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AHA Position Paper on Mitochondrial Function, Biology and Role in Disease

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Abstract

Cardiovascular disease is a major leading cause of morbidity and mortality in the United States and elsewhere. Alterations in mitochondrial function are increasingly being recognized as a contributing factor in myocardial infarction and in patients presenting with cardiomyopathy. Recent understanding of the complex interaction of the mitochondria in regulating metabolism and cell death can provide novel insight and therapeutic targets. The purpose of this statement is to better define the potential role of mitochondria in the genesis of cardiovascular disease such as ischemia and heart failure. To accomplish this we will define the key mitochondrial processes that play a role in cardiovascular disease, which are potential targets for novel therapeutic interventions. This is an exciting time in mitochondrial research. The past decade has provided novel insight into the role of mitochondria function and their importance in complex diseases. This Statement will define the key roles that mitochondria play in cardiovascular physiology and

disease, and provide insight into how mitochondrial defects can contribute to cardiovascular disease and it will also discuss potential biomarkers of mitochondrial disease and suggest potential novel therapeutic approaches.

Introduction:

The mitochondria are recognized as a key player in cardiomyocyte cell death following myocardial infarction and cardiomyopathies. Alterations in mitochondrial function are increasingly recognized in cardiovascular disease. Although it has been suggested that the failing heart is energy starved [1], the recent understanding of the complex interaction of the mitochondria in regulating metabolism and cell death provide novel insight and therapeutic targets. This bioenergetics perspective of cardiomyopathy can be understood as one manifestation of an array of different common clinical phenotypes including myopathies, neuropathies, nephropathies, endocrine disorders and metabolic diseases, aging and cancer. This is because the organs that are affected in the common “complex” diseases are the very same organs that have the highest reliance on mitochondrial function [2].

The purpose of this statement is to better define the potential role of mitochondria in the genesis of cardiovascular disease such as ischemia and heart failure (see Figure 1). To accomplish this we will define the key mitochondrial processes that play a role in cardiovascular disease, which are potential targets for novel therapeutic interventions. This is an exciting time in mitochondrial research. The past decade has provided novel insight into the role of mitochondria function and their importance in complex diseases. In section I this Statement will define the key roles that mitochondria play in cardiovascular physiology and disease. Section II will provide insight into how mitochondrial defects can contribute to cardiovascular disease and it will also discuss potential biomarkers of mitochondrial disease and suggest potential novel therapeutic approaches.

Mitochondria are well-known as the powerhouse of the cell and as discussed in Section I.A (Bioenergetics and Metabolism) in an active tissue such as heart they are responsible for generating most of the ATP in the cell. The role of post-translational modifications (PTMs) in the regulation of metabolism is also discussed (Section I.B). It has long been known that in addition to generating ATP, the mitochondria electron transport chain is also important in regulation of mitochondrial calcium. The recent identification of the proteins involved in regulating mitochondrial matrix calcium is providing new insights into the regulation and role of mitochondrial calcium (I.C). As discussed in Section I.D mitochondrial are also key regulators of cell death. In the process of electron transport to generate ATP, mitochondria can be a major source of reactive oxygen species (ROS) that can both contribute to cell death, and also serve as a signaling molecule (Section I.E). Because the generation of ROS can lead to damage to mitochondrial DNA and proteins it is important for the mitochondria to have mechanisms to assure quality control (Section I.F). Quality control can occur by fission/fusion to allow segregation of damaged mitochondria (Section I.F.1), mitophagy to remove damaged mitochondria (Section I.F.2) and ultimately cell death if the damage is too severe (Section I.D). Although mitophagy is important for quality control and for the removal of damaged mitochondria, based on measurement of mitochondrial protein turnover

(Section I.F.4) it appears that mitochondrial proteins turnover at different rates, suggesting that under normal circumstances mitophagy is not the main driver of mitochondrial protein turnover. In fact it is suggested that the dynamics of protein turnover can provide an assessment of the physiological state. Alterations in these mitochondrial functions are important in many cardiac diseases as discussed in Section II. Section II.A discusses the mitochondrial etiology of cardiomyopathy and Section II.B discusses the role of mitochondria in cardiotoxicity. Section II.C discusses potential biomarkers for mitochondrial diseases. Taken together this AHA Statement provides a state-of-the-art assessment of the current status of basic mitochondrial biology and how alterations in mitochondria can be major contributors to complex cardiovascular diseases.

I. Mitochondrial Function

A) Generation of ATP – Bioenergetics and Metabolism

Energy demands of the heart: The incessant energy requirements of the heart are sustained by the consumption of a mass of ATP daily that surpasses cardiac weight itself by approximately 5–10 fold [3]. This perpetual demand for energy reflects the continuous contractile functioning of the heart to sustain systemic circulation and nutrient supply. This high energy flux translates into the cardiomyocyte having a mitochondrial volume between 23% and 32% of myocellular volume [4]. Interestingly, cardiac mitochondrial density increases from man to mouse in parallel with increasing heart rate and oxygen consumption [4]. Based on the role of mitochondrial in energy transduction, it is not surprising, that any perturbations in mitochondrial energy balance, production or propagation would result in the development of cardiac pathology and/or susceptibility to injury (see Section II.A and B). However, a linear or direct correlation between mitochondrial energy metabolism and heart pathology is not clear cut. In this section, we give a brief overview of metabolism and perturbations and its consequences on myocardial function.

Electron transfer chain biology – energetics and ROS: The final common pathway for oxidative metabolism, which generates the bulk of cardiac ATP, is the sequential passage of electrons from high (NADH or $FADH_2$) to low (molecular oxygen) redox potentials down the electron transfer chain (ETC – complexes I through IV). This step-wise electron transfer results in the active pumping of hydrogen ions out of the mitochondrial matrix into the inter-membranous space. The ensuing electrochemical gradient generated across in the inner mitochondrial membrane facilitates the translocation of protons from the inter-membranous space through the F_o/F_1 ATPase (ATP-synthase) back into the mitochondrial matrix. This proton translocation is coupled to the phosphorylation of ADP to generate ATP. Collectively these reactions constitute oxidative phosphorylation and the direct synthesis of ATP from electron-transfer encapsulates coupled respiration [5]. Being cognizant to the high-energy demand of the heart, it is not surprising that mutations in genes that encode for ETC proteins are linked to the development of cardiomyopathy (See Section II.A) [6–8]. However, it should also be recognized that the dysfunction in the electron transport chain (ETC) not only affects ATP production but can concordantly impair intracellular Ca^{2+} flux (see section I.C), increase the generation of reactive oxygen species (ROS) (see section I.E), and alter redox balance by altering the NAD^+/NADH ratio [9, 10].

Fuel substrates and cardiac energetics in health and disease: Mitochondrial fatty acid β-oxidation is the most efficient and predominant substrate for energy production in the normal adult human heart with glucose oxidation, glycolysis, lactate and ketones additionally contributing toward myocardial ATP production [11]. Regulation of cardiac energy metabolism is complex and determined by the summation of intracellular substrate concentrations, transcriptional rates and activity of metabolic enzymes, and metabolic demands of the myocardium [12, 13]. However, as the heart remodels in response to hypertrophy and ischemia, marked changes in cardiomyocyte substrate metabolism can occur with an ultimate effect on ATP levels in the decompensating heart (Reviewed [14–16]). The relative contribution of fatty acids diminishes with enhanced reliance on glucose utilization during the development of cardiac hypertrophy. The regulatory programs attenuating fat utilization have been extensively investigated and include regulation at the transcriptional and post-transcriptional levels [17–20]. Moreover, this reduction involves coordinate downregulation of proteins controlling fatty acid uptake by the heart and mitochondria as well as of the enzymes controlling mitochondrial fatty-acid β-oxidation (FAO); see Section I.F.3 on Protein Turnover [17, 21–24]. Enzymes involved in glycolytic pathways are up-regulated even during early stages of cardiac dysfunction in response to increased adrenergic signaling, up-regulation of fetal gene programs, and/or hypoxia [25, 26]. The shift toward glucose metabolism improves myocardial contractile efficiency by increasing the stoichiometric ratio of ATP production to oxygen consumption in addition to minimizing oxidative losses through mitochondrial respiratory chain uncoupling associated with free fatty acid metabolism [27, 28]. Abnormally high myocardial dependence on fatty acid metabolism, as seen during ischemia or high adrenergic states, increases cardiac oxygen consumption by 30–50% adjusted for equivalent stroke work indices [29, 30]. Strategies to increase glucose oxidation and decrease fatty acid metabolism may improve myocardial energy efficiency by up to 30% [31]. Although the initial shift towards glucose metabolism at progressively more advanced stages of cardiac dysfunction is physiologically adaptive, the magnitude and impact of this adaption can be significantly limited by extra-cardiac factors, specifically the development of insulin resistance. Whole body insulin resistance can affect cardiac energy metabolism, even in structurally normal hearts. Type 2 diabetic patients who have otherwise normal cardiac function regenerate phosphocreatine at a significantly lower rate after exercise compared with non-diabetic controls [32]. In patients with cardiomyopathy, the development of insulin resistance is linked to increased sympathetic signaling leading to liberation of free fatty acids from adipose tissue into the bloodstream [33]. Thus, in the failing myocardium, decreases in insulin sensitivity can lead to further reductions in glucose oxidation and deteriorations in cardiac function by depriving the heart of access to a more metabolically efficient substrate.

TCA biology and anaplerosis: The capacity to use multiple substrates and the plasticity to switch substrate utilization enables continuous cardiac work under a wide variety of biological and pathological circumstances. Interestingly, under some pathological conditions such as severe hypertrophy, the coupling of glycolysis and pyruvate oxidation becomes disrupted with an increase in glucose oxidation that is insufficient to completely compensate for the reduced fat oxidation [24, 34]. These perturbations in substrate partitioning and selection may become associated with reduced contractile reserve and increased

susceptibility to ischemia-reperfusion injury [34–36]. Partial compensation for this energy-substrate oxidation deficit has recently been identified to occur via recruitment of alternative intermediary pathways (anaplerosis) to enhance flux through the tricarboxylic acid (TCA) cycle [23]. The necessity of anaplerosis in the heart is well established, in that the mechanical performance of isolated rat hearts when exclusive precursors of acetyl-CoA are used as substrate shows progressive deterioration with rapid restoration following introduction of anaplerotic substrates [37]. Whether the disruption of anaplerosis plays a significant role in cardiac maladaptation, and or whether it may be a therapeutic target for therapy is currently unknown [27].

