac events<sup>36</sup>. Thus, the clinical development of L-carnitine for the treatment of CVD seems to be at an impasse.

The β-oxidation inhibitor etomoxir has also been investigated in patients with congestive HF, with inconclusive results<sup>37,38</sup>. Conversely, perhexiline and trimetazidine — which resemble etomoxir in their capacity to inhibit β-oxidation (although to different degrees) — are currently approved in multiple countries (including Australia and Canada) as antianginal agents<sup>39</sup>. The therapeutic efficacy of perhexiline and trimetazidine has been proposed not to reflect a switch from fatty acid-driven to glucose-driven catabolism<sup>40</sup> but instead to entail an entire rebalancing of carbon and nucleotide phosphate fluxes<sup>41</sup> linked to autophagy activation<sup>42</sup> (see below).

Perhexiline is also effective (at least to some extent) in a subset of patients with cardiomyopathy<sup>40,43</sup>, but not in patients with left ventricular hypertrophy undergoing cardiac surgery<sup>44,45</sup>. Trimetazidine has been tested in multiple cohorts of patients with distinct cardiovascular disorders beyond angina, with variable degrees of efficacy<sup>46–49</sup>. Nonetheless, in the USA (but not in other countries), the clinical development of perhexiline and trimetazidine has been discontinued, presumably owing to a fairly narrow therapeutic index<sup>39</sup>.

5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR; also known as acadesine) is an intermediate in the synthesis of inosine monophosphate that potently activates 5'-AMP-activated protein kinase (AMPK), a metabolic sensor regulating mitochondrial biogenesis, dynamics, and metabolism<sup>50</sup>. Despite some promising preliminary results<sup>51,52</sup>, the clinical development of acadesine as a cardioprotective intervention in patients undergoing CABG surgery has been abandoned, at least in part owing to the lack of long-term efficacy<sup>53</sup>. In summary, despite a robust rationale to target mitochondrial metabolism for the prevention or treatment of CVD, this therapeutic strategy remains largely unrealized.

**Sirtuins.** Sirtuins are a family of NAD<sup>+</sup>-dependent deacetylases and deacylases that control multiple aspects of cellular metabolism, including mitochondrial function and redox balance<sup>54</sup>. The mammalian genome encodes seven different sirtuins, three of which (SIRT3, SIRT4, and SIRT5) are localized to mitochondria<sup>54</sup>. Pharmacological sirtuin activation mediates lifespan-extending functions in multiple experimental models<sup>55–57</sup>, and defects in both mitochondrial and extramitochondrial sirtuins have been associated with a variety of cardiovascular disorders<sup>58</sup>. *Sirt1*<sup>-/-</sup> mice are viable but have considerable developmental heart defects<sup>59</sup>. In *Sirt1*<sup>+/-</sup> hearts, ischaemic preconditioning does not preserve cardiac function after ischaemia-reperfusion injury, potentially linked to hyperacetylation of cytosolic proteins and consequent inhibition of autophagy<sup>60,61</sup>, whereas myocardial *Sirt1* overexpression has cardioprotective effects along with deacetylation of cytoplasmic proteins<sup>60,62</sup>. *Sirt3*<sup>-/-</sup> mice show signs of cardiac hypertrophy and interstitial fibrosis at 8 weeks of age, spontaneously develop age-related cardiomyopathy, and are more sensitive than their wild-type littermates to hypertrophic stimuli, including aortic constriction<sup>63,64</sup>. Such a susceptibility to cardiac hypertrophy reflects, at least in part, an increased propensity of the *Sirt3*<sup>-/-</sup> myocardium to undergo regulated necrosis upon mitochondrial permeability transition (MPT) as a consequence of cyclophilin D (CypD; also known as PPIF) hyperacetylation<sup>63,64</sup> (see below). Conversely, transgenic *Sirt3* overexpression has robust cardioprotective effects in mice<sup>63</sup>. Similar results to those observed in *Sirt3*<sup>-/-</sup> mice have been obtained with *Sirt2*<sup>-/-</sup>, *Sirt5*<sup>-/-</sup>, *Sirt6*<sup>-/-</sup>, and *Sirt7*<sup>-/-</sup> mice, and as shown with *Sirt3* overexpression, overexpression of *Sirt2* specifically in the myocardium had cardioprotective effects<sup>65–68</sup>. By contrast, *Sirt4*<sup>-/-</sup> mice seem to be less susceptible to angiotensin-II-induced cardiac hypertrophy than their wild-type counterparts, whereas cardiomyocyte-specific

Table 1 | Genetic studies implicating mitochondrial functions in cardiovascular physiology in mice

Mouse model	Specificity	Phenotype	Refs
<i>Atg5</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• In adults or nonregulated</li> </ul>	Cardiac hypertrophy and contractile dysfunction leading to premature death, accompanied by pronounced mitochondrial defects	120,121
<i>Bnip3l</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Cardiac hypertrophy with reduced left ventricular contractile function at 60 weeks of age	119
<i>Bnip3l</i> <sup>-/-</sup> <i>Bnip3</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Cardiomyocytes (<i>Bnip3l</i><sup>-/-</sup>) and whole body (<i>Bnip3</i><sup>-/-</sup>)</li> <li>• Nonregulated</li> </ul>	Cardiac hypertrophy with reduced left ventricular contractile function at 30 weeks of age	119
<i>Dnm1l</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• In adults</li> </ul>	Lethal dilated cardiomyopathy associated with PARK2 accumulation, which can be partially rescued by deletion of <i>Park2</i>	117
DNM1L-C452F	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Monogenic dilated cardiomyopathy associated with considerable mitophagic defects	93
<i>Fbxo32</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Premature death due to cardiac degeneration associated with deficient autophagic responses	122
<i>Lamp2</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Vacuolar myopathy affecting cardiac and skeletal muscle, similar to Danon disease	123
<i>Mfn1</i> <sup>-/-</sup> <i>Mfn2</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• In adults</li> </ul>	Cardiomyocyte dysfunction associated with lethal dilated cardiomyopathy, attributed to defects in mitochondrial fusion	90,91
<i>Mfn1</i> <sup>-/-</sup> <i>Mfn2</i> <sup>-/-</sup> <i>Dnm1l</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• In adults</li> </ul>	Cardiac hypertrophy associated with accumulation of mitochondria and severely distorted sarcomeric architecture	91
<i>Mfn2</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• Nonregulated</li> </ul>	Progressive cardiomyopathy leading to premature death, associated with impaired cardiac contractility and insensitivity to β-adrenergic stimulation	94,95
MFN2-AA	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• At birth</li> </ul>	Perinatal cardiomyopathy leading to premature death owing to a failure in the switch from fetal to adult mitochondria in cardiomyocytes	97
miR-212–132 cluster overexpression	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• Nonregulated</li> </ul>	Cardiac hypertrophy leading to heart failure and premature death	296
<i>Park2</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• At birth</li> </ul>	Perinatal cardiomyopathy leading to premature death owing to a failure in the switch from fetal to adult mitochondria in cardiomyocytes	97
	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• In adults</li> </ul>	No obvious phenotype	117
<i>Pink1</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Left ventricular dysfunction and cardiac hypertrophy at 2 months of age	116
<i>Sirt1</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Developmental heart defect and perinatal lethality	59
<i>Sirt1</i> overexpression	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• Nonregulated</li> </ul>	Attenuated age-associated cardiac hypertrophy (with moderate <i>Sirt1</i> overexpression) or spontaneous cardiomyopathy (with robust <i>Sirt1</i> overexpression)	62
<i>Sirt5</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Spontaneous hypertrophic cardiomyopathy linked to increased protein succinylation and altered β-oxidation	66
<i>Sirt6</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• In adults</li> </ul>	Spontaneous cardiac hypertrophy and heart failure	67
<i>Sirt7</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Hypertrophy and inflammatory cardiomyopathy characterized by extensive fibrosis and associated with premature death	68
<i>Slc8b1</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• In adults</li> </ul>	Heart failure associated with left ventricular remodelling	177
<i>Tfrc</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• Nonregulated</li> </ul>	Cardiac hypertrophy and premature death, accompanied by defects in mitochondrial respiration and ineffective mitophagy	124
<i>Trp53</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Decelerated cardiac ageing associated with improved mitophagic responses	125
<i>Txnrd2</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• Nonregulated</li> </ul>	Fatal dilated cardiomyopathy	204
	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• In adults</li> </ul>	Accelerated cardiac ageing linked with dysregulated autophagy	205
<i>Yme1l</i> <sup>-/-</sup>	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• Nonregulated</li> </ul>	Spontaneous cardiomyopathy associated with mitochondrial hyperfragmentation	88

DNM1L, dynamin-1-like protein; MFN2, mitofusin 2; PARK2, parkin RBR E3 ubiquitin protein ligase.



overexpression of *Sirt4* reportedly mediates detrimental effects in this model<sup>69</sup>. However, these findings have not yet been confirmed. At least in part, the cardioprotective effects of sirtuin activation originate from an antioxidant transcriptional programme orchestrated by forkhead box protein O3A (FOXO3A; also known as FOXO3)<sup>63</sup>, proficient autophagic responses<sup>70</sup>, and potentially the inhibition of MPT-driven regulated necrosis<sup>64,71</sup> (see below). Thus, sirtuins support cardiac fitness by affecting mitochondrial functions.

Sirtuins are activated by caloric restriction, which is also a potent inducer of autophagy, and a vast amount of literature is available on the multipronged beneficial effects of caloric restriction on cardiovascular health in humans, at least part of which are thought to depend mechanistically on sirtuins<sup>72</sup>. Additional sirtuin activators include the rather nonspecific natural polyphenols butein, honokiol, piceatannol, quercetin, and resveratrol<sup>73,74</sup> as well as several synthetic sirtuin-activating compounds, including SRT1720, SRT2104, and SRT3025 (REF.<sup>57</sup>). All these molecules have been shown to mediate beneficial effects in rodent models of CVD, and both SRT1720 and SRT2104 extend mouse lifespan<sup>74–77</sup>. Similarly, dietary supplementation with nicotinamide mononucleotide (NMN; a precursor of NAD<sup>+</sup>) mediates potent cardioprotective effects in mouse models of cardiomyopathy and ischaemia–reperfusion injury via a SIRT1-dependent or SIRT3-dependent mechanism<sup>78–80</sup>. The capacity of dietary resveratrol to limit the incidence or severity of various cardiovascular disorders (mostly in the context of type 2 diabetes mellitus) has been investigated in multiple clinical trials<sup>81–83</sup>, with inconclusive findings (often due to problematic study design). Still, no fewer than 20 non-closed (status: not terminated, suspended, or withdrawn) clinical trials are currently registered at [clinicaltrials.gov](https://clinicaltrials.gov) to investigate dietary supplementation with resveratrol in individuals with age-associated morbidities (mostly type 2 diabetes) and cardiovascular conditions including non-ischaemic cardiomyopathy (NCT01914081), hypertension (NCT01842399), atherosclerosis (NCT02998918), and endothelial dysfunction (NCT02256540). Results from a small randomized clinical trial including 40 patients with psoriasis (NCT01154101) suggest that SRT2104 is well tolerated<sup>84</sup>. The safety of SRT3025 has been investigated in healthy volunteers (NCT01340911), but to the best of our knowledge the results of this study have not been disseminated. Finally, the effects of dietary NMN supplementation on cardiometabolic functions are currently being formally investigated (NCT03151239). Taken together, these observations suggest that, although multiple dietary interventions that activate sirtuins, including caloric restriction, resveratrol, and NMN (both of which are available over the counter), might mediate robust cardioprotective effects, additional clinical testing is required for the establishment of official treatment protocols enabling the use of these agents for the treatment of CVD.

**Mitochondrial dynamics.** The mitochondrial network constantly undergoes remodelling owing to the mutually antagonistic activity of multiple proteins that promote fission, such as mitochondrial fission factor (MFF),

mitochondrial fission 1 protein (FIS1), and dynamin-1-like protein (DNM1L), and fusion, such as mitofusin 1 (MFN1), MFN2, and optic atrophy protein 1 (OPA1)<sup>85</sup> (FIG. 2). This process is paramount for the preservation of optimal mitochondrial functions in both physiological and pathological conditions, at least in part because fission enables the mitophagic disposal of dysfunctional mitochondria<sup>86</sup>. Accordingly, multiple genetic defects impairing mitochondrial dynamics have been linked to CVD in experimental models.