Metabolic modulation as a strategy for cardiac muscle pathology: Despite the findings of altered metabolism and energetic capacity in experimental models and in patients with cardiac muscle injury and remodeling, the myriad of agents that have been directly assessed as metabolic modulators have not been found to have significant clinic benefit in the management of heart disease. The use of these agents has recently been reviewed [16], and only some of the studies are discussed here to illustrate the overall lack of efficacy, inadequate sample size and/or potential adverse effects associated with the administration of these metabolic modulators. Etomoxir is an irreversible inhibitor of mitochondrial carnitine palmitoyltransferase-1 (CPT-1) and thus results in a reduction in long chain fatty acid oxidation. An initial pilot study suggested that etomoxir may improve myocardial function in patients with heart failure [38]. However, a subsequent controlled study was stopped prematurely because the drug was associated with hepatic transaminitis [39]. Another inhibitor of CPT-1 and CPT-2 is perhexiline and one study showed improvement in MVO2 max and ejection fraction [40], however, larger studies are needed before a conclusion can be made regarding this drug in heart disease. Trimetazidine is a partial inhibitor of the β-oxidation enzyme 3-ketoacyl CoA thiolase, and is shown in small studies to improve symptoms and cardiac function in patients with heart failure [41]. Ranolazine has a similar structure to trimetazidine and is currently FDA approved for treatment of stable angina. Although it may affect FFA metabolism, its main mechanism of action may be related to inhibition of the late inward Na+ channel. In the TIMI-36 trial, ranolazine did not reduce hospitalization for HF in patients with acute coronary syndrome [42]. Finally, dichloroacetate (DCA) increases myocardial glucose utilization by inhibiting pyruvate dehydrogenase kinase, leading to increased activity of the mitochondrial pyruvate dehydrogenase. In a small study of patients with heart disease, DCA increased stroke volume and myocardial efficiency [43], however, more substantial studies are needed to characterize the role of DCA in patients with heart disease.

Creatine kinase and high energy transfer—The creatine kinase (CK) reaction is the prime energy reserve that provides a rapid source of adenosine triphosphate (ATP) and facilitates its delivery from the mitochondrial site of production to sites of use, including the myofibrils in the heart [44]. Although the heart is a high-energy consuming organ, human genetic mutations linked to creatine deficiency usually result in neurological deficits [45]. In parallel, the genetic disruption of whole body creatine synthesis in the mouse had no detrimental effects on exercise capacity, on cardiac workload or adaptation to ischemia-reperfusion injury [46], and the overexpression of the creatine transporter, or exogenous

creatine supplementation in mice, again shows no cardiac benefit [47]. Nevertheless, in a large animal model, reduced CK ATP delivery was associated with impaired myocardial contractile function [48] and in human heart failure morbidity and mortality are linked to impaired CK metabolism and flux [49, 50].

B. Regulation of Function and Metabolism—The role of PTMs

It has been increasingly clear that there is cross-talk and signaling between the mitochondria, the cytosol and the nucleus. Post-translational modifications are a primary mechanism by which the mitochondrial communicate with the rest of the cell.

Acetylation: Nutrient-overload is linked to mitochondrial dysfunction and to the cardiovascular risk factors of obesity and diabetes [27, 51]. Conceptually, perturbations in mitochondrial metabolic intermediates, such as acetyl-CoA, which itself can function as a direct post-translational substrate to modify mitochondrial proteins through acetylation, may link these pathophysiological effects [52, 53]. Additional short-chain carbon metabolic intermediates including succinyl groups and malonyl groups can also bind to, and modify protein lysine residues [45, 54]. Our knowledge of the regulation of these latter modifications is too preliminary to expand on [45, 54, 55], and this section will focus on the role acetylation, as a nutrient-dependent mechanism, in the regulation of mitochondrial function.

Enzymatic and non-enzymatic control of protein acetylation: There are three major acetyltransferase families, and member proteins from each group have been implicated in the control of cellular homeostasis [56]. Deacetylase proteins are similarly grouped into distinct classes [57]. Class III deacetylases are NAD^+-dependent, and function as sensors of the energetic status of the cell in response to the subcellular compartment levels of NAD^+ and nicotinamide and/or to the ratio of NAD^+:NADH [35, 58, 59]. Recent findings have highlighted novel mechanisms that regulate levels of NAD [60–62]. These enzymes are defined as sirtuins and 7 family members (Sirt1 through Sirt7) are evident in mammalia [63]. Sirt1, 2 and 3 have the most robust deacetylase activity and predominantly function in the nuclear (Sirt1), cytoplasmic (Sirt1 and 2) and mitochondrial (Sirt3) respectively. As the focus of this section is on mitochondrial PTMs, it will focus on Sirt3.

The counter-regulatory acetyltransferase enzyme system is less well characterized, although nuclear Gcn5 and p300 counter the actions of Sirt1 [64]. The process of protein acetylation in the mitochondria is even less well understood, although GCN5L1 has been identified as a critical molecular component of this program and its functional role is beginning to be explored [53, 65]. Recently, the mitochondrial protein acetyltransferase, acetyl-CoA acetyltransferase 1 (ACAT1), which functions in ketogenesis to combine two acetyl-CoA molecules [66] has been found to regulate the pyruvate dehydrogenase complex as a canonical acetyltransferase [67]. This finding may open the door to expanding our understanding of acetyltransferase functioning within mitochondria.

Concurrently, the recognition of non-enzymatic acetylation of proteins in the presence of acetyl-CoA is evident [68] and denatured mitochondrial proteins undergo acetylation in the presence of acetyl-CoA [65]. Furthermore, elevated levels of acetyl-CoA, coupled to the

alkaline mitochondrial pH, have been shown to promote non-enzymatic protein acetylation [69]. This concept of non-enzymatic protein acetylation may be operational in diabetes where metabolic inflexibility, which is defined as the inability to switch from fatty acid to glucose oxidation during the transition from the fasted to fed state, results in part from the allosteric inhibition of pyruvate dehydrogenase by increased mitochondrial acetyl-CoA levels [70, 71]. The role of non-enzymatic protein acetylation has not been extensively investigated, although its' potentially important regulatory role has been reviewed [72]. Interestingly, analysis of the mitochondrial acetylome, under various nutrient conditions and in the presence or absence of Sirt3, shows evidence compatible with non-enzymatic and enzymatic control of the mitochondrial acetylome [73].

Mitochondrial Sirt3 and the heart: Although Sirt3 functions predominantly in mitochondria [74], data do support extra-mitochondrial deacetylase activity [75–77]. The depletion of Sirt3 has a subtle phenotype [78] which is unmasked in response to prolonged fasting,[79] following chronic perturbations in caloric intake [80–82] and in response to redox stress [83]. Numerous proteomic approaches have been employed to identify substrates of Sirt3 deacetylation and the vast majority of proteins with alternations in acetylation are found within mitochondria [73, 84, 85]. The functional characterization of these proteins show that Sirt3 mediated deacetylation regulates numerous aspects of mitochondrial function including the regulation of enzymes controlling β-oxidation, branch-chain amino acid metabolism, ketone biology, the electron transport chain, ATP production, the urea cycle [73, 79, 85, 86] and ROS catabolism [74, 87].

In light of the high energy demand of the heart and based on the Sirt3 targets characterized to date, the disruption of Sirt3 would be expected to have cardiac consequences. Despite this, young Sirt3 knockout mice do not have any obvious phenotype [88] and furthermore display normal exercise performance [89]. However, consistent with a 'fine-tuning' function, aging Sirt3 knockout mice develop cardiac dilatation [88], and pressure-overload results in maladaptive cardiac hypertrophy [88, 90]. The mechanisms underpinning these pathologies align with established functions attributable to Sirt3 including increased generation of ROS [88, 90]. Conversely Sirt3 overexpression promotes anti-apoptotic programs in cardiomyocytes [76] and cardiac-restricted Sirt3 transgenic mice exhibit enhanced ROS scavenging [90]. An interesting additional mechanism whereby Sirt3 deficiency could potentially contribute to the pathophysiology of cardiac hypertrophy is its regulatory role in controlling fatty acid metabolism [79]. As the loss of metabolic plasticity with the downregulation of fatty acid oxidation (FAO) is synonymous with cardiac pressure-overload mediated decompensation [12, 17], it is conceptually possible that the downregulation of FAO in Sirt3 knockout mice may play a role in the pressure-overload and aging maladaptive phenotype in the heart. However, this needs to be delineated further, as high-fat feeding has been concurrently shown to increase cardiac FAO in parallel with downregulation of Sirt3 [91].

As the regulatory control of mitochondrial protein acetylation is nutrient-level and redox-potential dependent, it is conceivable that primary perturbations within mitochondria that may modulate metabolic intermediates or redox-potential could initiate changes in the acetylome. This concept has been explored in the heart in response to genetic perturbations

associated with cardiovascular pathology where disruption of frataxin, cyclophilin D and components of the electron transfer chain result in the either basal or excessive pressure-overload induced cardiac dysfunction, and are associated with reduced NAD^+/NADH ratio and increased mitochondrial protein acetylation [13, 92, 93]. In primary cardiomyocytes, the frataxin and Complex I disruption of the acetylome are corrected in parallel with improvement in mitochondrial function, following Sirt3 induction [13, 92]. Although, not completely characterized, these data support the concept that the control of acetylation by intrinsic mitochondria functioning may, via a feedback loop, affect global mitochondrial functioning via mitochondrial acetylome regulation.

Future directions in understanding the mitochondrial acetylome: Advances in proteomics have enhanced our understanding of both the static and dynamic alteration of the mitochondrial acetylome [73, 94]. Additionally, these studies have identified site-specific changes in lysine residue acetylation that modulate protein function, stability, localization, allosteric interactions and/or control synergistic PTM's [79, 84, 95, 96]. Moreover, the stoichiometry of proteins and the domains surrounding substrate protein lysine residues may play important regulatory roles in the interaction of acetylase and deacetylase enzymes [73] and the further characterization of the acetylome modifying enzymes themselves may further expand our understanding of the role of acetylation in controlling mitochondrial function [53, 74, 94].

An area of some functional discrepancy has also arisen with respect to the acetylation of specific targets within a pathway compared to the global functioning of the canonical pathway in response to acetylation. This is most vividly illustrated where fatty acid oxidation is increased in the presence of excess fat and mitochondrial protein acetylation [52, 97], in contrast to studies showing direct deacetylation of lysine residues on FAO enzymes resulting in the activation of enzyme activity [79, 98]. The mechanisms underpinning these effects and whether this may be a result of tissue distinct regulatory cues needs to be explored.

Finally, although the role of acetylation in modifying individuals proteins is the main focus of this section, data is emerging to show that the overall function of mitochondrial quality control and integrity, which are also modulated by nutrient levels and redox stress, including mitochondrial turnover (mitochondrial dynamics, mitophagy and biogenesis) [53, 64, 99, 100] and redox- and proteotoxic-stress amelioration effects [101, 102] may be regulated by the mitochondrial acetylome [53]. The complexity of this regulation is further underscored where cross-talk between different PTM's function in concert to regulate protein function, as has been shown by concomitant modifications in acetylation and phosphorylation [103].