The myocardium of *Opa1*<sup>+/-</sup> mice has clustered mitochondria with disorganized cristae and reduced mitochondrial DNA (mtDNA) content, and *Opa1*<sup>+/-</sup> mice are more susceptible to cardiac hypertrophy induced by transverse aortic constriction than their wild-type counterparts<sup>87</sup>. Cardiomyocyte-specific deletion of *Yme1l1* accelerates cardiac OPA1 proteolysis, thereby favouring mitochondrial hyperfragmentation and metabolic impairment, leading to HF<sup>88</sup>. Interestingly, angiotensin-II-induced cardiomyopathy leads to OPA1 acetylation and consequent mitochondrial fragmentation, a detrimental process that is inhibited by SIRT3 (REF.<sup>89</sup>). The co-deletion of *Mfn1* and *Mfn2* from adult cardiomyocytes imposes a robust defect in mitochondrial fusion that drives cardiac dysfunction associated with rapidly progressive (and ultimately lethal) dilated cardiomyopathy<sup>90</sup>. Such a detrimental phenotype cannot be fully rescued by the concomitant deletion of *Dnm1l*, but the cardiomyopathy manifesting in *Mfn1*<sup>-/-</sup>*Mfn2*<sup>-/-</sup>*Dnm1l*<sup>-/-</sup> hearts progresses with different kinetics than in *Mfn1*<sup>-/-</sup>*Mfn2*<sup>-/-</sup> hearts and mostly reflects a mitophagic blockage<sup>91</sup>. However, *Mfn1*<sup>-/-</sup>*Mfn2*<sup>-/-</sup> hearts have reduced sensitivity to ischaemia–reperfusion injury compared with their wild-type counterparts, potentially as a consequence of mitigated Ca<sup>2+</sup> overload<sup>92</sup> (see below).

Transgenic expression of DNM1L-C452F (a hyperactive DNM1L variant) also drives dilated cardiomyopathy accompanied by a considerable mitophagic defect<sup>93</sup>. Similarly, mouse *Mfn2*<sup>-/-</sup> hearts spontaneously develop dilated cardiomyopathy accompanied by mitochondrial hyperfragmentation, impaired contractile performance, and insensitivity to  $\beta$ -adrenergic stimulation<sup>94,95</sup>. Further corroborating the importance of mitochondrial fusion for the preservation of cardiovascular homeostasis, adenovirus-mediated delivery of *Mfn2* to the mouse myocardium inhibits angiotensin-II-induced cardiomyopathy<sup>96</sup>. Interestingly, transgene-driven overexpression of a non-phosphorylatable MFN2 variant (MFN2-AA) in the myocardium of newborn (but not adult) mice prevents normal mitochondrial maturation, accompanied by a switch from glucose-driven to fatty acid-driven metabolism, and leads to premature lethality, most probably as a consequence of impaired mitophagy<sup>97</sup> (see below). Of note, physiological DNM1L-dependent mitochondrial fragmentation is critical for cardiac adaptation to increased energy demands<sup>98</sup>. Moreover, conditional deletion of one copy of *Dnm1l* from the myocardium exacerbates pressure-overload-induced cardiomyopathy as well as ischaemia–reperfusion injury in mice as a consequence of mitophagy impairment<sup>99,100</sup>. Altogether, these observations suggest that a balanced interplay between fission

Table 2 | Genetic studies implicating mitochondrial functions in cardiovascular pathology in mice

Model	Specificity	Phenotype versus wild-type or control mice	Refs
<b>Atherosclerosis</b>			
<i>Atg5<sup>-/-</sup></i>	<ul style="list-style-type: none"> <li>• Monocytes</li> <li>• Nonregulated</li> </ul>	Accelerated atherosclerosis in mice fed a HFD and in <i>Ldlr<sup>-/-</sup></i> mice	127,128
<i>Il1r1<sup>-/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Reduced aortic atherosclerotic plaque areas in <i>Apoe<sup>-/-</sup></i> mice fed a HFD	245
<i>Il1m<sup>-/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Synergized with the <i>Apoe<sup>-/-</sup></i> genotype to cause aortic inflammation with destruction of the vascular architecture	246
<i>Il1m</i> overexpression	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Marked protection against atherosclerosis	246
<i>Parp1<sup>-/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Reduced aortic atherosclerotic plaque areas in <i>Apoe<sup>-/-</sup></i> mice fed a HFD	265
<i>Sod2<sup>+/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Accelerated progression of atherosclerosis in <i>Apoe<sup>-/-</sup></i> mice fed a HFD	202
<b>Cardiomyopathy</b>			
<i>Atg5<sup>+/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Aggravated angiotensin-II-induced cardiac hypertrophy	126
<i>Atg7</i> overexpression	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Decreased ventricular dysfunction and cardiac hypertrophy and improved survival in a model of desmin-related cardiomyopathy	133
<i>Becn1<sup>+/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Accelerated heart failure in a model of desmin-related cardiomyopathy	141
<i>Lclat1<sup>-/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Mitigated hypertrophic cardiomyopathy induced by thyroid hyperstimulation associated with improved mitophagic flux	136
<i>Ppif<sup>-/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Protection against angiotensin-II-induced cardiac hypertrophy	261
<i>Sirt2<sup>-/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Aggravated angiotensin-II-induced cardiac hypertrophy	65
<i>Sirt2</i> overexpression	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• Nonregulated</li> </ul>	Mitigated angiotensin-II-induced cardiac hypertrophy	65
<i>Sirt3<sup>-/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Aggravated angiotensin-II-induced cardiac hypertrophy	63
<i>Sirt3</i> overexpression	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• Nonregulated</li> </ul>	Mitigated angiotensin-II-induced cardiac hypertrophy	63
<i>Sirt4<sup>-/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Limited angiotensin-II-induced cardiac hypertrophy	69
<i>Sirt4</i> overexpression	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• In adults</li> </ul>	Aggravated angiotensin-II-induced cardiac hypertrophy	69
<b>Cardiotoxicity</b>			
<i>Becn1<sup>+/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Reduced pathological cardiac remodelling after chronic doxorubicin administration	144
	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• Nonregulated</li> </ul>	Accelerated decline in ventricular systolic function after chronic doxorubicin administration	144
<i>Ripk3<sup>-/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Protected against doxorubicin-driven heart failure, coupled with impaired CaMKII activation and MPT desensitization	266
<i>Trp53<sup>-/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Reduced sensitivity to doxorubicin, might be mediated by reduced mitophagic responses	125
<b>Myocardial infarction</b>			
<i>Bcl2</i> overexpression	<ul style="list-style-type: none"> <li>• Cardiomyocytes</li> <li>• Nonregulated</li> </ul>	Reduced infarct size after I/R injury	258
<i>Becn1<sup>+/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Reduced cardiac damage at reperfusion	143
<i>Cgas<sup>-/-</sup></i>	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Improved survival after I/R injury, coupled with diminished pathological remodelling, enhanced angiogenesis, and preserved ventricular contractile function	240
	<ul style="list-style-type: none"> <li>• Whole body</li> <li>• Nonregulated</li> </ul>	Attenuated decline in cardiac function coupled with decreased production of inflammatory cytokines and chemokines and decreased inflammatory cell infiltration into the myocardium after left coronary artery ligation	242

Table 2 (cont.) | Genetic studies implicating mitochondrial functions in cardiovascular pathology in mice

Model	Specificity	Phenotype versus wild-type or control mice	Refs
<b>Myocardial infarction (cont.)</b>			
<i>Dnm1</i> <sup>+/-</sup>	• Cardiomyocytes • Nonregulated	Exacerbated heart failure associated with defective mitophagy and mitochondrial dysfunction after transverse aortic constriction	99
	• Cardiomyocytes • In adults	Impaired autophagy and reduced left ventricular function after I/R injury	100
<i>Ifnar1</i> <sup>-/-</sup>	• Whole body • Nonregulated	Cardioprotective phenotype resembling that caused by the <i>Cgas</i> <sup>-/-</sup> genotype	242
<i>Irf3</i> <sup>-/-</sup>	• Whole body • Nonregulated	Cardioprotective phenotype resembling that caused by the <i>Cgas</i> <sup>-/-</sup> genotype	242
<i>Mcu</i> <sup>-/-</sup>	• Cardiomyocytes • In adults	Protected against Ca <sup>2+</sup> overload-driven MPT, decreased infarct size, and preserved cardiac function	174,175
	• Whole body • Nonregulated	Abolished sensitivity to cyclosporine A after I/R injury, with minimal effects on cardiac function	173
MCU DN	• Cardiomyocytes • Nonregulated	Preserved $\Delta\Psi_m$ and limited ROS generation after I/R injury, but did not mediate overt cardioprotection	173,176
<i>Mfn1</i> <sup>-/-</sup> <i>Mfn2</i> <sup>-/-</sup>	• Cardiomyocytes • In adults	Reduced infarct size along with a decrease in mitochondrial Ca <sup>2+</sup> overload and ROS generation	92
<i>miR-150</i> <sup>-/-</sup>	• Whole body • Nonregulated	Cardioprotection associated with reduced expression of genes associated with RCD and inflammation	300
<i>Slc8b1</i> overexpression	• Cardiomyocytes • In adults	Reduced sensitivity to heart failure after I/R injury, at least partially dependent on reduced propensity to MPT	177
<i>Opa1</i> <sup>+/-</sup>	• Whole body • Nonregulated	Increased cardiac hypertrophy after transverse aortic constriction, associated with altered ejection fraction	87
<i>Parp1</i> <sup>-/-</sup>	• Whole body • Nonregulated	Decreased myocardial damage linked to reduced NF- $\kappa$ B signalling and general protection against RCD	264
<i>Pgam5</i> <sup>-/-</sup>	• Whole body • Nonregulated	Increased infarct size, correlating with inhibition of mitophagy and necrotic RCD	139
<i>Ppif</i> <sup>-/-</sup>	• Whole body • Nonregulated	Reduced sensitivity to I/R injury, mechanistically associated with reduced propensity to MPT-driven regulated necrosis	259,260
<i>Rheb</i> overexpression	• Cardiomyocytes • Nonregulated	Increased infarct size, which could be reversed by systemic administration of rapamycin	129
<i>Ripk3</i> <sup>-/-</sup>	• Whole body • Nonregulated	Protected against heart failure after I/R injury, coupled with impaired CaMKII activation and MPT desensitization	266
<i>Sirt1</i> <sup>+/-</sup>	• Whole body • Nonregulated	Impaired IPC associated with hyperacetylation of cytoplasmic proteins and consequent autophagy inhibition	60,62
<i>Sirt1</i> overexpression	• Whole body • Nonregulated	Cardioprotection associated with deacetylation of cytoplasmic proteins and consequent autophagy activation	60,62
<i>Sirt3</i> <sup>-/-</sup>	• Whole body • Nonregulated	Aggravated cardiac hypertrophy induced by transverse aortic constriction, potentially linked to MPT sensitization	64
<i>Sirt6</i> overexpression	• Cardiomyocytes • Nonregulated	Inhibited cardiac hypertrophy induced by transverse aortic constriction, potentially linked to MPT desensitization	67
<i>Stk4</i> <sup>-/-</sup>	• Whole body • Nonregulated	Cardioprotection coupled to increased autophagic responses in the heart	135
<b>Pressure overload</b>			
<i>Atg5</i> <sup>-/-</sup>	• Cardiomyocytes • Nonregulated	Increased sensitivity to pressure overload	120
<i>Becn1</i> <sup>+/-</sup>	• Whole body • Nonregulated	Reduced pathological cardiac remodelling	142
	• Whole body • Nonregulated	Aggravated pathological cardiac remodelling	142
<i>Bnip3l</i> <sup>-/-</sup> <i>Bnip3</i> <sup>-/-</sup>	• Cardiomyocytes ( <i>Bnip3l</i> <sup>-/-</sup> ) and whole body ( <i>Bnip3</i> <sup>-/-</sup> ) • Nonregulated	Rapid functional cardiac decompensation	119
<i>Camk2a</i> <sup>-/-</sup>	• Cardiomyocytes • Nonregulated	Reduced ROS generation coupled with RCD inhibition and preserved systolic function	182
<i>Dnase2a</i> <sup>-/-</sup>	• Cardiomyocytes • Nonregulated	Severe myocarditis and dilated cardiomyopathy associated with premature death	131

$\Delta\Psi_m$ , mitochondrial transmembrane potential; CaMKII, calcium/calmodulin-dependent protein kinase II; DN, dominant-negative; HFD, high-fat diet; IPC, ischaemic preconditioning; I/R, ischaemia-reperfusion; MCU, calcium uniporter protein, mitochondrial; MPT, mitochondrial permeability transition; NF- $\kappa$ B, nuclear factor- $\kappa$ B; RCD, regulated cell death; ROS, reactive oxygen species; SIRT3, sirtuin 3.