Phosphorylation: As recently reviewed there is extensive phosphorylation of proteins in the mitochondrial matrix as well as in the mitochondrial electron transport complexes [104, 105]. A number of recent studies have reported that there are several hundred phosphorylated proteins in cardiac mitochondria [106, 107]. There are also sex differences in phosphorylation of mitochondrial proteins [108]. Many of the phosphorylated mitochondria proteins are outer mitochondrial proteins, which are likely phosphorylated by cytosolic kinases, and have been shown to regulate mitochondrial dynamics and cell death

pathways. As discussed previously [104], the occupancy or fraction of the protein that is phosphorylated may be low for many of these proteins, and it is possible that these many of these low level modifications are of little or no functional consequence. It is also unclear to what extent phosphorylation of mitochondrial matrix proteins occurs in the matrix as opposed to prior to import into the matrix. Furthermore, with the exception of the PDH and BCKDH kinase and phosphatase little is known about the kinases and phosphatases responsible for mitochondrial phosphorylation O'Rourke et al have recently reviewed the evidence for the mitochondrial localization of other kinases [105]. Furthermore, although a large number of phosphorylated mitochondrial proteins have been identified, very few phosphorylation sites have been demonstrated to alter enzyme or protein activity. It has been proposed that cAMP generated in the mitochondrial activates mitochondrial PKA to regulate ATP production [109]. However, recent studies have found that alterations in mitochondrial cAMP and PKA do not contribute significantly to acute calcium stimulation of oxidative phosphorylation [110].

Given that extensive phosphorylation has been identified in the mitochondria, it is tempting to speculate that changes in mitochondrial phosphorylation regulate mitochondrial function. However, it will be important for future studies to better define the function consequences of these sites of phosphorylation as well as defining the kinases and phosphatase that regulate their phosphorylation.

S-Nitrosylation: S-nitrosylation (SNO) is the covalent attachment of nitric oxide moiety to a protein thiol group. As recently reviewed [111–113], SNO is a redox dependent modification that suggested to alter cell function by altering protein or enzyme activity, by altering protein localization, by shielding critical cysteine residues from oxidation, by altering protein stability, by altering binding partners and by competing with other PTMs. An increase in oxidative stress leads to a decrease in protein SNO and thereby alters the SNO/ROS balance. ROS leads to the consumption of NO and thus cardiac specific overexpression of SOD leads to an increase in NO bioavailability [114]. Another mechanism by which an increase in oxidative stress reduces NO/SNO signaling is by uncoupling of NOS. Alterations in NOS signaling have been proposed to predispose one to cardiovascular disease [115]. Cardioprotection is associated with a modest increase in S-nitrosylation and the majority of the proteins that exhibit an increase in SNO are mitochondrial [116]. This may be related to the redox environment of the mitochondria. Changes in cell redox can alter the generation of NO, the lifetime or bioavailability of NO, and the reactions that lead to protein SNO and denitrosylation. A key cysteine in the mitochondrial ATP synthase was shown to undergo multiple redox modification and the extent of different modifications differed in dyssynchronous heart failure compared to cardiac resynchronization therapy [117].

C. Calcium transport

The electrochemical gradient across the inner mitochondrial membrane is the driving force for calcium transported across the mitochondria inner membrane by the recently identified [118, 119] mitochondrial calcium uniporter (MCU). Uptake into the mitochondria, of small physiological levels of calcium, is thought to regulate mitochondrial metabolism and ATP

production [120–123]. In heart, an increase in contractility is mediated by an increase in the cytosolic calcium transient. The increase in cytosolic calcium is transmitted to the mitochondria via Ca uptake into mitochondria, which leads to activation of the calcium sensitive mitochondrial dehydrogenases [124] and several complexes of electron transport thereby increasing ATP production as needed for the increase in work [120]. Under pathological conditions of high cytosolic calcium (calcium overload), mitochondria are capable of taking up large amounts of calcium, which lead to the opening of the mitochondrial permeability transition pore, a large conductance channel in the inner mitochondrial membrane [125, 126] (see section I.D). The sustained opening of this transition pore is a trigger for cell death [126].

As reviewed recently the MCU exist in a multiprotein complex with several proteins that regulate its activity [127–132]. Calcium efflux from cardiac mitochondria occurs via the Na-Ca exchanger (NCXL) (see [133] for a recent review). Calcium transits the outer mitochondrial membrane via the voltage dependent anion channel (VDAC). The mitochondrial Na-Ca exchange has been shown to regulate mitochondrial calcium levels and to connect mitochondrial Ca to intracellular Na, such that the rise in Na that occurs during hypertrophy and heart failure is reported to lead to alterations in mitochondrial Ca leading to altered redox and metabolism [134, 135]. There are recent data suggesting that alterations in mitochondrial calcium can contribution to the development of arrhythmias [134, 136].

Recently several groups developed MCU knockout (MCU-KO) or mice without a functional MCU (DN-MCU) to study the role of mitochondrial Ca in modulating metabolism and cell death [137–141]. Because it is generally assumed that mPTP opening and subsequent cell death is generally initiated by calcium influx into the mitochondria via the MCU, it was hypothesized that the MCU-KO hearts would have reduced mPTP opening and reduced cell death following ischemia. There was consistency among the different groups in that mitochondria from the MCU-KO hearts did not take up Ca and did not undergo Ca activated mPTP [137, 139, 140], however there were interesting differences regarding whether these mice were protected from ischemia and reperfusion mediated death. In the mice in which MCU was knocked out or mutated before birth, the hearts did not show a decrease in infarct size following ischemia-reperfusion [137, 141]. In contrast the mice in which loss of MCU was induced in adults by tamoxifen showed smaller infarcts following ischemia and reperfusion [139, 140]. One possible explanation for these differences is that when MCU is deleted before birth that compensatory mechanisms develop which somehow modify cell death pathways such that loss of MCU is not protective. A role for compensatory mechanisms is also consistent with the observation that loss of MCU is lethal on a C57B6 background.

D) Mitochondria and cell death

Prior to the 1980s, cell death was viewed as a passive process. At odds with this concept were long-standing observations that specific cells die at specific times during development in multicellular organisms ranging from worms to mammals [142]. However, it was not until the identification of a small network of genes which modulates developmental cell death in C. elegans that the concept of “regulated cell death” came into focus [143]. By the 1990s,

the descendants of these genes were recognized to also mediate apoptotic cell death in adult organisms including humans [144]. By the turn of the century, it became clear that a large proportion of necrotic cell deaths, thought to be the last bastion of passive cell death, are actually highly regulated [145–148]. In addition to apoptosis and necrosis, other regulated death programs – defined by morphology and/or the context they occur – likely exist [149], including a form of cell death associated with autophagy ("autosis") [150]. What regulated forms of cell death share in common is that process mediated by signaling pathways whose components are constitutively present in the cell. These hardwired pathways remain inactive, however, until receipt of a "death signal" originating from outside or inside the cell.

Apoptosis and necrosis have been most intensively studied. While they share inciting death stimuli and are mediated by overlapping pathways, they differ in morphology and consequences to surrounding tissue [151]. Specifically, apoptosis is a stealth form of cell death because plasma membrane integrity is maintained until the fragmented cellular corpses are eliminated by phagocytosis. In contrast, plasma and organelle membrane breakdown is a defining feature of necrosis and may be actively mediated. The end result in necrosis is the release of inflammatory mediators that cause collateral tissue damage in a paracrine manner and through the recruitment of leukocytes. Based on traditional pathological analysis, the major form of cardiomyocyte death in the infarct zone is thought to be necrosis [152], while a delayed wave of apoptosis takes place in the peri-infarct region especially with reperfusion [153, 154]. Genetic experiments in mice have established that regulated necrosis and apoptosis both play important roles in the generation of the infarct (examples include [95, 147, 155–163]). In dilated cardiomyopathy, low - but clearly elevated - levels of cardiomyocyte apoptosis take place and are an important component in pathogenesis of this syndrome. [164]. Necrosis has also been reported to contribute to heart failure, but has been less well studied [148].

Apoptosis and necrosis can each be induced through two general pathways, one involving cell surface "death" receptors and the other the mitochondria [144, 151, 165]. Even when the signals initiate through death receptors, the mitochondria are often part of a critical amplification loop. Regardless of initiating pathway, the end-game in apoptosis is to activate caspases, a class of cystinyl proteases that cut following aspartic acid residues. Caspases then proteolyze multiple cellular substrates to bring about the demise of the cell. The molecular goal in necrosis, on the other hand, depends on the initiating pathway. Induction of necrosis through the death receptor pathway ("necroptosis") is mediated through activation of Receptor Interacting Protein (RIP) 1 and RIP3, homologous serine/threonine kinases whose targets are an area of active investigation.

Mitochondria have been recognized as playing a central role in both apoptotic and necrotic cell death. The triggering event in mitochondrial-mediated apoptosis is permeabilization of the outer mitochondrial membrane (OMM) allowing the release of "apoptogens", including cytochrome c, SMAC/DIABLO, Omi/HtrA2, AIF, and EndoG [165]. What these proteins share in common is that they carry out healthy functions within the mitochondria, but are toxic in the cytosolic compartment. For example, in healthy cells, cytochrome c participates in electron transport at the inner mitochondrial membrane as part of oxidative phosphorylation. In contrast, once cytosolic during apoptosis, cytochrome c binds Apaf-1 to

trigger assembly of the apoptosome in which procaspase-9 is activated. OMM permeabilization during apoptosis is promoted by BAX and BAK, pro-cell death members of the BCL-2 family of proteins [166]. While it is not known precisely how these proteins bring about permeabilization (e.g. one model involves pore formation), it is clear that homo- and hetero-oligomerization is important. BAX and BAK are regulated primarily through changes in their conformations. In the case of BAX, which resides in the cytoplasm of healthy cells in an inactive conformation, conformational activation [167, 168] is brought about by direct binding of BIM or a truncated form of BID (tBid), which are members of the BH3-only arm of the BCL-2 family. The function of BH3-only proteins is to bring death signals to BAX and BAK from other pathways in the cell. Activation of BAX exposes a transmembrane domain in its 9th α-helix that has a predilection for the OMM and presumably facilitates BAX mitochondrial translocation. BAK resides constitutively in the OMM and is thought to be activated in a similar fashion, although this has been studied in less depth. Anti-apoptotic BCL-2 proteins such as BCL-2, BCL-xL, and MCL-1 inhibit BAX and BAK both by functioning as sinks for BIM and tBid and possibly also through direct interactions with BAX and BAK.

The triggering event in mitochondrial-mediated necrosis is the sustained opening of the mitochondrial permeability transition pore (mPTP) in the inner mitochondrial membrane (IMM) [151]. In healthy cells, the OMM is impermeant to apoptogens - but allows the passage of ions and small molecules. Opening of the mPTP during necrosis results in rapid dissipation of the proton gradient across the IMM that is generated by pumping of protons into the intermembrane space during oxidation of substrates in the Krebs cycle. Since this transmembrane proton gradient is needed to drive ATP synthesis, mPTP opening abruptly stops production of new ATP. To further compound this energetic deficit, ATP consumption continues largely unabated during necrosis [169]. In contrast, apoptotic cells shut down ATP-requiring functions such as DNA repair, translation, and proteasome function [170–172] and experience less reduction in ATP synthesis. A second consequence of mPTP opening during necrosis is the ingress of water down its osmotic gradient into the solute-rich mitochondrial matrix. This causes matrix swelling, resulting in expansion of the redundant IMM and sometimes rupture of the OMM, which lacks redundancy. Rupture of the OMM sets up the possibility that apoptogens may gain access to the cytoplasm in necrosis (albeit via OMM rupture rather than permeabilization) and trigger caspase activation [147]. Given the cataclysmic events resulting from cessation of ATP synthesis, the extent to which subsequent engagement of the downstream apoptosis signaling contributes to cell death in necrosis is unclear.

The composition of the mPTP has been an area of great controversy [173]. The pore has often been depicted as a complex involving the voltage dependent anion channel (VDAC) in the OMM and the adenine nucleotide translocase (ANT) in the IMM. Genetic studies, however, have demonstrated that neither VDAC [147] nor ANT [174] are required for pore opening. Similarly, the mitochondrial phosphate carrier in the IMM, more recently hypothesized to be part of the mPTP, has proven to be dispensable [175, 176]. What then is the mPTP? Recent work suggests the unanticipated result that a core component is the F_1-F_0 ATP synthase itself [177–179]. While these data are exciting, additional studies will be required for in vivo proof.

The best characterized stimulus for mPTP opening are increases in the concentration of Ca^{2+} in the mitochondrial matrix [180]. The effects of increased [Ca^{2+}] on mPTP opening are sensitized by oxidative stress, increases in phosphate, and decreases in ATP and ADP[180–182]. These conditions operate during ischemia and/or reperfusion [183]. The binding site through which Ca^{2+} triggers mPTP opening is not known, however. A critical facilitator of mPTP opening is cyclophilin D, a peptidyl prolyl isomerase in the mitochondrial matrix [160, 184]. While it is known that cyclophilin D binds the F_1-F_0 ATP synthase [178] and it has been reported that the cyclophilin D prolyl isomerase activity is required for facilitation of mPTP opening [184], the precise mechanism is not understood. The drug cyclosporin A, which binds cyclophilin D, inhibits mPTP opening and necrosis [185]. Although not an essential component of the mPTP, ANT also functions as a positive regulator of pore opening [174]. Recently, the pro-apoptotic proteins BAX and BAK were found to be critical mediators of primary necrosis [163, 186]. Mice lacking BAX and BAK or BAX alone exhibit markedly decreased cardiac necrosis, apoptosis, and infarct size following ischemia/reperfusion in vivo [158, 163]. Analysis of BAX mutants shows that its apoptotic and necrotic functions are distinct. Current evidence supports two non-mutually exclusive models in which BAX functions as an OMM component of the mPTP and/or facilitates necrosis indirectly by promoting mitochondrial fusion.

Many questions remain concerning the mitochondrial events that mediate cell death. First, the complete composition of mPTP is not clear at this point. Second, the upstream signaling that feeds into both necrotic and apoptotic programs at the mitochondria remains incompletely understood especially in the case of ischemia/reperfusion. Third, the molecular connections linking apoptotic and necrotic programs at the mitochondria and the factors that determine how a specific cell will die are not known in any depth.

Despite these deficits in knowledge, inhibition of mitochondrial-mediated cell death has been contemplated – especially for ischemic syndromes. We will limit the discussion here to two points. First, there has been a clinical trial of cyclosporin A in 58 patients with ST-segment elevation myocardial infarction [187]. Administration of the drug at the time of percutaneous coronary intervention reduced serum creatine kinase but not troponin I. Hyperenhancement on MRI, a measure of infarct size, was reduced at day 5. At 6 months, however, there were no statistically differences in left ventricular function [188]. This study is inconclusive, but encouraging. It suggests that inhibition of mPTP opening may be an effective treatment strategy for reperfused myocardial infarction in humans, but also raises the question as to whether compounds more potent than cyclopsporin A need to be developed. Second, given that necrosis and apoptosis both contribute to the pathogenesis of myocardial infarction, selection of a therapeutic target such as BAX, which mediates both forms of cell death [163, 186], should be considered.

E. Generation of ROS

During electron transport if there is any leak of electrons it can lead to the generation of reactive oxygen species (ROS) and mitochondria are one of the major cellular sources of ROS. Mitochondria also contain antioxidant mechanisms to remove ROS. In low levels ROS

can act as a signaling molecule, whereas higher levels can lead to irreversible damage to mitochondria and cells and is a major contributor to cardiovascular disease.

In mitochondria ROS formation results from sporadic, possibly undesired, reactions occurring especially at the level of the electron transport chain (ETC) [189–191]. Besides these occasional processes, mitochondria also contain enzymes that catalyze H2O2 generation as the obligatory product [192].

The ETC drives electrons from reduced coenzymes ($NADH(H^+)$ and $FADH_2$) to oxygen that undergoes the complete reduction to water in the terminal reaction catalyzed by Complex IV (i.e., cytochrome c oxidase). A minor fraction (about 0.1%) of the electrons flowing through the ETC is suggested to cause the partial reduction of O2 into superoxide [189]. In particular, flavins or quinones of the first three complexes are able to act as single electron donors resulting in superoxide formation, especially under conditions that decrease the flow of electrons towards Complex IV where O2 is fully reduced to H_2O [189, 190]. Notably, ROS formation can also result from reverse electron flow. Recently, this concept has been supported by demonstrating in vivo that succinate accumulated during ischemia is oxidized during reperfusion resulting in large ROS formation that is likely due to the reverse electron flow within complex I [193].

ROS formation is favored by high mitochondrial membrane potential (i.e., low ATP synthesis), large $NADH(H^+)$ or when electron flow is hampered by alterations in respiratory complexes. Conversely, a decrease in ROS levels should follow the acceleration in electron flow caused by mitochondrial uncoupling [194], yet conditions have been reported in which mitochondrial uncoupling and $\Delta\psi_m$ dissipation are associated with an increased ROS formation.[135, 195] According to the model of “redox-optimized ROS balance” [196] this apparent paradox might be explained by a concomitant depletion of the antioxidative capacity that would result in H2O2 accumulation despite a decreased formation of superoxide by the ETC [197].

An increased ROS formation is also associated with the uncoupling-like effect generated by opening of the mPTP. Indeed, this process has been proposed to amplify an initial oxidative stress through the so-called ROS-induced ROS release (RIRR) [191]. ROS can trigger PTP opening through oxidative modifications of mitochondrial proteins involved in PTP formation and control, such as FoF1 ATP synthase e[198] or cyclophilin D [199]. However, despite evidence that ROS formation follows PTP opening [200, 201], the underlying mechanisms have not yet been elucidated. On the other hand, a slight increase in ROS formation resulting from opening of mitochondrial K^+ATP channels has been proposed to prevent mPTP opening and elicit cardioprotection [202–204]. A similar process could contribute to protection induced by preconditioning or postconditioning that is abrogated by antioxidant treatment [188, 205, 206]. Therefore, the notion that a mild ROS accumulation increases the resistance to oxidative stress [207] might be explained by opposite effects on the susceptibility to PTP opening elicited by slight and large ROS formation, respectively.

Superoxide that does not cross the inner mitochondrial membrane is rapidly dismutated into the freely permeable H2O2 by Mn-superoxide dismutase (Mn-SOD). The finding that Mn-

SOD deficient mice develop ROS toxicity and dilated cardiomyopathy [208, 209], underlines the importance of ROS in this pathology and mitochondria as their source and target. This concept is further supported by the beneficial effects afforded by targeting catalase expression in mitochondria [210–212].

Besides respiratory chain complexes, several other mitochondrial enzymes have been described as potential ROS producers. These include the flavin containing glycerol-3-phosphate-, proline- and dihydroorotate-dehydrogenase at the outer leaflet of the inner mitochondrial membrane, the electron transfer flavoprotein-ubiquinone (ETF:Q) oxidoreductase system of fatty acid β-oxidation within the inner mitochondrial membrane, and pyruvate- and 2-oxoglutarate dehydrogenase within the mitochondrial matrix [213]. However, the contribution of these enzymes to the overall ROS production of mitochondria within a given cell is difficult to establish. In fact, as is also the case with respiratory complexes, loss-of-function approaches (i.e., pharmacological inhibition or genetic deletion) would inevitably hamper the physiological functions of these vital proteins jeopardizing energy metabolism, ionic homeostasis and cell viability. Convincing demonstration that mitochondria generate ROS in vivo is also provided by interventions targeting mitochondrial enzymes, such as p66Shc and monoamine oxidases (MAO) that generate H_2O_2 as a direct and obligatory product.

In response to various stress stimuli, the cytosolic adaptor protein p66Shc translocates to mitochondria where it catalyzes H_2O_2 formation by means of electron transfer from cytochrome c to oxygen [214]. Indeed, ROS generation is reduced in cells lacking p66Shc and in p66Shc−/− mice, whose lifespan is increased by 30% [215, 216]. Furthermore, genetic deletion of p66Shc protects against ischemia/reperfusion (I/R) injury and diabetes-induced cardiovascular derangements [192, 217].

The two isoforms of MAO, A and B, are flavoenzymes located in the outer mitochondrial membrane. MAOs catalyze the oxidative deamination of catecholamines, serotonin and biogenic amines generating the corresponding aldehydes, H2O2 and ammonia. H2O2 and aldehydes [206]produced by MAO have been shown to synergize in disrupting mitochondrial function associated with loss of function and viability of the heart [192]. In addition, ammonia might stimulate ROS formation by dihydrolipoyl dehydrogenase, the E3 component of pyruvate and oxoglutarate dehydrogenase [218]. Interestingly in human atrial biopsies MAO has been shown to produce 10 times more H_2O_2 than the respiratory chain, and its expression is correlated with an increased risk for postoperative atrial fibrillation [219]. Major advantages with investigating the role of MAO in oxidative stress are given by a defined molecular structure, specific substrates and clinically available inhibitors. However, the substrates used and the mechanisms of activation under injury conditions are still not clear. In addition, the clinical use of MAO inhibitors in cardiovascular diseases is perceived as problematic due to a hypertensive reaction occurring when selective MAO-A inhibition is combined with intake of tyramine-rich food, such as aged cheese and alcoholic beverages. Conversely, MAO-B inhibition is devoid of this potential risk [220].