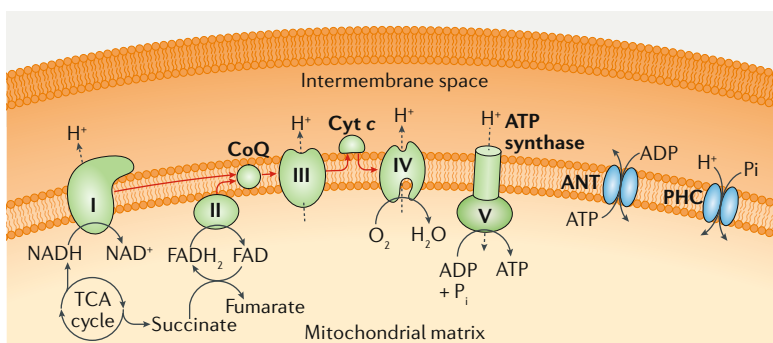
and fusion is paramount for cardiovascular health as it preserves mitochondrial fitness in both physiological and pathological conditions. Further corroborating this notion, the levels of various factors involved in the regulation of mitochondrial dynamics, including FIS1, MFN2, and OPA1, are altered in the course of CVD<sup>101–103</sup>. Of note, MFN2 is also aetiologically involved in the proliferative arrest and death of vascular smooth muscle cells elicited by oxidative stress in rats<sup>104</sup>. In line with this notion, transgene-driven *Mfn2* overexpression reportedly prevents vascular smooth muscle cell proliferation and restenosis in rat models of

arterial injury induced by balloon denudation of the left common carotid artery<sup>105</sup>. However, these effects seem to be independent of the role of MFN2 in the regulation of mitochondrial dynamics<sup>104</sup>.

The chemical DNM1L inhibitor mdivi-1 mediates cardioprotective effects in rodent models of cardiac ischaemia–reperfusion injury<sup>106–108</sup> and cardiomyopathy<sup>109,110</sup>, but the specificity of mdivi-1 has been questioned<sup>111</sup>. Nonetheless, similar observations have been made with other DNM1L inhibitors such as P110 (REFS<sup>112,113</sup>) and dynasore<sup>114</sup>. A cell-permeant peptide enabling MFN2-dependent mitochondrial fusion has also been developed<sup>115</sup>, but its biological activity in the cardiovascular system remains to be investigated. To the best of our knowledge, none of these agents has been tested in clinical settings thus far.

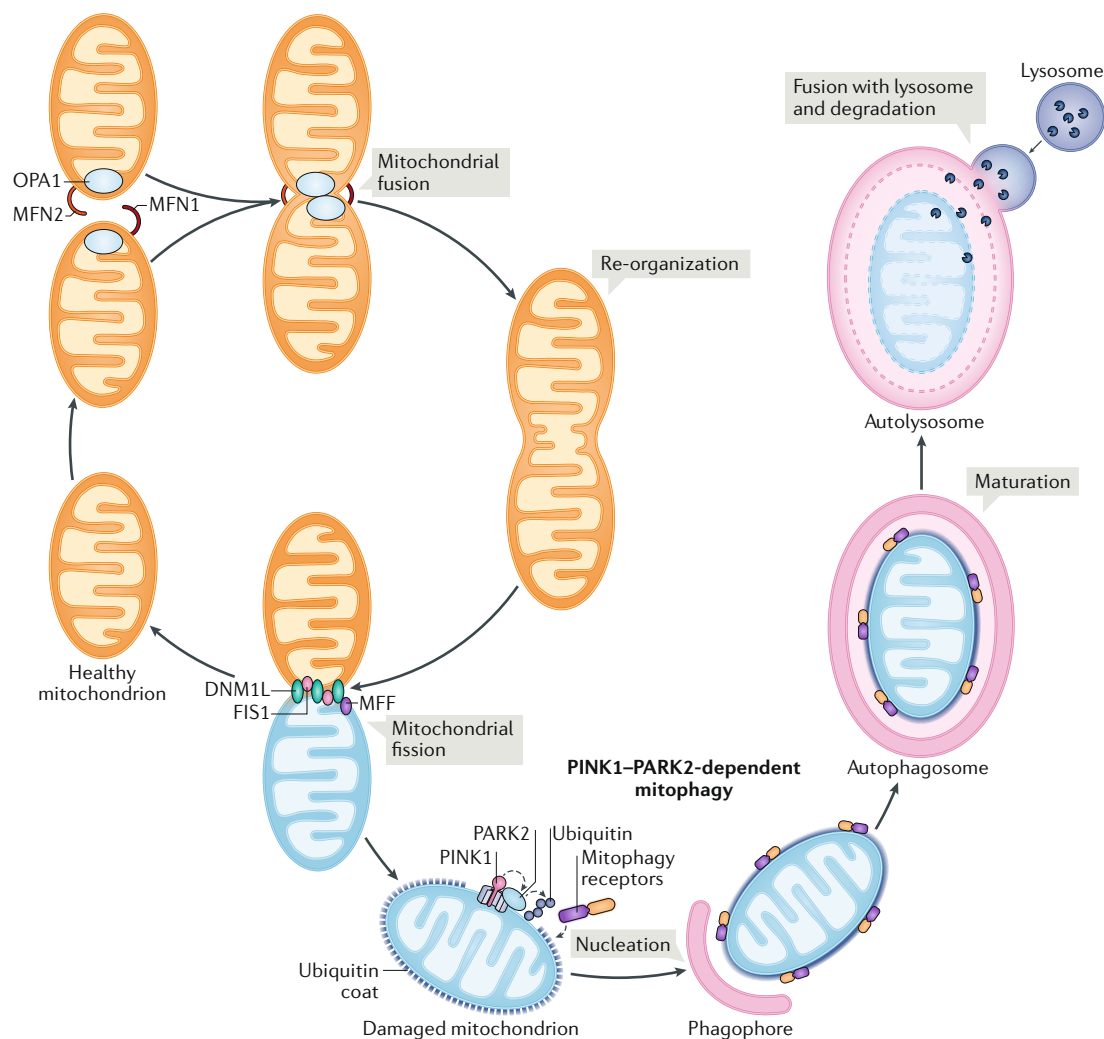
### Box 1 | Principles of oxidative phosphorylation

Oxidative phosphorylation is a core bioenergetic process whereby reducing equivalents present in the mitochondrial matrix are sequentially used by four multiprotein complexes (generally referred to as respiratory complexes I–IV) and two electron shuttles (namely, coenzyme Q (CoQ) and cytochrome c (Cyt c)) to generate an electrochemical H<sup>+</sup> gradient across the inner mitochondrial membrane that is harnessed in a controlled manner by the F<sub>1</sub>F<sub>o</sub> ATP synthase (also known as respiratory complex V) to catalyse the phosphorylation of ADP into ATP. The main substrates for oxidative phosphorylation are NADH, which provides electrons to complex I (also known as NADH dehydrogenase), and succinate, which provides electrons to complex II (also known as succinate dehydrogenase) via FADH<sub>2</sub>. Accordingly, FADH<sub>2</sub> can also fuel oxidative phosphorylation at the level of complex II. Both complex I and II deliver electrons to complex III (also known as CoQ:Cyt c oxidoreductase) via CoQ. However, only complex I transfers electrons onto complex III while also extruding H<sup>+</sup> ions from the mitochondrial matrix to the intermembrane space. Complex III transfers electrons to complex IV (also known as Cyt c oxidase) via Cyt c, culminating with the reduction of O<sub>2</sub> into H<sub>2</sub>O. This last step is the reason why O<sub>2</sub> is critical for oxidative phosphorylation. Both complex III and complex IV directly contribute to the generation of the mitochondrial transmembrane potential ( $\Delta\psi_m$ ). Finally, the F<sub>1</sub>F<sub>o</sub> ATP synthase uses a well-described rotatory mechanism to dissipate the  $\Delta\psi_m$  in a controlled manner, coupled with phosphorylation of ADP into ATP. This reaction requires ADP and inorganic phosphate (P<sub>i</sub>), which are provided by the permeability transition pore components adenine nucleotide translocator (ANT) and phosphate carrier (PHC; also known as SLC25A3), respectively (see the figure; please note that stoichiometry is not respected for the sake of simplification). Importantly, the reaction catalysed by the F<sub>1</sub>F<sub>o</sub> ATP synthase is reversible. This reversibility implies that in ischaemic conditions the capacity of oxidative phosphorylation to drive ATP synthesis is impaired, owing to limited oxygen availability, and that high amounts of ATP are consumed by the F<sub>1</sub>F<sub>o</sub> ATP synthase to preserve the  $\Delta\psi_m$ . All metabolic intermediates entering the tricarboxylic acid (TCA) cycle, including (but not limited to) glucose-derived pyruvate and branched-chain amino acid-derived and fatty acid-derived acetyl-CoA and succinyl-CoA, can drive the synthesis of NADH and succinate in the mitochondrial matrix, thereby supporting oxidative phosphorylation. Fatty acid oxidation also supports oxidative phosphorylation via FADH<sub>2</sub> synthesis. Of note, the cellular efficiency of oxidative phosphorylation depends on a variety of parameters, including the number of mitochondria per cell and their fragmentation state, the amount of respiratory complexes per mitochondrion, the supramolecular organization of respiratory complexes, substrate and O<sub>2</sub> availability, the expression of endogenous inhibitors, and local redox and pH conditions<sup>339,340</sup>.



**Mitophagy.** Mitophagy constitutes a pillar in the maintenance of mitochondrial homeostasis in both the healthy and diseased cardiovascular system<sup>56</sup>. Accordingly, multiple defects in the molecular apparatus underlying proficient mitophagic responses have been associated with spontaneous CVD in experimental models<sup>17</sup>. *Pink1*<sup>-/-</sup> mice (lacking a kinase involved in the recognition of depolarized mitochondria) develop left ventricular dysfunction and cardiac hypertrophy by 2 months of age<sup>116</sup>. Deletion of *Park2* (also known as *Prkn*; encoding parkin RBR E3 ubiquitin protein ligase, a functional mitochondrial interactor of serine/threonine protein kinase PINK1, which is required for multiple variants of mitophagy) from the myocardium of adult mice causes a very mild cardiac phenotype in unstressed animals<sup>117</sup>. Conversely, *Park2* ablation from the myocardium of neonate mice causes premature and rapidly lethal cardiomyopathy associated with failed mitochondria maturation (strikingly similar to the phenotype associated with MFN2-AA expression)<sup>97</sup>. Similarly, knockout of *park* (the fly orthologue of *Park2*) in *Drosophila melanogaster* causes dilated cardiomyopathy that can be rescued by cardiomyocyte-specific re-expression of *park*<sup>95,118</sup>. *Bnip3l*<sup>-/-</sup> mice lack a core component of the molecular apparatus for mitophagy and spontaneously develop cardiomegaly and contractile depression by 60 weeks of age, a pathological phenotype that is further accelerated by the concomitant deletion of *Bnip3* (coding for yet another protein involved in mitophagy)<sup>119</sup>. Genetic defects affecting autophagy also compromise cardiovascular homeostasis owing to the accumulation of dysfunctional mitochondria. This observation holds true for: cardiomyocyte-specific deletion of *Atg5* in adult mice, which causes lethal cardiac hypertrophy accompanied by disorganized sarcomere structure as well as mitochondrial misalignment and aggregation<sup>120,121</sup>; whole-body deletion of *Fbxo32* in mice, which is associated with premature death owing to cardiac degeneration associated with deficient autophagic responses<sup>122</sup>; and the *Lamp2*<sup>-/-</sup> genotype, which causes a major lysosomal dysfunction that, in mice, drives a vacuolar myopathy that affects cardiac and skeletal muscles, resembling Danon disease<sup>123</sup>. Of note, multiple genetic and pharmacological interventions that impair mitochondrial dynamics impose at least some degree of mitophagic





**Fig. 2 | Overview of mitochondrial dynamics.** The mitochondrial network is constantly reshaped by the antagonistic activity of proteins that mediate fission, such as mitochondrial fission factor (MFF), mitochondrial fission 1 protein (FIS1), and dynamin 1-like protein (DNM1L), and proteins that promote fusion, such as mitofusin 1 (MFN1), MFN2, and optic atrophy protein 1 (OPA1). One of the essential roles of fission is to segregate dysfunctional mitochondria, thereby enabling their uptake by the autophagic machinery and consequent degradation in lysosomes. PARK2, parkin RBR E3 ubiquitin protein ligase; PINK1, PTEN-induced putative kinase 1.

incompetence<sup>86</sup>. These two processes are so intimately interconnected that mechanistically ascribing the phenotype to either of the alterations is difficult. Additional genetic alterations that trigger CVD in rodents, such as cardiac deletion of *Tfrc* (coding for the transferrin receptor)<sup>124</sup>, are associated with mitophagic defects. Moreover, genetic defects that improve mitophagic proficiency, such as whole-body absence of *Trp53* (also known as *Tp53*; coding for a master regulator of cellular biology that inhibits autophagy in physiological settings), decelerate spontaneous cardiac ageing<sup>125</sup>. Taken together, these observations exemplify the critical role of mitophagy in the preservation of physiological cardiovascular homeostasis. That said, *Park2* deletion seems to rescue, at least in part, the lethal phenotype of *Dnm1l* deletion in the adult myocardium<sup>117</sup>, suggesting a role for uncontrolled mitophagy in the detrimental phenotype imposed by defects in mitochondrial fission (see above).