The list of dedicated enzymes for ROS formation in mitochondria includes nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4) [221]. At variance from other NOX

isoforms localized to the plasma membrane, NOX 4 displays a preferential localization to intracellular sites, appears to be constitutively active and generates H_2O_2 in preference to superoxide [222, 223]. Since the localization to cardiac mitochondria has been established based upon reactivity to antibodies that were not tested in NOX4−/− cardiomyocytes, further studies are necessary. In addition, the role in the cardiovascular system is debated, since NOX4 appears to cause both beneficial and detrimental effects in models of cardiac pressure overload [28, 29, [77, 221]

As in the rest of the cell, in mitochondria ROS generation is counterbalanced by efficacious removal systems. Besides superoxide dismutation by Mn-SOD, peroxide handling is carried out by a thiol redox system centered on glutathione (GSH and GSSG in its reduced and oxidized form, respectively) and thioredoxin (Trx) [224–226]. H2O2 is reduced into water by glutathione peroxidases (Gpx1 and 4) and peroxiredoxin 3 (Prx3) that is maintained into the active reduced form by Trx. In addition, GSH is used by the mitochondrial glutathione-S-transferase K to detoxify products of oxidative damage, such as α,β unsaturated aldehydes and alkyl hydroperoxides, and by glutaredoxin 2 that catalyzes the formation and reversal of protein-GSH mixed disulfides [225].

The oxidized forms of glutathione and Trx resulting from Gpx and Prx catalysis are reduced by the corresponding reductases at the expense of $NADP(H^+)$. The mitochondrial $NADP^+$ pool is reduced by malic enzyme, $\Delta\psi$m-dependent nicotinamide nucleotide transhydrogenase, and Ca^{2+}-modulated isocitrate deghydrogenase. Therefore, the $NADP^+$/$NADP(H^+)$ ratio links oxidative metabolism and mitochondrial function with ROS signaling and antioxidant activities [197]. Prx3 is responsible for more than 90% of H2O2 removal in mitochondria [227]. However, since Prx3 is highly susceptible to oxidation, under conditions of severe oxidative stress such as myocardial ischemia and reperfusion [228], Gpx1 might become the major sink for H_2O_2.[225] Prx3 overexpression confers increased resistance to ischemia-reperfusion injury [229] lending further support to the relevance of mitochondrial-generated ROS in cardiac diseases.

The occurrence and the relevance of ROS formation in mitochondria is supported by direct methods for the in vivo detection [230], effects of targeted antioxidants and enzymes [231], and decreased ROS accumulation following inhibition or deletion of mitochondrial ROS sources [232]. The majority of studies relate mitochondrial ROS formation with cell injury suggesting that beneficial effects are afforded by preventing ROS accumulation in a wide array of cardiovascular diseases, such as ischemia/reperfusion injury, heart failure, ageing and diabetic cardiomyopathy [190, 192, 217, 233, 234].

Although it is undeniable that high levels of ROS impair function and viability of any cell type, a large body of evidence indicates that ROS generated within mitochondria are involved in signaling processes that are crucial for the optimal response to physiological and pathological stimuli [192, 235–237]. Indeed, several reports document the crucial role of mitochondrial ROS generation in a wide variety of cardiomyocyte functions. The physiological role of mitochondrial ROS is likely to be linked to post-translational modifications of proteins, especially at the level of cysteine residues [238]. For instance, S-nitrosylation has been recognized as a cardioprotective mechanism that prevents irreversible

oxidation of proteins [225, 230, 239]. In addition, signaling pathways involving protein phosphorylation are modulated by oxidation of critical cysteines, especially in protein phosphatases [240]. Besides these short-term responses mitochondrial ROS are involved in long-lasting changes by acting on transcriptional factors, such as hypoxia-inducible factors (HIFs) and nuclear factor erythroid 2–related factor 2 (Nrf2) [226, 241–243].

Far from always being beneficial, a decrease in mitochondrial ROS levels could be detrimental. A large increase in glutathione content or the administration of N-acetylcysteine was shown to elicit mitochondrial oxidation and cytoxicity despite a decrease in ROS levels [244]. Suppression of mitochondrial ROS generation by mitochondrial-targeted catalase hampered autophagy worsening heart failure caused by deletion of mitofusin 2 [245]. Interventions aimed at reducing mitochondrial ROS levels, such as expression of dominant negative Nox [149], deletion of p66Shc [246], or ablation of thioredoxin-interacting protein [247], were found to exacerbate mild injury induced by ischemia-reperfusion protocols of short duration. This paradoxical notion that contrasts with protection by antioxidant treatments in prolonged episodes of ischemia-reperfusion suggests that mitochondrial ROS are involved in triggering self-defense mechanisms. Supporting this concept antioxidants abrogate the powerful protection of both ischemic pre- and post-conditioning [203, 204, 206]. In this respect, the term mitohormesis has been introduced to describe the j-shaped curve whereby low doses of mitochondrial ROS trigger beneficial adaptive responses that are replaced by detrimental processes at high doses [241, 248]. Although this concept appears to accommodate the protective effects of exercise and calorie restriction [248], especially in clinical settings methods are not available to define the threshold separating beneficial from harmful ROS levels. Other relevant issues to address are the interactions among the various ROS sources and the conditions involved in local compartmentalized ROS formation as compared to diffusion of ROS and oxidized products to the entire cell and surrounding tissues.

F) Mitochondrial Quality Control

1. Fission/Fusion/Mitochondrial Dynamics—As cells and organisms reproduce their mitochondria divide to repopulate the progeny. Mitochondria also divide and fuse back together in non-dividing, quiescent and postmitotic cells such as cardiomyocytes and neurons; however the rates in cardiomyocytes appear to be quite low. This continual fission and fusion cycle, a process also called mitochondrial dynamics, is known to be essential for the healthy maintenance of mitochondria and their host cells and organisms. Mitochondrial dynamics participate in mitophagy, apoptosis, differentiation and a variety of stress responses. The adverse consequences that interrupting *in vivo* cardiomyocyte mitochondrial dynamics has on mitochondrial stress, mitochondrial biogenesis, and programmed cardiomyocyte death were recently demonstrated in side-by-side comparative studies after conditional genetic deletion of either cardiac mitochondrial fusion or fission pathways [249].

Mitochondrial morphology reflects the relative rates of fission and fusion and can be visualized in fixed cells and tissues by immunostaining. Perturbing the ratio of fission and fusion rates will lead to either fragmented, punctiform mitochondria or excessively long or interconnected mitochondria. However, as mitochondrial morphology is dynamic, fission

and fusion rates are best visualized in live cells. Quantification of mitochondrial fusion rates can be performed by using photoactivatable green fluorescent protein (PAGFP) that is targeted to the mitochondrial matrix. When the mito-PAGFP is activated with a laser targeted to one mitochondrion, the PAGFP fluorescence is increased about 100 times and as this mitochondrion fuses with others, the fluorescence is diluted [250]. Quantification of this dilution rate reflects the organelle fusion rate independent of the fission rate and shows remarkable difference among cell types and changes for example, early during apoptosis. An alternate approach to assess fusion utilizes two cell populations, one expressing GFP in mitochondria and another expressing red fluorescent protein (RFP) in the mitochondria. When cells from these two populations are fused with polyethylene glycol the rate that the GFP and RFP merge to form yellow fluorescence reflects the mitochondrial fusion rate [251]. These techniques have been used extensively to characterize proteins that mediate the fusion process [252] and the physiological consequences of mitochondrial dynamics [253].

The molecular machinery that mediates mitochondrial fusion utilizes large GTPases in the dynamin family [252, 254]. Mitofusins (Mfn1, Mfn2) mediate fusion of the outer mitochondrial membrane, whereas Opa1 mediates fusion of the inner mitochondrial membrane. Mfn1, Mfn2 and Opa1 all require GTPase activity for fusion activity. Mfn1 and Mfn2 span the outer mitochondrial membrane with most of the protein and the GTPase domain facing the cytosolic compartment. Opa1 is localized within the intermembrane space anchored to the inner mitochondrial membrane. Mitofusins and Opa1 usually work in concert to coordinately fuse both mitochondrial membranes. Opa1 activity is regulated by proteolytic processing but how Mfn1 and Mfn2 are regulated is not yet clear.

Mitochondrial fission utilizes a large GTPase called Drp-1, a homologue of dynamin that is well understood to mediate fission of endocytic vesicles from the plasma membrane. Like dynamin during endocytosis, Drp1 assembles into spirals that wrap around mitochondria and appear to constrict the inner and outer membranes during GTP cleavage to start the fission process. Drp1 exists free in the cytosol from where it docks to mitochondrial fission sites by interacting with outer mitochondrial membrane spanning proteins Mff, Mid49 and Mid52 [252, 254]. Fis1 is a protein required for Dnm1 (a Drp1 orthologue) mediated fission in yeast, but is not required for fission in metazoans. Instead, Fis1 in metazoans participates in mitophagy [255]. Drp1 activity is regulated by phosphorylation at several sites on the protein. Phosphorylation at some sites stimulates fission, for example during the cell cycle, and at other sites phosphorylation inhibits fission. Interestingly, endoplasmic reticulum tubules wrap around sites of mitochondria prior to their fission and may play a role in defining the site of mitochondrial fission or in assembling the fission complex at the proper location [256].

Identification of these fission and fusion proteins has allowed generation of animal models and cell culture lines for the exploration of the physiological significance of mitochondrial dynamics. Mfn1, Mfn2 and Opa1 knock out mice are all embryonic lethal suggesting that both fission and fusion are required for maintenance of mammalian embryos. However, fibroblasts generated from the embryos survive in culture although with altered mitochondrial morphology and in some cases, metabolic deficits [252]. Cardiac myocyte specific knockout of Mfn1 and 2 in adults causes cardiomyopathy. Interestingly, myocytes

die after only 3–4 cycles of mitochondrial fission without opposing fusion [83]. Surprisingly, mitochondrial fusion is also linked to cardiac development. Through regulating calcium levels and calcineurin, mitochondrial dynamics control Notch signaling and stem cell differentiation into cardiomyocytes [257].

Mutations in several of the mitochondrial fusion genes have been identified to cause human disease [252, 254]. Dominant optic atrophy, the most common form of hereditary blindness, is caused by haploinsufficiency in Opa1. Thus, retinal ganglion cells are highly dependent, and more so than other human tissues, on fusion of the inner mitochondrial membrane. Another example is mutations in Mfn2 that cause Charcot-Marie-Tooth Disease Type 2A. Understanding the intriguing tissue specificity of defects from mutations in mitochondrial dynamics genes, that have what may be considered housekeeping duties, remains a major challenge in discerning the roles of mitochondria *in vivo*. Because of the links of mitochondrial dynamics to human and animal health, there are efforts to drug the pathway. For example, mDIVI is a small molecule that inhibits Drp1 and mitochondrial fission, which has been reported to have protective activity in numerous animal disease models including heart ischemia-reperfusion injury [258].

It is clear that mitochondria have to continually divide and fuse but what are the essential roles of mitochondrial dynamics at the molecular level? Mitochondrial fission has been linked to damage avoidance through segregation of debris within mitochondria. As discussed later in this Statement (Section I.F.2), mitophagy following fission, allows the clearance of damaged mitochondria and selective elimination of damaged proteins [253, 259]. Mitochondrial fission has been linked to apoptosis that can also function as a severe form of stress response. Mitochondrial dynamics are also required for proper transport of mitochondria to proper locations within cells. On the other hand, fusion between mitochondria is thought to allow compensation to help rescue organelles from damage by the exchange of proteins and RNAs from one mitochondrion to another [260]. Mitochondria accumulate mtDNA deletions and mutations over time and these mutations can generate mitochondrial proteins that are dysfunctional or misfolded. If mitochondria did not fuse, dysfunctional proteins could lead to serious loss of function consequences. However, fusion with another mitochondrion, that may have mutations in other genes, would allow compensation between organelles and avoid serious consequences of mutation accumulation. Mitochondrial hyperfusion is a broadly active stress response that may facilitate such compensation [261].

2. Mitophagy and mitochondrial autophagy*: mechanisms, overlap, and distinctions*—Mitophagy, literally meaning “eating mitochondria”, is the term applied to the cellular mechanism for identifying and selectively eliminating dysfunctional mitochondria as part of the overall mitochondrial quality control process [262]. Mitophagy is essential to sequester and remove senescent or damaged mitochondria that could otherwise accumulate and become sources of cytotoxic ROS (see Section I.E). Whereas the distal components of the mitophagy pathway, ie autophagosomal engulfment of mitochondria and their transfer to lysosomes for degradation and component recycling, are shared with macroautophagy, the proximal events that detect and select dysfunctional organelles for targeted elimination are highly specific for mitophagy. Two central proteins

driving this detection/selection process are the cytosolic E3 ubiquitin ligase Parkin [263] and the mitochondrial kinase PINK1[56], encoded by genes (*PARK2* and *PARK7*, respectively) for which loss of function mutations have been linked to autosomal recessive forms of Parkinson's disease [264].

The discovery that PINK1 and Parkin interact to promote mitochondrial fitness [265, 266], and elucidation of mitochondrial stabilization of PINK1 as the initiating event in mitophagy [267, 268], were central to our current understanding of the mechanisms underlying mitochondrial quality control. Briefly, healthy mitochondria maintain an electrochemical inner membrane gradient, $\Delta\psi_m$ that drives ATP production by the electron transport complex (see Section I.A). Senescent mitochondria are unable to sustain a normal $\Delta\psi_m$, and damaged mitochondria may completely dissipate $\Delta\psi_m$, resulting in depolarization. Mitochondrial $\Delta\psi_m$ status is a key determinant of PINK1-Parkin pathway activity: Healthy fully polarized mitochondria import and rapidly degrade PINK1, maintaining low kinase activity. Upon depolarization however, PINK1 degradation is suppressed [268], thereby increasing its abundance and promoting multiple PINK1 kinase-mediated events including Parkin translocation to [269], and activation at, [270, 271] mitochondrial outer membranes. At the mitochondrion Parkin ubiquitylates dozens of mitochondrial proteins [272], thereby promoting autophagosomal engulfment of the damaged organelle. The overall result for the cell is selective mitophagic destruction of depolarized mitochondria.

Mitophagy is inextricably linked to mitochondrial dynamism, i.e. mitochondrial fission and fusion. In many cells mitochondria form highly interconnected reticular networks that are constantly remodeling through periodic fission and fusion. However, in adult cardiac myocytes mitochondria fission/fusion are rare [273]. For this reason, in hearts it is likely that mitochondrial dynamism is dispensable for morphometric remodeling, but nevertheless plays an important role in cardiac mitophagic quality control through the process of dynamin-related protein (Drp)1-mediated asymmetric fission [274]. Accordingly, a mitochondrion in the early stages of senescence or one that has sustained moderate damage will segregate its dysfunctional components into one of the two daughter organelles generated by a fission event. The damaged (and therefore depolarized) daughter mitochondrion will be promptly identified as such and removed via PINK1-Parkin mediated mitophagy, whereas the healthy daughter will rejoin the cellular mitochondria pool, likely by fusing with other similarly fit mitochondria. The particular role for Parkin-dependent versus Parkin-independent or "alternate" mitophagy mechanisms in healthy and diseased hearts is only beginning to be investigated [275, 276].

Mitofusins (Mfn) 1 and 2, so designated because they promote physical tethering between mitochondria and subsequent GTPase-dependent mitochondrial fusion, also have a role in mitophagy. In addition to promoting fusion of the healthy daughters after asymmetric fission (see above), PINK1 kinase stabilization in damaged mitochondria results in phosphorylation of Mfn2 on two domains, thus conferring Parkin binding activity to this mitochondrial outer membrane protein and facilitating both Parkin translocation and its subsequent ubiquitination of mitochondrial proteins [55]. For this reason, hearts deficient in Mfn2 that do not exhibit defects in mitochondrial fusion (because Mfn1 is still present) instead develop a defect in mitochondrial quality control [55, 277].

There are surprising consequences of the mechanistic involvement of mitochondrial dynamism in mitochondrial quality control. For example, if mitophagy is malfunctioning but dynamism is intact, then the process of asymmetric fission will generate a highly dysfunctional daughter organelle that cannot be removed through the usual quality control process. Instead, the improperly retained damaged mitochondrion can fuse with, and by exchanging cellular components thereby damage, normal mitochondria within the same cell. An example of fusion-mediated mitochondrial contagion was recently uncovered in Parkin-deficient *Drosophila* fruit fly heart tubes [245]. In this model, because fusion contributed to the spread of mitochondrial damage, heart failure was attenuated by cardiomyocyte-specific suppression of *Drosophila* mitofusin (MARF).

An important role for PINK1-Parkin mediated mitophagy in normal functioning of the nervous system is clear from Parkinson's disease [278]. Surprisingly, while genetic suppression of PINK1 or Parkin in fruit flies is detrimental to mitochondrial fitness and normal functioning of neural tissue, skeletal muscle, and myocardium, [279] germ-line gene ablation of the orthologous mouse genes evokes only modest phenotypes [280]. In mouse hearts, germ line ablation of PINK1 and Parkin appears to produce only mild and slowly progressive basal cardiac dysfunction, but increased sensitivity to ischemic injury [84, 93, 281]. Likewise, cardiomycyte-specific Parkin ablation in adult mice provoked no detectable phenotype, and conditional cardiac Parkin overexpression had no detectable adverse consequences (Song M et al Circ Res 2015, epub ahead of print). Thus, it is possible that PINK1-Parkin mediated mitophagy is relatively unimportant to mitochondrial homeostasis in normal mammalian hearts. On the other hand, the absence of notable nervous system dysfunction (i.e. Parkinson's disease phenotypes) in these same mice [280], evidence of compensatory upregulation of alternate E3 ligases in hearts of germ-line Parkin knockout mice [245], and the cardiomyopathy that is evoked by cardiomyocyte-specific interruption of PINK1-Parkin signaling (through Mfn2 ablation) [55, 277] suggest that this pathway of mitochondrial quality control may indeed be important under specific and as yet incompletely understood circumstances. The true role of PINK1 and Parkin in mammalian hearts may only be uncovered by creating new experimental models and/or by additional human genetic testing for rare PINK1 and Parkin mutations in clinical cardiac disease. It is also worth mentioning that Parkin can also regulate fat uptake [282].

If it is correct that maintaining mitochondrial quality is essential to cell health, then the absence of damaging mouse phenotypes in PINK1 and Parkin knockout mice, and focal degeneration of dopaminergic neurons (rather than larger system-wide effects) in Parkinson's disease linked to human PINK1 or Parkin mutations, suggest the presence of one or more alternate pathways of mitochondrial quality control [283]. Indeed, mitochondria can be eliminated independent of PINK1 and Parkin by an autophagic mechanism that employs pro-apoptotic Bcl2 family proteins Nix and Bnip3 to target dysfunctional mitochondria and connect them to autophagosomes [284]. Conceptually, this mechanism resembles so-called "mitoptosis", in which opening of the mitochondrial permeability transition pore activates mitochondrial autophagy [285]. In this context, Nix and Bnip3 accumulate on damaged mitochondria, facilitate the permeability transition, and promote mitochondrial autophagy by serving as mitochondrial adaptor proteins that bind to autophagosomal LC3 or GABARAP [286–289]. Although Nix and Bnip3 are widely

recognized for their pro-apoptotic effects in cardiac failure following pressure overload hypertrophy and myocardial infarction, respectively [290–293], the possibility that they also promote homeostatic mitochondrial autophagy in hearts merits further investigation.

Dissipation of $\Delta\psi_m$, aka mitochondrial depolarization, contributes to the signal for PINK1 stabilization and initiation of Parkin-mediated mitophagy. Evidence is accumulating that ROS, which are also markers of mitochondrial dysfunction, can play a similar role in Parkin-independent mitochondrial autophagy [277, 294]. *In vivo* disruption of cardiomyocyte Parkin signaling by ablating its Mfn2 mitochondrial receptor evokes a cardiomyopathy. As expected, normalization of ROS with mitochondrial-directed catalase improves this mitophagic cardiomyopathy. In contrast, super-suppression of ROS (with mitoCAT expressed at higher levels) is detrimental, both accelerating and exacerbating the cardiomyopathy [277]. These findings reveal an essential signaling function for mitochondria-derived ROS in compensatory mitochondrial autophagy pathways induced when the Parkin pathway is interrupted.

3. Protein turnover independent of mitophagy—As discussed in Section I.F.2, damaged mitochondria can be removed by mitophagy. However individual mitochondrial proteins can also be damaged and may underlie several pathologic phenotypes [295]. It is therefore paramount that the renewal, or turnover, of proteins within mitochondria is sustained in times of enhanced cellular stress, as failure to maintain normal protein turnover may lead to accumulation of damaged/misfolded proteins and may underlie various disease etiologies. Protein turnover has been deemed “a missing dimension” in proteomics [296], as quantitative proteomic measurements typically involve the profiling of static protein abundance between different disease states or conditions. Recent advances in protein dynamics methodologies have enabled the simultaneous measurement of individual proteins comprising entire mitochondrial [244, 297–299] proteomes. A recent study provided the first assessment of global mitochondrial proteome kinetic signatures in a disease model of cardiac remodeling, which demonstrated that protein turnover rates are under independent control indicative of diverse regulatory processes driving remodeling of the mitochondria in disease [297].