Multiple genetic defects impairing mitophagic proficiency aggravate disease severity in experimental models of CVD<sup>17</sup>. *Bnip3l*<sup>-/-</sup>*Bnip3*<sup>-/-</sup> hearts are highly sensitive to decompensation induced by pressure overload<sup>119</sup>. Homozygous or heterozygous deletion of *Atg5* from the mouse myocardium exacerbates cardiomyopathy driven by pressure overload<sup>120</sup> and angiotensin II administration<sup>126</sup>. Similarly, mice bearing *Atg5*<sup>-/-</sup> monocytes are more susceptible to develop atherosclerotic lesions in response to a high-fat diet or *Ldlr* deletion than mice with wild-type monocytes<sup>127,128</sup>. Mice engineered to overexpress *Rheb*, which encodes the endogenous autophagy inhibitor RAS homologue enriched in brain (RHEB), in the myocardium are more susceptible to cardiac ischaemia-reperfusion injury than wild-type mice, a detrimental phenotype that can be partially rescued by administration of the pharmacological autophagy activator rapamycin<sup>129,130</sup>. *Dnase2a*<sup>-/-</sup> mice, which lack a lysosomal nuclease (deoxyribonuclease 2α) that is involved

#### Transferrin

Iron-binding plasma glycoprotein that controls the level of free iron ions in biological fluids.

in the autophagic degradation of mtDNA released upon mitochondrial damage, are extremely sensitive to pressure-overload-induced cardiomyopathy, at least in part owing to exaggerated inflammatory responses in the myocardium<sup>131</sup> (see below). Interestingly, cathelicidin antimicrobial peptide (CAMP) can bind mtDNA to limit its degradation by DNase 2 $\alpha$  (DNASE2 $\alpha$ ), which has been associated with exacerbated atherosclerosis in *ApoE*<sup>-/-</sup> mice<sup>132</sup>.

Whole-body overexpression of *Atg7* (encoding a core component of the autophagic machinery) restrains cardiac hypertrophy and extends survival in a mouse model of desmin-related cardiomyopathy<sup>133</sup>. The *Trp53*<sup>-/-</sup> genotype limits both ischaemia–reperfusion injury and doxorubicin cardiotoxicity in mice, potentially owing to reduced myocardial susceptibility to RCD (see RCD section below), and improved mitophagy<sup>125,134</sup>. Multiple other genetic alterations that mediate beneficial effects in experimental models of CVD are associated with superior mitophagic responses (although precise mechanistic links are missing), including the *Stk4*<sup>-/-</sup> genotype, which limits cardiac ischaemia–reperfusion injury<sup>135</sup>, and the whole-body deletion of *Lclat1*, which mitigates hypertrophic cardiomyopathy induced by thyroid hyperstimulation<sup>136</sup>. Moreover, multiple cardioprotective interventions including hypothermia and the administration of glucagon-like peptide 1 receptor (GLP1R) agonists have been shown to promote autophagy (at least in some cell types), correlating with reduced amounts of RCD<sup>137,138</sup>. Conversely, *Pgam5*<sup>-/-</sup> mice are more susceptible to cardiac ischaemia–reperfusion injury than their wild-type littermates along with a whole-body defect in mitophagy, potentially linked to the capacity of phosphoglycerate mutase family member 5 (PGAM5) to regulate DNML-dependent fission<sup>139</sup>. Similarly, mice with an endothelial cell-specific deletion of *Pdcd10* spontaneously develop a syndrome resembling cerebral cavernous malformations, accompanied by robust autophagic defects<sup>140</sup>. Thus, the optimal elimination of damaged mitochondria by mitophagy is fundamental for the cardiovascular system to control potentially pathogenic challenges.

Interestingly, the role of beclin 1 (BECN1), a core component of the autophagic machinery that participates in multiple instances of mitophagy<sup>7</sup>, in the preservation of cardiovascular homeostasis in pathological settings is rather controversial. Indeed, whereas BECN1 has been attributed a cardioprotective role in some models of CVD<sup>99,141</sup>, *Becn1*<sup>+/-</sup> rodents consistently exhibited low sensitivity to potentially cardiotoxic challenges<sup>142–144</sup>. Although the reasons for this apparent discrepancy remain to be formally elucidated, linking them to emerging autophagy-independent functions of BECN1 in RCD regulation is tempting<sup>145</sup>. Further corroborating the critical role of mitophagy in cardiovascular homeostasis, ischaemic preconditioning has been associated with the translocation of PARK2 to depolarized mitochondria and consequent initiation of their autophagic disposal<sup>146</sup>. Moreover, the expression levels of components of the mitophagic apparatus such as PINK1 decrease in patients with CVD<sup>116</sup>, and HF is more frequent in individuals with mitophagy defects (as in patients with Parkinson disease)<sup>147</sup>.

Sirtuin activators such as caloric restriction and resveratrol are potent activators of autophagy, adding to multiple lines of evidence intimately linking the sirtuin system and autophagic responses. Additional pharmacological agents that promote mitophagy or autophagy have been shown to mediate beneficial effects in rodent models of CVD<sup>17</sup>. These include the natural polyamine spermidine, an inhibitor of the acetyltransferase E1A-associated protein p300 (EP300)<sup>148–150</sup>, and the natural macrolide rapamycin (also known as sirolimus), which inhibits the master suppressor of autophagy mechanistic target of rapamycin (mTOR)<sup>151–154</sup>. Conversely, systemic administration of nonspecific inhibitors of autophagy such as 3-methyladenine, which targets multiple variants of phosphatidylinositol 3-kinase (PI3K), and bafilomycin A1, which suppresses lysosomal functions, generally increases disease severity in rodent models of CVD, including ischaemia–reperfusion injury<sup>153,155,156</sup>. Interestingly, sirolimus is largely employed in drug-eluting stents to prevent restenosis after percutaneous coronary intervention<sup>157</sup>. Although this use originated from the potent antiproliferative and anti-inflammatory activity of sirolimus<sup>158</sup>, it cannot be excluded that the therapeutic benefits of this strategy involve, at least in part, the induction of autophagy, which reportedly stimulates the degradation of oxidized LDL<sup>159</sup> and might also favour the clearance of macrophages from the atherosclerotic plaque<sup>160,161</sup>. Moreover, multiple FDA-approved agents that mediate beneficial effects on the cardiovascular system, including aspirin (which is widely used as an anti-inflammatory and anticoagulant)<sup>162</sup>, statins (which are currently used to lower circulating levels of cholesterol and triglycerides)<sup>163</sup>, and suberanilohydroxamic acid (SAHA; a histone deacetylase inhibitor used for the treatment of cutaneous T cell lymphoma)<sup>164,165</sup>, trigger proficient autophagic responses in the myocardium.

Despite the robust links between mitophagy and/or autophagy activation and improved cardiovascular homeostasis in health and disease, targeting the underlying molecular apparatus with specific pharmacological intervention has proved to be challenging<sup>130</sup>. Accordingly, no clinical trials are currently investigating the therapeutic potential of mitophagy and/or autophagy modulators beyond caloric restriction and sirolimus in patients with CVD.

**Ca<sup>2+</sup> homeostasis.** In cardiomyocytes, mitochondria participate (to some extent) in the buffering of cytosolic Ca<sup>2+</sup> ions. Depolarization of the plasma membrane activates voltage-dependent L-type Ca<sup>2+</sup> channels, and Ca<sup>2+</sup> enters into the cytosol, which causes Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release from the sarcoplasmic reticulum via ryanodine receptor 2 (RYR2); Ca<sup>2+</sup> is removed from the cytosol predominantly by members of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) family and by solute carrier family 8 member A1 (SLC8A1; also known as NCX1)<sup>166</sup>. In physiological conditions, mitochondrial Ca<sup>2+</sup> uptake is mediated by calcium uniporter protein, mitochondrial (MCU)<sup>167,168</sup>. Conversely, Ca<sup>2+</sup> efflux from the mitochondrial matrix relies primarily on the Na<sup>+</sup>/Ca<sup>2+</sup> antiporter SLC8B1

#### Cerebral cavernous malformations

Cerebrovascular disease characterized by enlarged and leaky capillaries that predispose to seizures, focal neurological deficits, and fatal intracerebral haemorrhages.

#### Histone deacetylase inhibitor

Member of a fairly new class of targeted anticancer agents that operate by derepressing histone acetylation, resulting in the epigenetic reconfiguration of multiple transcriptional modules.

(also known as NCLX)<sup>169</sup>. Although mild, transient elevations of mitochondrial Ca<sup>2+</sup> levels support oxidative phosphorylation and ATP synthesis<sup>170</sup>, persistent Ca<sup>2+</sup> overload favours MPT<sup>171</sup>. In line with this notion, the transgene-driven overexpression of a leaky variant of RYR2 in the mouse myocardium exacerbates the cardiotoxic effects of ischaemia–reperfusion injury and causes mitochondrial Ca<sup>2+</sup> overload in cardiomyocytes<sup>172</sup>. Moreover, in multiple cell types, including cardiomyocytes, MCU deficiency confers resistance to MPT driven by mitochondrial Ca<sup>2+</sup> overload<sup>173,174</sup>, and the conditional deletion of *Mcu* from adult cardiomyocytes mediates cardioprotective effects against ischaemia–reperfusion injury in vivo<sup>174,175</sup>. However, the hearts from *Mcu*<sup>-/-</sup> mice, as well as mouse hearts expressing a dominant-negative variant of MCU, are as susceptible to ischaemia–reperfusion injury ex vivo as their wild-type counterparts<sup>173,176</sup>. The reasons underlying this apparent discrepancy remain to be elucidated. As a possibility, the contribution of mitochondrial Ca<sup>2+</sup> overload to MPT might be limited when ischaemia–reperfusion injury is imposed ex vivo. Irrespective of this conundrum, MCU seems to be required for optimal cardiac responses to acute physical demands<sup>174,175</sup>. Importantly, deletion of *Slc8b1* from adult mouse cardiomyocytes provokes sudden death as a consequence of mitochondrial Ca<sup>2+</sup> overload leading to widespread MPT-driven necrosis of the myocardium<sup>177</sup>. Conversely, *Slc8b1* overexpression mediates robust cardioprotection in mouse models of cardiac ischaemia–reperfusion injury<sup>177</sup>. These observations exemplify the importance of mitochondrial Ca<sup>2+</sup> fluxes for cardiovascular homeostasis in health and disease.