Protein turnover measurements rely on the ability to track the rate at which individual proteins are being replaced by de novo synthesized proteins. Several methodologies have been utilized for these measurements, and all involve the introduction of an isotope precursor into a living system in order to mark individual proteins and determine their longevity in cells. For an excellent, comprehensive review on strategies used to measure protein turnover, including the experimental model, stable isotope label, labeling protocol, relative isotope abundance transition, and calculation of turnover rate, see [300].

Protein turnover rate is evaluated by tracking the integration or loss of a label into a protein pool. Proteins exhibit a diverse range of half-lives, with housekeeping proteins tending towards longer half-lives and regulatory proteins towards shorter half-lives. Thus, sampling times following initiation of labeling must cover an adequate range of time in order to accurately model proteins exhibiting both fast and slow rates of turnover. Moreover, the number of sampling timepoints is directly correlated to the accuracy of the labeling

trajectory. An additional consideration for complex organisms is the slow equilibration of the stable isotope label (e.g., ^{2}H) in a precursor pool (e.g., body water), which is incompletely labeled in *in vivo* labeling strategies and requires complex analysis to determine precursor pool enrichment. While each stable isotope methodology has its strengths and weaknesses, heavy water labeling has distinct advantages for translational research in that at low enrichment levels it is safe for humans over years [301], it is easy to maintain constant enrichment levels of ^{2}H in body water following $^{2}H_2O$ intake[302, 303], and it is the most cost effective stable isotope. Detailed methods and equations underlying computational analysis are outlined in [244], and the automated software, ProTurn[297] is available at http://www.heartproteome.org/proturn/.

Four recent studies have interrogated protein dynamics in mitochondria in whole animals or humans, using oral consumption of either deuterated leucine-labeled protein [298] or drinking water [244, 297, 299]. Two recent studies investigated mitochondrial protein turnover changes is cardiac pathologies. A translational study by Lam et al. [297], in which $^{2}H_2O$ labeling was employed to investigate changes in protein dynamics of cardiac proteins in mouse and blood proteins in both mouse and human. Turnover rates for 496 human plasma proteins, spanning a 50-fold range of turnover rates from albumin (half-life 18.3 days) to IGF2 (half-life 8 hours) were determined. Mice undergoing cardiac remodeling via chronic isoproterenol infusion were compared to controls, and turnover rates for 2,964 mouse proteins were assessed in mouse cardiac mitochondrial and cytosolic fractions and in blood. Rates were highly diverse and ranged from <1 day to >3 weeks (100-fold range). Cytosolic proteins turned over approximately 10% per day (average half-life of 6.5 days) and mitochondrial proteins approximately 5% per day (average half-life 15 days). Consistent with enhanced protein synthesis occurring in cardiac hypertrophy during remodeling, turnover rates in isoproterenol-treated mice averaged 1.2 times faster than in wild-type hearts with turnover rate increases detected in 972 proteins (>1.3 fold) and decreases in 216 proteins. In contrast, isoproterenol withdrawal, or reverse remodeling, led to an average of 20% decrease in protein turnover rate. Importantly, this study identified proteins exhibiting supernormal elevations or attenuations in protein turnover during cardiac remodeling, suggesting that these proteins may be pathogenic in the cardiac remodeling process. Proteins involved in mitochondrial dynamics showed heterogeneous results, in that some mitochondrial dynamics proteins exhibited elevated turnover (MIRO1/2, LONP and PHB), while others were unchanged (MFN1/2 and FIS1). Subunits residing in the same respiratory complex (ETC I-V) displayed widespread changes, thereby giving insight into proteins that may be rate-limiting factors in complex formation and highlighting the important finding that synthesis-degradation cycles of proteins within the same functional group (i.e. complexes, organelles) are under independent control. Interestingly, turnover measurements also unveiled markedly enhanced turnover in virtually all glycolytic enzymes (HKI half-life decreased from 16.7 to 9.8 days; GAPDH 10.8 to 6.7 days; phosphoglycerate mutase-1 12.2 to 6.9 days) in the absence of any changes in static protein abundance, thus providing mechanistic insights for supporting the alteration in substrate utilization, from fatty acids to glucose, known to occur in cardiac disease. Importantly, these novel mechanistic insights into cardiac remodeling are masked in measurements of static protein abundance, highlighting the unique biological dimension unveiled by protein turnover measurements.

Lastly, a study by Shekar et al. [299], used $^{2}H_2O$-labeling in rats to measure turnover in heart failure from two cardiac mitochondrial subpopulations, subsarcolemmal (SSM) and interfibrillar (IFM). This group investigated the hypothesis that mitochondrial protein synthesis (and thus, oxidative capacity) is decreased in transverse aorta constriction (TAC)-induced heart failure, and IFM exhibit more pronounced detriments than SSM. Results from this study showed an overall decrease in mitochondrial content in IFM, but not SSM mitochondrial populations, coordinate with a more pronounced detriment in basal and stimulated respiratory rate in IFM.

The physiological and pathophysiological implications gleaned from turnover measurements of individual proteins within functional mitochondrial subproteomes are many. Dynamic equilibria of proteins fluctuate in times of cellular stimulation or altered cellular stress, and protein turnover measurements provide a missing dimension of protein behavior that will enable mechanistic studies on the governance of protein synthesis and degradation. Very little is known regarding the interplay of these processes in tuning the abundance of individual proteins or protein complexes in the cell. Global turnover measurements with individual protein resolution, now possible for all detectable proteins within the mitochondrial proteome, have indicated that diverse regulatory mechanisms exist in metabolic pathways. Furthermore, it is now understood that protein half-life is an exquisitely regulated cellular parameter that is correlated with phenotype, however is mostly disassociated with static protein abundance. Protein dynamics provide a unique understanding of biological regulation of mitochondrial proteins, that when related to static protein abundance and mRNA expression, can inform on the poorly understood process of protein degradation in basal and diseased conditions.

Mitochondrial protein dynamics measurements have high translational significance regarding mitochondrial biomarker discovery and treatment of mitochondrial diseases. Mitochondrial diseases are a heterogeneous class of conditions that need sensitive and specific biomarkers for their accurate diagnosis and prognostic assessment. The advantages of using protein turnover measurements, rather than the commonly-employed protein abundance, for early detection of pathophysiological states has been discussed [304] and clearly demonstrated [305, 306]. The sensitivity of protein dynamics is in many ways superior to static protein abundance, in that alterations in turnover measurements will likely be more pronounced and may precede alterations in static protein abundance in pathologic progression of diseases. Protein turnover rate is also a critical factor in biomarker discovery (see Section II.C), as markers with lower clearance rates within the systemic circulation would be more robust indicators of disease states. Protein dynamics measurements will also unveil novel disease mechanisms (i.e., protein degradation insufficiencies), that will spawn the development of novel therapeutic classes.

Moreover, questions underlying the relationship between protein turnover and abundance remain. For example, certain mitochondrial proteins exhibit rapid turnover rates (and thus, consume a sizeable amount of ATP for their renewal) with little to no change in static abundance. Observations such as these may begin to challenge the conventional school of thought that the abundance of a protein present in a cell directly correlates with its

magnitude of impact in the cell. While several educated guesses have been put forth regarding questions like these, scientific evidence is lacking.

G) Signaling by release of mitochondrial DNA and proteins

As discussed in Section I.D, mitochondria are involved in the intrinsic pathway of apoptosis where they release soluble proteins, including cytochrome c from the intermembrane space into the cytosol to initiate caspase activation [307]. The release of these proteins is a consequence of mitochondrial outer membrane permeabilization. Other examples for functional molecules released from mitochondria include 1) ROS to activate hypoxic gene expression, ROS-dependent MAP kinase and damaged macromolecules including DNA and proteins, 2) calcium which participates calcium crosstalk between mitochondria and the plasma membrane and between mitochondria and the endoplasmic/sarcoplasmic reticulum and ATP from apoptotic and necrotic cells as a danger signal [308]. Recently, it has been reported that mitochondria DNA and proteins are involved in innate immunity. In this section, we will focus on the role of mitochondria in inflammation [309].

The immune system is activated not only by microorganisms infection, but also by endogenous molecules [310]. The endogenous molecules are separated from immune system sensors by plasma membrane and compartmentalization within the cell. However, endogenous molecules are released into circulation during necrosis or into the cytoplasm during degradation of organelles. These endogenous molecules that can induce inflammatory responses are referred to as damaged-associated molecular patterns (DAMPs). Pattern recognition receptors (PPRs) are responsible for sensing the presence of microorganisms by recognizing structures that are conserved among microbial species, which are called pathogen-associated molecular patterns (PAMP). Moreover, PRRs are attributable to recognizing DAMP. To date, four different classes of PRR families have been identified including transmembrane proteins such as Toll-like receptors (TLR) and C-type lectin receptors, as well as cytoplasmic sensors such as Retinoic acid-inducible gene (RIG)-I-like receptors and NOD-like receptors (NLRs). In addition to PRRs, inflammasomes are multiprotein complexes which contribute to the intracellular identification of potentially harmful substances, bacteria, or viruses. Inflammasomes are comprised of three major components, a characteristic scaffolding protein, the small adapter molecule ASC, and procaspase-1 which is responsible for the activation of pro-inflammatory cytokines. Those scaffolding proteins include NOD-like receptor family, pyrin domain containing 1 (NLRP1), Absent in melanoma 2 (AIM2), NLR family CARD domain containing protein 4 (NLRC4), RIG-I and NLRP3, and each protein forms the corresponding inflammasome in concert with ASC and procaspase 1.

Since mitochondria are evolutionary endosymbionts derived from bacteria, mitochondria still have many morphological and biochemical features of their bacterial ancestors, including a double-membrane, membrane lipid (cardiolipin), unmethylated CpG motifs in mitochondrial DNA, absence of histones and the ability to form N-formyl peptides, which are synthesized by the use of separate sets of rRNAs and tRNAs encoded by the mitochondrial genome [311]. Unmethylated CpG motifs and N-formyl peptides (NFPs) are inflammatogenic and mitochondrial DAMPs.