Further corroborating the crucial role for intracellular Ca<sup>2+</sup> homeostasis in cardiac physiology, genetic defects in plasma membrane L-type Ca<sup>2+</sup> channels are known to impair cardiac signal conduction, potentially favouring the development of arrhythmia<sup>178</sup>. Moreover, hyperactivation of the cytosolic Ca<sup>2+</sup>-responsive enzyme calcium/calmodulin-dependent protein kinase II (CaMKII) has been aetiologically linked to a variety of cardiovascular disorders, often reflecting the ability of CaMKII to regulate mitochondrial functions. Mice engineered to overexpress an endogenous inhibitor of CaMKII in cardiomyocytes are protected from ischaemia–reperfusion injury in vivo<sup>179</sup>, presumably reflecting the capacity of CaMKII to trigger MCU-dependent mitochondrial Ca<sup>2+</sup> overload, blunt antioxidant defences, and trigger DNM1L-dependent mitochondrial fragmentation<sup>179–182</sup>. Deletion of *Camk2d* (encoding one of the CaMKII subunits) attenuates pathological maladaptation in a genetic mouse model of decompensating cardiac hypertrophy<sup>182</sup>. Moreover, CaMKII seems to participate in the pathogenesis of atherosclerotic plaques<sup>183</sup>, although the underlying molecular mechanisms remain to be unveiled.

Although pharmacological regulators of cellular Ca<sup>2+</sup> homeostasis are commonly available for the treatment of some cardiovascular disorders (for example, verapamil, a blocker of plasma membrane Ca<sup>2+</sup> channels used virtually worldwide for the treatment of arrhythmia and some forms of hypertension)<sup>184</sup>, mitochondrial Ca<sup>2+</sup> fluxes have

been rather elusive drug targets. NCLX inhibitors such as CGP-37157, KB-R7943, and SEA0400 mediate promising cardioprotective effects in animal models of HF<sup>169,185</sup>. These results are at odds with the findings obtained with *Nclx*<sup>-/-</sup> mice<sup>177</sup>, most likely reflecting the capacity of chemical NCLX inhibitors such as CGP-37157 to preserve redox homeostasis<sup>169</sup>. That said, NCLX inhibitors never entered clinical development, presumably owing to specificity issues, because these compounds also inhibit the plasma membrane Na<sup>+</sup>/Ca<sup>2+</sup> antiporter SLC8A1<sup>186</sup>. Chemical inhibitors of MCU including DS16570511 have also been identified<sup>187</sup>, but whether MCU inhibition constitutes a valid therapeutic objective for the treatment of CVD remains controversial. Supporting caution over this approach, the anticancer agent mitoxantrone, which is associated with robust cardiotoxic effects in some patients, potently inhibits MCU (potentially contributing the adverse effects of this chemotherapeutic)<sup>188</sup>. The necroptosis inhibitor NecroX-5 has also been suggested to mediate beneficial effects via MCU inhibition<sup>189</sup>, but the specificity of this molecule remains to be determined. Finally, a panel of CaMKII inhibitors is available for investigational purposes, including competitive and noncompetitive inhibitors of ATP or substrate binding, agents that disrupt calmodulin binding, and agents that mimic endogenous CaMKII blockers<sup>190</sup>. Although many of these agents mediate consistent beneficial effects in animal models of CVD (reviewed previously)<sup>190</sup>, none of them has entered clinical development.

**Oxidative stress.** Mitochondria generate reactive oxygen species (ROS) as a normal by-product of oxidative phosphorylation, and physiological ROS levels regulate multiple cardiovascular processes, including (but not limited to) metabolic functions in the myocardium and endothelial permeability in vessels<sup>191</sup>. However, mitochondrial dysfunction is generally associated with massive ROS overgeneration (BOX 2), which (especially when cellular antioxidant defences are lowered) causes oxidative damage to macromolecules, thereby favouring the establishment of local inflammation<sup>2</sup> and initiating multiple variants of RCD including MPT-driven regulated necrosis and ferroptosis<sup>171,192</sup>. The human failing myocardium reportedly has more than twofold higher levels of superoxide anion than the healthy myocardium<sup>193</sup>. Similar observations have been made in the context of diabetic and hypertensive cardiomyopathy<sup>194,195</sup>. Moreover, markers of oxidative damage to lipids<sup>196</sup>, nucleic acids<sup>197</sup>, and proteins<sup>198</sup> have been documented in the circulation or in the myocardial tissue of patients with MI or HF (and in animal models of these conditions)<sup>199</sup>. Finally, myocardial mitochondria exhibit increased oxidative damage in aged versus young rats<sup>200</sup>, and the mitochondrial network of rat endothelial cells produces increased levels of H<sub>2</sub>O<sub>2</sub> with ageing<sup>201</sup>. These observations suggest that oxidative stress is involved in multiple forms of CVD, including ageing-associated cardiovascular disorders. Corroborating an aetiological role for ROS overproduction in at least some variants of CVD, the absence of one copy of *Sod2* (which encodes a mitochondrial superoxide dismutase) aggravates atherosclerosis

#### Necroptosis

Form of RCD that depends on mixed lineage kinase domain-like protein (MLKL), receptor-interacting serine/threonine-protein kinase 3 (RIPK3), and, at least in some settings, the kinase activity of the RIPK3 homologue RIPK1.

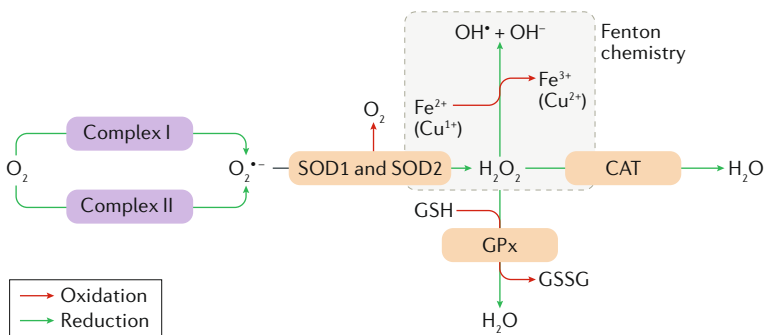
#### Ferroptosis

Iron-dependent form of RCD that obligatorily relies on lipid peroxidation and is tonically inhibited by glutathione peroxidase 4 (GPx4).

Box 2 | Mitochondrial generation of reactive oxygen species

In physiological conditions, an estimated 0.2–2.0% of molecular O<sub>2</sub> taken up by mitochondria is not used as a terminal electron acceptor in the respiratory chain (see BOX 1) but forms superoxide anion (O<sub>2</sub><sup>•-</sup>) at the level of complex I or complex III (a process known as electron leak). O<sub>2</sub><sup>•-</sup> can be rapidly metabolized by mitochondrial and mostly extramitochondrial variants of superoxide dismutase (SOD2 and SOD1, respectively), which catalyse the formation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and O<sub>2</sub>. In turn, H<sub>2</sub>O<sub>2</sub> can have different fates: it can be metabolized by catalase (CAT), resulting in H<sub>2</sub>O formation; it can be metabolized by multiple peroxidases (including glutathione peroxidase (GPx)), coupling the reduction of H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O with the oxidation of a nucleophilic species, such as reduced glutathione (GSH); and it can be converted into the hydroxyl radical (OH<sup>•</sup>) and hydroxyl anion (OH<sup>-</sup>) in the presence of Fe<sup>2+</sup> or Cu<sup>1+</sup> (Fenton reaction) (see figure; please note that stoichiometry is not respected for the sake of simplification). Physiological levels of reactive oxygen species (ROS) are involved in the regulation of several biological processes, including intracellular signalling, adaptation to hypoxia, autophagy, and both adaptive and innate immunity<sup>341</sup>. However, ROS levels that exceed endogenous antioxidant capacities cause extensive macromolecular damage to DNA, proteins, and lipids, generally leading to cellular senescence (the permanent proliferative inactivation of a cell damaged beyond repair) or regulated cell death.

In the hypoxic myocardium, electrons cannot flow normally through the respiratory chain because O<sub>2</sub> availability is limited. This impairment favours the acquisition of a reduced state by respiratory complexes, which enables electron leak, O<sub>2</sub><sup>•-</sup> synthesis, and oxidative damage to the respiratory chain. At tissue reperfusion, restored oxygen availability drives an abrupt increase in electron flow through damaged respiratory complexes, which is associated with a burst in O<sub>2</sub><sup>•-</sup> production. Reperfusion is the phase at which mitochondria are most sensitive to ROS-mediated mitochondrial permeability transition because the low pH associated with ischaemia potentially inhibits mitochondrial permeability transition. It has been proposed that uncoupling, the process whereby the transfer of electrons along the respiratory chain occurs in the absence of net extrusion of H<sup>+</sup> ions from the mitochondrial matrix, leading to decreased mitochondrial transmembrane potential and therefore to reduced sensitivity of respiratory complexes to hypoxia-mediated reduction, might have evolved as a physiological barrier against oxidative damage rather than as a thermogenic process<sup>342</sup>.



GSSG, glutathione disulfide.

**Damage-associated molecular patterns (DAMPs).** Endogenous molecules that exert potent immunomodulatory functions upon binding to cellular receptors that evolved to control microbial pathogens.

**Inflammasome**  
 Supramolecular complex containing caspase 1 (CASP1), which, among other functions, catalyses the proteolytic processing of IL-1β and IL-18, thereby enabling their release in a bioactive form.

progression in *ApoE*<sup>-/-</sup> mice<sup>202</sup>. Placing mice under progressive respiratory hypoxia after ischaemia-reperfusion limits ROS production because hypoxia induces a robust regenerative response with decreased myocardial fibrosis and improvement of left ventricular systolic function<sup>203</sup>. Moreover, cardiomyocyte-specific deletion of *Txnrd2* (which encodes thioredoxin reductase 2) from mouse embryos leads to fatal dilated cardiomyopathy<sup>204</sup>. Interestingly, imposing the same genetic defect on adult mice generates a much milder cardiac phenotype resembling accelerated cardiac ageing<sup>205</sup>. This finding suggests that the embryonic and neonatal myocardium and its adult counterpart have different sensitivity to oxidative stress.

The possibility to use antioxidants (including molecules available over the counter as dietary supplements)

for the treatment of CVD drove an intense wave of preclinical and clinical investigation spanning the past 2 decades. Coenzyme Q<sub>10</sub>, α-tocopherol (vitamin E), ascorbic acid (vitamin C), and β-carotene (the precursor of vitamin A) have all been clinically tested for the treatment or prophylaxis of HF<sup>206,207</sup>, high-risk heart surgery<sup>208</sup>, acute MI<sup>209–214</sup>, and atherosclerosis<sup>215,216</sup>. The majority of these studies confirmed that active levels of antioxidants can be achieved in the circulation of patients with CVD, although most often this is not associated with measurable clinical benefits, perhaps with the exception of coenzyme Q<sub>10</sub> supplementation for the treatment of moderate-to-severe HF<sup>206</sup>. Some clinical trials are ongoing to test the clinical activity of coenzyme Q<sub>10</sub> or its reduced counterpart (ubiquinol) in patients with HF (NCT03133793, NCT01925937, NCT02779634, and NCT02847585), cardiac arrest (NCT02934555), and atherothrombosis (NCT02218476) as well as the capacity of ascorbic acid to prevent atrial fibrillation after CABG surgery (NCT03123107).

Promising preclinical results have been obtained with mitochondria-targeted antioxidants, including elamipretide (also known as Bendavia, MPT-131, and SS-31), mitoQ, and mito-TEMPO, in animal models of MI<sup>217</sup>, hypertensive cardiomyopathy<sup>195,218–220</sup>, HF<sup>221</sup>, ischaemia-reperfusion injury<sup>222–224</sup>, pathological tissue remodelling after MI<sup>225</sup>, and atherosclerosis<sup>226</sup>, fostering the initiation of multiple clinical trials. Both the EVOLVE (NCT01755858) and the EMBRACE STEMI (NCT01572909) studies, evaluating the capacity of elamipretide to limit restenosis after angioplasty of the renal or coronary artery, respectively, did not report clinical benefits<sup>227,228</sup>. Conversely, high-dose elamipretide decreased left ventricular end-diastolic volume and end-systolic volume in HF<sup>218,221</sup> with reduced ejection fraction, pointing to (at least some degree of) clinical efficacy<sup>229</sup>. Elamipretide is still being investigated in Europe for its therapeutic effects in patients with HF (NCT02914665 and NCT02788747), whereas in the USA, increased attention is being dedicated to the possibility of using elamipretide for the treatment of mitochondrial disorders (such as myopathies and retinopathies). Along similar lines, mitoQ is mostly being investigated in clinical settings other than CVD.

**Inflammation.** The major role of mitochondria in the establishment of innate inflammatory responses that contribute to the pathogenesis of CVD is now clear<sup>230</sup>. This observation reflects the key contribution of mitochondrial metabolism and ROS production to multiple immune functions (which is beyond the scope of this Review)<sup>231</sup>, and the fact that mitochondria contain a large amount of endogenous molecules that can act as damage-associated molecular patterns (DAMPs) upon release<sup>232,233</sup>. These molecules include (but potentially are not limited to) ROS, mtDNA, ATP, and cardiolipin<sup>233</sup>. Both ROS and mtDNA (alone or complexed with TFAM) can stimulate inflammatory responses from the cytosol, owing to their capacity to stimulate the release of IL-1β, IL-18, and type I interferon upon activation of the inflammasome and the stimulator



of interferon genes protein (STING; also known as TMEM173)<sup>234,235</sup>. Moreover, extracellular mtDNA can drive granulocyte degranulation upon binding to Toll-like receptor 9 (TLR9)<sup>236</sup>. Extracellular ATP released in the course of RCD operates both as a chemoattractant and as an immunostimulant for myeloid cells<sup>237</sup>. Finally, cardiolipin can favour the activation of  $\gamma\delta$  T lymphocytes via a CD1D-dependent mechanism<sup>238</sup>. Although not all these processes have been implicated in the pathophysiology of CVD, these observations exemplify well how mitochondrial dysfunction in the cardiovascular system, especially in the presence of a mitophagic defect, can drive detrimental inflammatory responses.

In line with this notion, mice lacking the cytosolic DNA sensor cyclic GMP-AMP synthase (CGAS)<sup>239</sup> have improved early survival after MI along with reduced cardiac immune infiltration and consequent pathological tissue remodelling<sup>240</sup>. *Cgas*<sup>-/-</sup> mice, *Irf3*<sup>-/-</sup> mice (lacking an effector of CGAS signalling), and *Ifnar1*<sup>-/-</sup> mice (lacking one of the subunits of the type I interferon receptor)<sup>241</sup> are protected against MI compared with wild-type mice, a cardioprotective phenotype that is accompanied by decreased cardiac expression of inflammatory cytokines and chemokines and decreased inflammatory cell infiltration in the myocardium<sup>242</sup>. Similar cardioprotective effects have been documented with hearts from *Nlrp3*<sup>-/-</sup> mice (which lack a core component of the inflammasome) subjected to ischaemia–reperfusion injury *ex vivo*<sup>243</sup>. Moreover, mtDNA escaping mitophagic degradation as a consequence of *Dnase2* deletion aggravates disease symptoms and progression in a mouse model of pressure-overload-induced cardiomyopathy, a detrimental phenotype that can be partially rescued by *Tlr9* co-deletion or administration of TLR9-inhibiting oligodeoxynucleotides<sup>131</sup>. NLRP3, CGAS, and STING have also been aetiologically involved in the endothelial inflammatory response driven by diet-induced obesity and in some models of atherogenesis<sup>235,244</sup>. Moreover, atherogenesis caused by a high-fat diet is inhibited in *ApoE*<sup>-/-</sup>*Il1r1*<sup>-/-</sup> mice (which lack both apolipoprotein E and the receptor for IL-1 $\beta$ , IL-1R1) compared with *ApoE*<sup>-/-</sup> mice<sup>245</sup>. By contrast, deletion of *Il1rn* (encoding an endogenous inhibitor of IL-1R1) aggravates considerably the disease pathogenesis in *ApoE*<sup>-/-</sup> mice, whereas *ApoE*<sup>-/-</sup> mice engineered to overexpress *Il1rn* are largely protected from high-fat-diet-induced atherogenesis<sup>246</sup>. Finally, a common loss-of-function variant in *P2RX7* (coding for one of the receptors for extracellular ATP) is associated with reduced risk of cardiovascular events in smokers<sup>247</sup>. These studies are only a few examples of how genetic defects in the pro-inflammatory signalling pathways elicited by mitochondrial DAMPs reduce disease incidence, severity, or progression in rodent models of CVD as a consequence of quenched inflammatory responses.

In line with this notion, pharmacological inhibitors of the signal transduction cascades activated by mitochondrial DAMPs provided beneficial effects in multiple experimental models of CVD. For instance, administration of a type I interferon-neutralizing antibody protected mice against MI to a similar extent as the absence of *Irf3* or *Ifnar1* (REF.<sup>242</sup>). Similarly, wild-type mice

subjected to ischaemia–reperfusion while receiving a pharmacological inhibitor of NLRP3 (16673-34-0) had a significant reduction in infarct size afterwards compared with their control counterparts<sup>248,249</sup>. Some degree of cardioprotection has also been observed with the P2RX7 inhibitor Brilliant Blue in rat hearts subjected to ischaemia–reperfusion *ex vivo*<sup>250</sup> as well as with the TLR9-targeting oligodeoxynucleotide ODN 2088 in rats with spontaneous hypertension<sup>251</sup>. Interestingly, elamipretide binds to and prevents the peroxidation of cardiolipin<sup>252</sup>, and blocking  $\gamma\delta$  T lymphocytes with a monoclonal antibody specific for killer cell lectin-like receptor subfamily K member 1 (KLRK1) reportedly attenuates ischaemia–reperfusion injury in a cardiac transplantation model in rats<sup>253</sup>. However, whether elamipretide influences the capacity of cardiolipin to activate  $\gamma\delta$  T lymphocytes remains to be elucidated. Although multiple anti-inflammatory agents are currently available for the treatment of CVD, they all operate by either inhibiting immune cell activation (as in the case of corticosteroids) or by blocking the production of pro-inflammatory eicosanoids (as in the case of NSAIDs)<sup>254,255</sup>. To the best of our knowledge, no therapeutic agent designed to intercept DAMP emission from mitochondria or specifically block the downstream signalling cascades has reached clinical development.

**Regulated cell death.** A prominent aetiological component of multiple cardiovascular disorders, including HF, MI, and atherosclerosis, is the demise of cells damaged beyond recovery<sup>15</sup>, generally occurring via multiple, highly interconnected signalling cascades<sup>4</sup> (BOX 3). Widespread and irreversible mitochondrial dysfunction culminating with the permeabilization of mitochondrial membranes has a central role in apoptosis, MPT-driven regulated necrosis, and parthanatos<sup>256</sup>, *de facto* contributing to pathological tissue loss in the context of CVD<sup>15</sup>. In line with this notion, mice bearing genetic alterations of the molecular apparatus for RCD are protected (at least to some degree) against multiple cardiovascular pathologies. For instance, *Bbc3*<sup>-/-</sup> mice lack one of the upstream activators of intrinsic apoptosis and have increased resistance to ischaemia–reperfusion injury compared with their wild-type littermates<sup>257</sup>. Similarly, mice overexpressing *Bcl2*, encoding the apoptosis regulator BCL-2, have mitigated MI upon ischaemia–reperfusion injury<sup>258</sup>. *Ppif*<sup>-/-</sup> mice, which lack the crucial component for MPT-driven regulated necrosis CypD, are protected against cardiac ischaemia–reperfusion injury<sup>259,260</sup>, angiotensin-II-induced cardiomyopathy<sup>261</sup>, and arrhythmia (in this last case, perhaps also linked to preserved Ca<sup>2+</sup> fluxes)<sup>262</sup>. The deletion of *Parp1*, which encodes poly(ADP-ribose) polymerase 1 (a nuclear DNA repair enzyme that is required for parthanatos), mediates beneficial effects in mouse models of ischaemia–reperfusion injury<sup>263,264</sup> and atherogenesis<sup>265</sup>. Moreover, both *Ripk3*<sup>-/-</sup> mice (which lack a critical regulator of necroptosis) and mice engineered to overexpress dominant-negative CaMKII in the heart are protected against ischaemia–reperfusion injury and the cardiotoxic effects of doxorubicin<sup>266</sup>. These findings link the molecular machinery for necroptosis, which normally proceeds independently

#### $\gamma\delta$ T lymphocytes

Small subsets of T cells expressing a rather invariant variant of the T cell receptor and mostly operating at the interface between innate and adaptive immunity.

#### Eicosanoids

Large family of arachidonic acid derivatives involved in the regulation of multiple biological processes, including the recruitment and activation of immune cells.

#### Apoptosis

Form of RCD initiated by extracellular or intracellular cues that is precipitated by the sequential activation of various members of the caspase protein family.

#### Parthanatos

Necrotic variant of RCD driven by PARP1 hyperactivation and precipitated by the consequent bioenergetic catastrophe coupled to enzymatic DNA degradation.

## microRNAs

(miRNAs). Small non-coding RNA molecules that regulate the expression of target genes at the transcriptional or post-transcriptional level.

of mitochondria<sup>256</sup>, to mitochondrial dysfunction and consequent MPT-driven necrosis.

Extraordinary efforts have also been dedicated to the development of clinically useful inhibitors of RCD for cardioprotective purposes<sup>267</sup>, with rather dismal results. Indeed, although dozens of compounds targeting distinct modules of the molecular machinery for RCD have been successfully synthesized and demonstrated to mediate beneficial effects in experimental models of CVD<sup>267</sup>, none of these agents is currently approved for clinical use. Among other approaches, promising preclinical results in animal models of CVD have been obtained with caspase inhibitors, including: the pan-caspase blockers Z-VAD-FMK<sup>268</sup> and MX1013 (REF.<sup>269</sup>); inhibitors of the core activator of intrinsic apoptosis, apoptosis regulator BAX (BAX), including a cell-permeant peptide derived from the endogenous BAX inhibitor BCL-2-like protein 1 (BCL-2L1)<sup>270</sup>, as well as the two small molecules Bcl1 and Bcl2 (although these compounds were tested only in rodent models of brain ischaemia)<sup>271</sup>;

inhibitors of serine protease HTRA2, mitochondrial (HTRA2), such as the small molecule UCF-101 (REFS<sup>272,273</sup>); molecules that preserve the integrity of the respiratory chain in the course of RCD, including multiple 2-sulfonyl-pyrimidinyl derivatives (although these compounds have been investigated only in rodent models of neurodegeneration)<sup>274,275</sup>; PARP1 inhibitors such as 3-aminobenzamide<sup>263</sup>; and inhibitors of MPT-driven necrosis, including TRO40303 (a small molecule specific for translocator protein (TSPO)<sup>276</sup>), cinnamic anilides (the precise molecular target of which remains to be determined<sup>277</sup>), and the CypD-targeting compounds cyclosporine A, Debio-025, NIM811, and sangliferrin A (REF.<sup>278</sup>). Most of these molecules never reached clinical development, often owing to specificity or bioavailability issues<sup>267</sup>. Conversely, both TRO40303 and cyclosporine A have been investigated for their clinical benefits in patients undergoing percutaneous coronary intervention for acute MI<sup>279,280</sup>. However, despite considerable enthusiasm elicited by the release of efficacy data from the first randomized clinical trial to test cyclosporine A for this indication<sup>280</sup>, subsequent studies did not document clinical benefits<sup>281,282</sup>. Similarly, TRO40303 seems to be well tolerated but devoid of clinical efficacy<sup>283,284</sup>. To the best of our knowledge, the clinical development of TRO40303 has been discontinued. By contrast, a large number of clinical trials are ongoing to test the therapeutic effects of cyclosporine A. The vast majority of these studies, however, are aimed at investigating the activity of cyclosporine A as an immunosuppressant rather than as an MPT inhibitor. Indeed, cyclosporine A is approved by the FDA to prevent and treat graft-versus-host disease after bone marrow transplantation, the rejection of kidney, heart, and liver transplantation, and a panel of autoimmune disorders<sup>285,286</sup>. Of note, the vasodilator nicorandil, which is approved in several countries for the treatment of angina, reportedly potentiates ischaemic preconditioning, at least in some experimental models, by inhibiting MPT<sup>287</sup>. Clinical data from a few studies indicate that nicorandil (which was not conceived as an MTA) might confer cardioprotection after MI<sup>288–290</sup>, a possibility that remains under scrutiny.

### Box 3 | Mechanistic notions on regulated cell death

Mammalian cells exposed to very harsh microenvironmental conditions (such as extreme temperatures and elevated osmotic pressures) die in a virtually uncontrollable manner, reflecting the physical breakdown of the plasma membrane. However, this unregulated cell death is fairly uncommon in the context of human pathophysiology. Instead, human cells generally succumb to pathological cues in the context of failing adaptation to stress via regulated cell death (RCD), which ensues the activation of a genetically encoded machinery that determines the kinetics of the process and its immunological correlates. Indeed, according to current models, mammalian cell death is not caused by the activation of specific proteolytic or nucleolytic pathways, as was thought until the early 2010s, but rather by a lethal shortage of ATP coupled to the accumulation of unrepairable oxidative damage to macromolecules, leading to irreversible loss of plasma membrane integrity. Therefore, actual cytoprotection (that is, a reduction in the percentage of cells succumbing to a cytotoxic cue, as opposed to a simple delay in RCD) might not be achievable after cells are committed to death (that is, when cellular functions are compromised beyond recovery)<sup>4,330</sup>.

Irrespective of this (rather debated) point and its major therapeutic implications (see main text), multiple molecular cascades precipitating RCD in mammals have been identified. These signal transduction cascades rely on a dedicated molecular machinery, meaning that they can be retarded (or accelerated) by specific pharmacological or genetic interventions, and include the following:

- Extrinsic and intrinsic variants of apoptosis: a caspase 3-dependent pathway optionally involving mitochondrial outer membrane permeabilization.
- Mitochondrial permeability transition-driven necrosis: a cyclophilin D-dependent process elicited at the inner mitochondrial membrane.
- Necroptosis: another form of regulated necrosis culminating with plasma membrane permeabilization dependent on mixed lineage kinase domain-like protein (MLKL).
- Ferroptosis: an iron-dependent pathway mediated by uncontrolled lipid peroxidation.
- Parthanatos: a poly(ADP-ribose) polymerase 1-dependent process resulting in a lethal bioenergetic crisis coupled to DNA degradation.
- Pyroptosis: an inflammatory variant of RCD linked to plasma membrane permeabilization by gasdermin protein family members.
- Lysosome-dependent cell death: RCD that is initiated by lysosomal breakdown and precipitated by lysosomal hydrolases.
- Autophagy-dependent cell death: a form of RCD aetiologically linked to components of the molecular machinery for autophagy.
- NETotic cell death: a reactive-oxygen-species-dependent form of RCD restricted to haematopoietic cells and linked to neutrophil extracellular trap (NET) production.
- Entotic cell death: referring to the lysosomal degradation of living cells internalized by other, nonphagocytic cells via an actomyosin-dependent mechanism (entosis)<sup>4,330</sup>.

**Mitochondrial microRNAs.** Most (if not all) aspects of mitochondrial biology are now known to be subjected to epigenetic regulation by microRNAs (miRNAs)<sup>291</sup>. Importantly, this process occurs not only in the nucleus but also in the mitochondrial matrix, where all the components of the molecular apparatus for miRNA-dependent gene silencing are present<sup>292</sup>. Both nuclear miRNAs and mitochondrial miRNAs (also known as mitomiRs) have been implicated in the pathogenesis of multiple cardiovascular disorders<sup>291</sup>. The co-deletion of the sequences encoding miR-181c and miR-181d mediated cardioprotective effects in a mouse model of ischaemia–reperfusion injury, potentially linked to preserved levels of the mitochondrially encoded cytochrome *c* oxidase subunit 1 (MT-CO1) and ameliorated respiratory functions<sup>293</sup>. Overexpression of miR-30b in mouse cardiomyocytes decreases infarct size after ischaemia–reperfusion injury, reflecting the ability of miR-30b to downregulate CypD levels and

thereby impair MPT<sup>294</sup>. Similarly, miR-2861 knock-down protects the mouse heart from ischaemia–reperfusion injury in vivo, a beneficial phenotype potentially linked to upregulation of solute carrier family 25 member 4 (SLC25A4)<sup>295</sup>. Co-deletion of the genes encoding miR-212 and miR-132 provides cardioprotection against pressure-overload-induced cardiomyopathy along with the activation of FOXO3A-dependent autophagy<sup>296</sup>. Consistently, cardiomyocyte-specific overexpression of miR-132, miR-199a, miR-212, or miR-421 in rodents triggers or aggravates CVD along with the induction of mitophagic defects<sup>296–298</sup>. Nanoparticle-based delivery of a miR-181c coding vector also leads to cardiac dysfunction by provoking mitochondrial impairment<sup>299</sup>, as does the deletion of *mir-150* and the co-deletion of *miR-181a* and *miR-181b*<sup>293,300</sup>. Altogether, these observations exemplify the intimate links between the epigenetic regulation of gene expression at both mitochondrial and nuclear levels, mitochondrial biology, and CVD.

Several miRNA-targeting strategies have been shown to mediate beneficial effects in preclinical models of CVD. The mitochondrial pool of miR-378 increases in the course of diabetic cardiomyopathy in mice, and intraperitoneal administration of a miR-378 antagonist mediates cardioprotection, linked with the preservation of mitochondrially encoded ATP synthase subunit a (MT-ATP6) synthesis<sup>301</sup>. The mitochondrial levels of mitochondrially encoded cytochrome *b* (MT-CYB) are significantly lower in hearts from rats with spontaneous hypertension than in control hearts from Wistar rats, associated with an upregulation in the mitochondrial pool of miR-21 (which promotes *Cytb* translation)<sup>302</sup>. In line with the hypothesis that miR-21 upregulation constitutes a compensatory response to decreased MT-CYB levels and consequent ROS overgeneration, intravenous delivery of an adenoviral vector for the overexpression of *miR-21* in rats with spontaneous hypertension mediates short-term beneficial effects on systolic blood pressure and long-term cardioprotection<sup>302</sup>. miR-106a is robustly upregulated in the hypertrophic myocardium, along with a profound downregulation of MFN2, and data from cultured cardiomyocytes exposed to miR-106a mimics or antagonists suggest that antagonizing miR-106a might contribute to the restoration of MFN2 levels and consequent rescue of mitochondrial functions<sup>303</sup>. miR-324-5p and miR-761 are negative regulators of mitochondrial fission, and intravenous delivery of a miR-324-5p or miR-761 mimic limits apoptotic RCD and tissue damage in the myocardium of mice exposed to ischaemia–reperfusion<sup>304,305</sup>. Similarly, administration of a miR-499 antagonist (which also inhibits mitochondrial fission) exacerbates infarct size in mice exposed to ischaemia–reperfusion<sup>306</sup>. Expression of miR-33a and miR-33b is markedly increased in human carotid atherosclerotic plaques compared with normal arteries, and treatment of *ApoE*<sup>-/-</sup> mice with miR-33 antagonists reduces arterial atherosclerotic lesions along with the normalization of mitochondrial functions<sup>307</sup>. Additional progress is required for miRNA-targeting agents to enter clinical development<sup>308</sup>.

### Obstacles in the development of MTAs

Despite an extraordinary experimental effort spanning over the past 3 decades, virtually no MTAs are currently approved for use in patients with CVD. We surmise that such a dismal situation is linked (at least in part) to pharmacodynamic and pharmacokinetic issues, a hitherto fragmentary knowledge of the molecular mechanisms behind mitochondrial processes, and a rather simplistic appreciation of the pathophysiology of some cardiovascular disorders.

**Pharmacological issues.** Multiple MTAs have limited pharmacological specificity for their mitochondrial targets. Cyclosporine A and other CypD-targeting agents are perhaps the most representative examples of this problem. Cyclosporine A and sanglifehrin A potentially inhibit MPT by binding to CypD, de facto mediating robust cytoprotective effects in rodent models of CVD and other pathologies associated with MPT-dependent tissue loss<sup>278</sup>. However, both cyclosporine A and sanglifehrin A also enable the binding of peptidyl-prolyl cis-trans isomerase A (PPIA) to the heterodimeric phosphatase calcineurin, resulting in potent calcineurin inhibition and consequent complete blockage of T cell activation<sup>309</sup>. With systemic administration, the immunosuppressive effect of cyclosporine A and sanglifehrin A are prominent, as demonstrated by the fact that cyclosporine A is approved for use in various clinical settings as an immunosuppressant<sup>285,286</sup>. Novel CypD inhibitors that lack immunosuppressive activity such as Debio-025 and NIM811 are currently being developed<sup>278</sup>. In addition, attention is being focused on strategies for the targeted delivery of cyclosporine A to the myocardium. In this setting, promising results have been obtained with poly(lactic-co-glycolic acid) (PLGA) nanoparticles incorporating cyclosporine A, which were more potent than cyclosporine A at limiting ischaemia–reperfusion injury in mice in the absence of alterations in the myocardial recruitment of inflammatory monocytes<sup>310</sup>.

Untargeted antioxidants also have specificity issues because, on entering the cell, antioxidants can quench ROS from multiple (not necessarily mitochondrial) sources, which limits the purely mitochondrial activity of these compounds. Multiple strategies have been successfully used to target antioxidants specifically to mitochondria, most of which harness the capacity of cationic molecules to accumulate spontaneously within the mitochondrial matrix mediated by the mitochondrial transmembrane potential ( $\Delta\Psi_m$ )<sup>311,312</sup>. One of the major issues with this approach, potentially decreasing its therapeutic value, is that dysfunctional mitochondria often have decreased  $\Delta\Psi_m$  and, consequently, are unable to accumulate cationic molecules<sup>313</sup>. Alternative techniques for mitochondrial delivery, including the use of lipophilic cationic peptides<sup>314</sup>, also rely on the  $\Delta\Psi_m$  and, therefore, cannot circumvent this issue. Similarly, mitochondrial proteins encoded by the nuclear genome enter the mitochondrial matrix by a  $\Delta\Psi_m$ -dependent mechanism<sup>315</sup>. Thus, devising a strategy for the targeted delivery of molecules to dysfunctional mitochondria will be important. The surface properties of permeabilized

#### Nanoparticle

Particle of 1–100 nm in size surrounded by an interfacial layer consisting of ions, inorganic molecules, or organic molecules that determines the biological and biophysical properties of the particle.

#### Mitochondrial transmembrane potential ( $\Delta\Psi_m$ )

Electrochemical gradient built across the inner mitochondrial membrane by the respiratory chain. The  $\Delta\Psi_m$  drives multiple mitochondrial functions, including ATP synthesis and protein transport.

mitochondria (including PINK1 and PARK2 accumulation, as well as extensive ubiquitylation)<sup>6</sup> could be useful but remain unexplored in this context.

Another pharmacological obstacle in the development of clinically useful MTAs relates to pharmacokinetics and biodistribution. In the absence of a tissue-targeting strategy, systemically administered MTAs are confronted by large numbers of mitochondria outside the cardiovascular system, which operate (at least to some degree) as a sink to limit bioavailability at diseased sites. Cardiomyocytes contain more mitochondria than many other cell types<sup>316</sup>, which could potentially favour MTA accumulation, but so do myocytes and neurons, and the skeletal muscle largely exceeds the myocardium in terms of mass. These considerations suggest that some MTAs delivered systemically at safe doses cannot reach bioactive levels at the mitochondrial compartment of diseased cells from the cardiovascular system. Strategies to target MTAs to specific cells of the cardiovascular system, such as PLGA nanoparticles<sup>310</sup>, might (at least partially) circumvent this obstacle.

**Lack of precise mechanistic knowledge.** Despite considerable advances in the understanding of many mitochondrial processes involved in the pathogenesis of CVD, precise mechanistic knowledge is often lacking. Perhaps the best example of our lack of knowledge of mitochondrial processes comes from MPT<sup>317</sup>. The concept that MPT results from the activity of a supra-molecular entity assembled at the interface between the inner and outer mitochondrial membranes, generally referred to as the permeability transition pore complex (PTPC), is widely accepted<sup>171</sup>. However, the precise molecular composition of the PTPC remains obscure, and multiple other aspects of the PTPC biology (including its potential links with the F<sub>1</sub>F<sub>0</sub> ATP synthase) are still a matter of intense debate, despite >2 decades of experimental work on this topic<sup>317,318</sup>. This lack of precise mechanistic knowledge of mitochondrial processes reflects an intrinsic complexity of the system and the lack of good indicators of mitochondrial (dys)function for use in vivo.

Several mitochondrial proteins are strictly required for embryonic development or adult survival, generally owing to their essential bioenergetic functions. One notable example is cytochrome c, somatic (CYCS), which functions as an electron shuttle of the respiratory chain<sup>319</sup>. Because *Cytc*<sup>-/-</sup> mice die in utero, investigating the role of CYCS in RCD in vivo called for the development of refined genetic models<sup>320</sup>. Similar models have not yet been generated for the vast majority of mitochondrial proteins with a prominent vital function<sup>319</sup>. Another large group of mitochondrial proteins exists in multiple isoforms that have a large degree of genetic redundancy<sup>317</sup>. For instance, the mouse genome encodes at least three distinct variants of the PTPC component adenosine nucleotide translocase (*Slc25a4*, *Slc25a5*, and *Slc25a31*) and of the ATP synthase F<sub>0</sub> complex subunit C (*Atp5g1*, *Atp5g2*, and *Atp5g3*)<sup>321,322</sup>. This genetic redundancy complicates considerably the generation of functional knockout models for in vivo studies, although

it also presumably reflects the critical requirement for mitochondrial ATP synthesis for life (implying that complete knockout models might not be viable). In addition, some mitochondrial proteins have functional redundancy, meaning that they can substitute for each other in a specific activity. This functional redundancy seems to be the case for multiple components of the PTPC, at least in some experimental models<sup>317</sup>. These observations exemplify the intrinsic complexity of multiple mitochondrial processes.

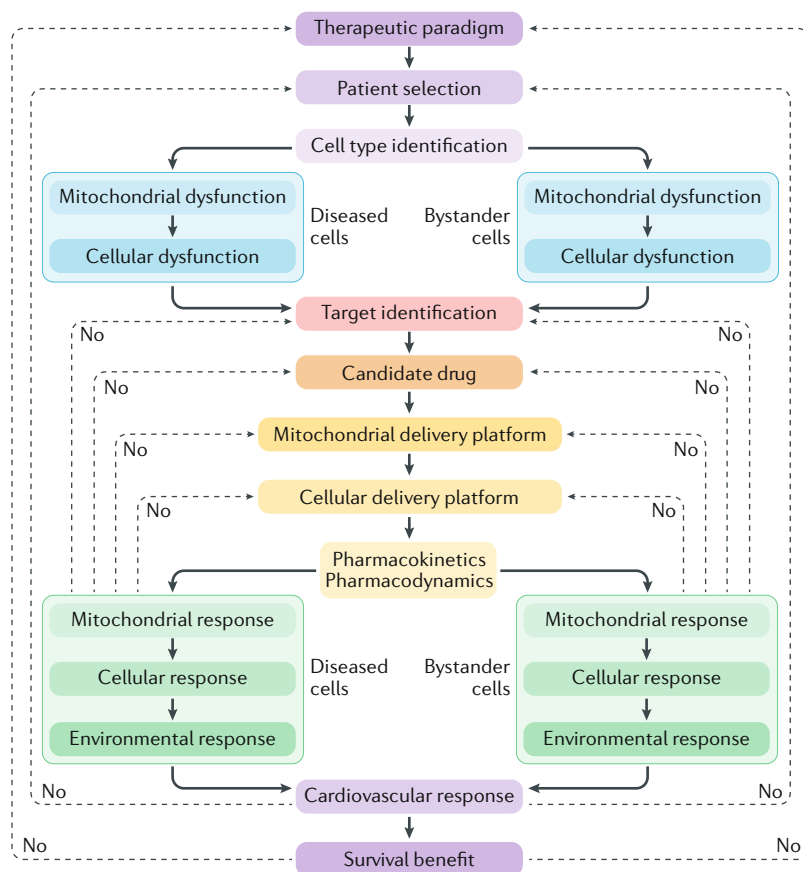
Despite the existence of a variety of probes for in vitro use, monitoring mitochondrial function in vivo thus far has proved challenging. Carbonylation of circulating proteins or lipoproteins has been used to monitor oxidative stress in the context of CVD<sup>323</sup>. However, this technique per se does not enable the identification of the tissue experiencing oxidative damage, nor the precise source of ROS. Measuring the carbonylation of cardiac proteins, such as myosin-binding protein C, cardiac-type (MYBPC), might constitute an improved alternative, although this approach also does not enable the identification of the ROS source and it can be performed only post-mortem<sup>324</sup>. Mass-spectrometry-based profiling of energy metabolites in blood has been proposed as a surrogate biomarker of mitochondrial dysfunction in the context of HF<sup>325</sup>, but the wide applicability of these findings remains untested. One promising approach to monitor mitochondrial dysfunction in preclinical models of CVD is provided by the so-called MitoTimer mouse, a mouse strain engineered to express a mitochondria-targeted mutant of the DsRed fluorescent protein (which shifts to red fluorescence when oxidized) under the control of a cardiomyocyte-specific promoter<sup>326,327</sup>. MitoTimer enables the study of mitochondrial structure, redox state, and mitophagic disposal by fluorescence microscopy on fixed tissue<sup>326,327</sup>. Finally, multiple radioactive tracers are being developed to monitor mitochondrial functions in real time in the setting of CVD<sup>26,328</sup>. These molecules, some of which are already approved for use in humans (for different applications), might constitute preferential tools to study the links between mitochondrial dysfunction and multiple forms of CVD in patients.

**Limited appreciation of the multifactorial nature of CVD.** All cardiovascular disorders are complex pathological entities that develop in the context of multiple cellular, histological, and systemic processes including (but not limited to): an initial attempt of cells to cope with potentially detrimental perturbations of their micro-environment for the restoration of cellular homeostasis; the failure of such an adaptive mechanism, culminating with the initiation of RCD coupled to inflammatory responses; the establishment of acute local inflammation after the recruitment of immune cells, at least partly linked to the disposal of dead cells and cell remnants; and the initiation of repair processes, either culminating with resolved inflammation and fibrosis (if the initial perturbation of homeostasis is relieved) or proceeding chronically along with a continuous wave of RCD and low-degree inflammation (if the initial perturbation of homeostasis persists).

#### Carbonylation

Term generally referring to the metal-catalysed oxidation (primary carbonylation) or addition of reactive aldehydes (secondary carbonylation) to amino acid side chains.





**Fig. 3 | Pharmacological audit trail for the development of novel mitochondria-targeting agents for clinical applications.** To develop novel, clinically useful mitochondria-targeting agents for the treatment or prevention of cardiovascular disease, it is paramount to delineate upfront: the therapeutic paradigms in which mitochondrial dysfunctions cause or aggravate cardiovascular disease; specific patient subsets in which such alterations might have a predominant role in disease pathogenesis; the cell populations that are affected by mitochondrial dysfunction (the diseased cells, which do not necessarily overlap with the cell populations that are commonly linked to disease pathogenesis); and the nature of mitochondrial dysfunction and how such a dysfunction affects the biology of diseased and/or other cells from the cardiovascular or immune system (bystander cells). This analysis will potentially enable the identification of a mitochondrial target for pharmacological interventions and a candidate drug. Delivery platforms tailored to the mitochondrial compartment of diseased cells will have to be developed and characterized in conventional pharmacokinetic and pharmacodynamic studies, followed by an assessment of mitochondrial, cellular, and microenvironmental parameters in both the diseased and bystander cell populations. In the absence of biological efficacy, the choice of molecular target, drug candidate, and/or delivery platforms will have to be re-evaluated, with particular attention for immunological disease correlates. Otherwise, a cardiovascular response followed by improved patient survival might emerge. In the absence of either or both, the entire therapeutic paradigm and/or patient selection should be fully reconsidered.

This process is further complicated by at least four additional elements. First, the entire process involves not only cells from the cardiovascular system (the main target of clinically available drugs) but also stromal cells and, to a greater extent, immune cells<sup>230</sup>. Although the contribution of immune cells to some forms of CVD such as atherosclerosis was appreciated long ago<sup>14</sup>, the role of innate immune mechanisms such as dysregulated type I interferon signalling in HF has just begun to emerge<sup>242</sup>. Second, there is a critical, and we believe often underestimated, time component in the pathogenesis of

most, if not all, cardiovascular disorders. As an example, ischaemia–reperfusion injury is often viewed (and experimentally modelled) as a rather uniform entity, and potential therapeutic interventions administered at reperfusion are tested for their capacity to decrease infarct size or improve survival. Although these models are widely viewed as clinically relevant (patients with acute MI indeed enter intensive care during the ischaemic phase), they are intrinsically unable to dissect the sequence of events initiated at reperfusion, many of which have a direct effect on patient survival. Third, CVD generally develops in elderly individuals, along with a variety of comorbidities, including (but not limited to) obesity, diabetes, and declining immune functions<sup>329</sup>. These disorders affect not only the natural progression of CVD but also its sensitivity to treatment<sup>329</sup>. However, only a few animal models of CVD that are currently available recapitulate such comorbidities. Fourth, many cellular processes involved in the pathogenesis of CVD have a considerable degree of redundancy. For instance, after mammalian cells commit to RCD, inhibiting one single variant of the process only delays (rather than prevents) cellular demise, and it has been argued that actual cytoprotection can be achieved only in the course of adaptive responses to perturbation of homeostasis<sup>330</sup>. This concept casts doubts on the hypothesis that pharmacologically blocking RCD in diseased cardiovascular cells provides clinical benefits (which has been intensively tested with dismal results) and suggests that improving the ability of healthy cells to cope with perturbations of homeostasis constitutes a robust prophylactic strategy. Interestingly, an abundant literature established a robust interconnection between various components of the molecular machineries for RCD and inflammation<sup>331</sup>. This finding opens the intriguing possibility that modulating RCD pathways in diseased cardiovascular cells might affect the consequent inflammatory responses, de facto mediating beneficial effects via cell-extrinsic circuitries<sup>332</sup>. Such a possibility awaits urgent experimental validation. In support of this notion, cyclosporine A, one of the few MTAs currently approved for use in clinics (although not for the treatment of CVD), robustly inhibits MPT and mediates potent anti-inflammatory effects.

Altogether, these observations indicate that improved pharmacodynamic and pharmacokinetic properties, a refined mechanistic knowledge of mitochondrial processes, and a reconsideration of the pathogenesis of (at least some) cardiovascular disorders, together with a redesigned pharmacological audit trail (FIG. 3), are instrumental for the development of novel MTAs with clinical use.

## Conclusions

Robust genetic data demonstrated a crucial role for mitochondrial dysfunction in the pathogenesis of multiple cardiovascular disorders. Nonetheless, the development of MTAs for use in patients with CVD has been rather dismal. Thus far, great attention has been focused on modulating a single mitochondrial process in cells from cardiovascular compartments, and the immunological correlates of RCD and RCD-driven inflammation have

**Pharmacological audit trail**  
Rational framework to guide the development of novel therapeutic agents that involves assessing the risk of failure at any specific stage.

been fairly overlooked. We firmly believe that systematically addressing CVD as a complex phenomenon that is intimately connected with inflammatory responses will be instrumental for the development of novel agents with clinical applications. Alongside, endowing MTAs with superior pharmacological specificity and acquiring additional knowledge on the precise molecular mechanisms linking mitochondrial dysfunction to CVD pathogenesis, potentially aiming at strategies that simultaneously modulate multiple aspects of the disease, will be paramount. In this context, it will be important to evaluate carefully the cardiovascular effects (or lack thereof) of precise genetic interventions targeting mitochondrial functions on the basis of the age and sex of the animals and the potential existence of compensatory pathways, especially based on functional (rather than genetic) redundancy, as well as evaluate the effects in the context of pathologically relevant comorbidities.

Deleting specific mitochondria-relevant genes from the embryonic myocardium has consequences that the same intervention does not provoke in the adult<sup>97</sup>,

which is particularly relevant for the development of pharmacological interventions. Data accumulating over the past decade point to considerable differences in the sensitivity of male versus female rodents to experimental CVD, and epidemiological data in humans support similar conclusions<sup>333,334</sup>, but little work has been done with specific reference to mitochondrial dysfunction<sup>335</sup>. Moreover, whereas the effect of genetic redundancy on a specific mitochondrial pathway can be addressed with (relatively complex, but feasible) co-deletion and/or depletion strategies<sup>336–338</sup>, identifying (and investigating) functional redundancy is far more complex. Finally, an unmet need exists for new rodent models that faithfully recapitulate the comorbidities that normally accompany CVD in humans<sup>329</sup>. In conclusion, although the route to the identification of clinically useful MTAs is long and tortuous, a large amount of evidence suggests that mitochondrial dysfunction remains a promising target for the treatment of multiple forms of CVD.

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#### Author contributions

M.B., M.R.W., and L.G. researched data for the article and wrote the manuscript. D.A.S., G.K., P.P., and L.G. reviewed and/or edited the manuscript before submission. All authors made substantial contributions to discussion of the content.

#### Competing interests

D.A.S. is a consultant to and inventor on patents licensed to CohBar, GlaxoSmithKline, Jumpstart Fertility, Liberty Biosecurity, Life Biosciences, and MetroBiotech. The other authors declare no competing interests.

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