In response to pressure overload, mitochondria are damaged and damaged mitochondria are degraded by autophagy or mitophagy (see section I.F.2). Among PRRs, TLR9 senses unmethylated CpG motifs in bacteria and virus. Mitochondrial DNA is degraded by DNase II in the autolysosome, which is an acidic DNase and localized in the lysosome. When the induction of autophagy is insufficient in pressure-overloaded hearts, mitochondrial DNA escapes from autophagy-mediated degradation and binds to TLR9 to induce inflammatory responses cell-autonomously in cardiomyocytes, myocarditis, and dilated cardiomyopathy. However, it is reported that depletion of autophagic proteins promotes cytosolic translocation of mitochondrial DNA and caspase-1-dependent cytokines mediated by the NALP3 inflammasome in response to lipopolysaccharide in macrophages [312]. In addition to TLR9, the NALP3 inflammasome might be involved in inflammation in the failing heart. The role of autophagy in innate immunity may be cell type-dependent.

In the case with myocardial infarction, where necrosis is a main feature of cell death, mtDNA is released into circulation. The serum from patients after coronary intervention contains mitochondrial DNA [313]. Cellular disruption by trauma releases mitochondrial molecules including DNA into circulation to activate neutrophils and cause systemic inflammation [314]. Thus, it is possible that mitochondrial DNA in circulation after myocardial infarction may contribute to the inflammatory responses in infarct hearts.

Mitochondrial NFPs are released from degenerating mitochondria at tissue damage [314, 315] and recognized by formyl peptide receptors (FPRs), which have evolved to mediate phagocyte migration to sites of tissue injury. Although the role of NFPs-FRPs signaling pathway in heart diseases, specially myocardial infarction remains to be elucidated, it is possibility that the pathway may play a role in inflammation in infarct hearts.

The newly discovered role of mitochondria as DAMP-containing organelles places mitochondria in a central position as initiators and modulators of sterile inflammation in failing or infarct hearts. In case of pressure-overloaded hearts, autophagy regulates degradation of mitochondrial DNA and resultant inflammation in cardiomyocytes. In infarct hearts accompanied by necrosis, mitochondrial DNA released into circulation could activate and recruit various inflammatory cells in the lesion.

II) Mitochondria and Cardiovascular Disease

A) Mitochondrial myopathies-Mitochondrial etiology of cardiomyopathy

Cardiomyocytes have among the highest concentrations on mitochondria of any human cell. Because of the high mitochondrial ATP demands of the heart, relatively subtle defects in the mitochondrial ATP generating apparatus, oxidative phosphorylation (OXPHOS), can preferentially affect cardiac function.

If we rearrange the classification of the common diseases based on bioenergetics rather than anatomy, it becomes clear that all of the complex diseases can be envisioned as having the same underlying pathophysiological basis, partial bioenergetics dysfunction. Since the mitochondria are assembled from between 1000 and 2000 nuclear DNA (nDNA) genes plus thousands of copies of the maternally-inherited mitochondrial DNA (mtDNA) genes and the

mitochondria both process the calories in our diet into usable energy and are acutely sensitive to a wide range of toxins, it follows that perturbation of mitochondrial bioenergetics can readily explain the etiology of the full range of common clinical disease symptoms (Figure 2)[2].

The nDNA coded genes relevant to mitochondrial function include the approximately 1000 proteins located within the mitochondrion [316] plus all of the genes involved in regulating cellular bioenergetics including the signal transduction enzymes (AMPK, SIRTUINs, etc.); nuclear receptor transcription factors (peroxisome proliferator-activated receptor (PPAR) family, the heteromeric partners of the PPARPs (RXR), the PPAR gamma coactivator-1-alpha (PGC-1alpha) family of co-activators, etc.); environmentally regulated transcription factors (HIF1α, NFκB, etc.), nutrient sensing systems (PTEN, TSC, mTOR, etc.); regulators of mtDNA biogenesis (replication [POLG & Trinkle], transcription [POLRMT] and translation [mitochondrial ribosomal proteins and elongation factors]); and those chromatin remodeling systems that permit nDNA-mitochondrial gene expression. Because of their high energetic demand the tissues most commonly affected by partial mitochondrial dysfunction are the heart, brain, muscle, renal, and endocrine systems [2, 317–320].

While the nDNA codes for the great majority of mitochondrial proteins, the mtDNA codes for the thirteen most critical OXPHOS polypeptides plus the 22 tRNAs and two rRNAs for their expression. OXPHOS generates energy by coupling electron transport (complexes I, II, and IV) with ATP synthesis (complex V) through the electrochemical gradient.

Severe mitochondrial diseases can result from homozygous mutations in nDNA-coded mitochondrial genes [318, 321, 322] or severe mtDNA mutations. Milder mitochondrial disease can result from heterozygous nDNA mutations, from mild mtDNA mutations, or from severe mtDNA mtations that are heteroplasmic, the mixture within the cytoplasmic of mutant and normal mtDNA. Milder mtDNA mutations result in symptoms when approximating homoplasmic (pure mutant) while more severe mutations an lead to disease when heteroplasmic.

There are three classes of clinically relevant mtDNA variants: ancient adaptive variants, recent deleterious mutations, and somatic mutations that accumulate in tissues during development and with age. Ancient adaptive mutations have accumulated along radiating maternal lineages as women migrated out-of-Africa to populate Eurasian and the Americas. A subset of these mtDNA mutations changed OXPHOS function and human physiology permitting descendent populations to adaptation of the new environments. mtDNAs harboring these locally beneficial variants became regionally enriched by adaptive selection, and as their descendants acquired additional mutations a group of related haplotypes developed known collectively as a haplogroup. While beneficial in one environment, these variants can be maladaptive in anther environment. Various mtDNA haplogroups have now been correlated with a broad spectrum of diseases including Alzheimer and Parkinson disease, macular degeneration, psychiatric disorders, stoke, diabetes, cardiovascular disease, sepsis, asthma, AIDs progression, various forms of cancer, types of athletic performance, and longevity [2, 320, 322–324].

Recent deleterious mutations continually arise within modern female lineages. Hundreds of such pathogenic mutations have been identified and are catalogued in our mtDNA database, MITOMAP[325]. An example of a frequently homoplasmic “mild” pathogenic mutation is the common Leber Hereditary Optic Neuropathy (LHON) complex I gene missense mutation, *ND4* nt 11778 G>A (R340H) [322, 326, 327]. Examples of more severe heteroplasmic mtDNA mutations are the $tRNA^{Lys}$ nt 8344 A>G mutations which can manifest as hearing loss at low heteropmasmy but cardiomyopathy and Myoclonic Epilepsy and Ragged Red Fiber (MERRF) disease at higher heteroplasmy [328, 329] and the $tRNA^{Leu(UUR)}$ nt 3243A>G mutation which at 50–90% mutant heteroplasmy can manifest as myopathy and cardiomyopathy [330] or stereotypically as Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-Like Episodes (MELAS) [331] but at 10–30% mutant can present as Type I or Type II diabetes [332, 333] or autism [334]. Somatic mtDNA mutations accumulate throughout life, progressively eroding mitochondrial function. The rate of accumulation of these mutation can be modulated by nDNA gene variants [40, 335–337] resulting in degenerative diseases such as Alzheimer and Parkinson Disease [338–340], and like can be induced by environmental challenges.

A major reason for the complexity of mitochondrial diseases is the reliance of every aspect of cellular function on energy flux. Thus, the energy status of the mitochondrion must be monitored by every functional change in the cell. This is accomplished by all cellular functions being either driven or regulated my mitochondrial high energy or metabolic intermediated. The importance of this mitochondrial signaling to the nuclear-cytosolic epigenome and signal transduction systems has been demonstrated by examining the cellular and transcriptional changes that occur in cells with the same nucleus by different percentages of the $tRNA^{Leu(UUR)}$ nt3243G mutation. This revealed cellular structure and gene transcription changed in discrete phases in response to the progressive increase in percentage of mutant mtDNA, relative to homoplasmic normal mtDNAs, with 20–30% 3243G mutant having one transcriptional profile, 50–90% mutant a second, and 100% a third. Thee phase shifts in the transcriptome exactly correspond to the changes in patient phenotypes that are associated with this mutation, 10–30% diabetes and autism, 50–90% cardiomyopathy and MELAS, 100% perinatal lethality [341].

mtDNA Variation and Cardiomyopathy: Cardiomyopathy has been associated with all three classes of mtDNA variants, ancient adaptive mutations, recent maternal mutations, and somatic mutations. Ancient adaptive mutations have been linked to increased risk of metabolic and cardiovascular disease [342, 343] and mice heterozygous for the mitochondrial antioxidant enzyme, MnSOD (*Sod2*) are prone to hypertension [344].

Recent deleterious mtDNA mutations have repeatedly been linked to cardiomyopathy [322]. While heteroplasmic mtDNA mutations are commonly accepted as causal for cardiomyopathy, homoplasmic mutations are more difficult to distinguish from ancient adaptive variants which may or may not be contributory. One approach to identify potentially pathological homoplasmic mutations is to first determine the mtDNA haplogroup using MITOMAP and MITOMASTER [325] in association with PHYLOTREE [345]. Then determine if the putatively deleterious homoplasmic mutation has been previously observed with that haplogroup in the normal population. If then it is increasingly likely that the

homoplasmic variant may be contributory to the disease [346]. The pathogenicity of the mutation must still be confined by functional tests in cybrids [347]. However, it is also possible that a variant has been observed before but in a different haplogroup context. Such variants may be deleterious on the wrong mtDNA background and thus might contribute to the diseases [348]. Proof that a homoplasmic mtDNA mutation can cause cardiomyopathy comes from the generation of a mouse that harbors an mtDNA missense mutation in the *COI* nt 6598T>C V421A and develops hypertrophic cardiomyopathy [349].

De novo and somatic mtDNA mutations also cause cardiac disease. Spontaneous single event heteroplasmic deletions in the mtDNA can cause the Kearns-Sayre Syndrome which frequently presents with cardiac conduction defects and heart block in association with chronic external ophthalmoplegia (CPEO) [322]. The accumulation of a heterogenous array of somatic mtDNA mutations has also been shown to be associated with cardiomyopathy in the hearts of heart transplantation patients, as monitored using the common mtDNA 5 kb deletion [6, 350].

nDNA Mitochondrial Gene Mutations and Cardiomyopathy: Cardiomyopathy is one of the primary presentations of boys with Barth Syndrome which results from mutations in the nDNA-coded X-linked Tafazzin gene required for cardiolipin metabolism [351]. Cardiomyopathy is also the primary symptom of homozygous mutations that inactivate the chromosome 4 heart-muscle-brain isoform of the adenine nucleotide translocator (*ANT1*) [352, 353]. The ANTs, of which there are four in humans, exchange mitochondrial ATP for cytoplasmic ADP across the mitochondrial inner membrane. *ANT1* inactivating mutations result in mitochondrial cardiomyopathy and myopathy [354–356]. This has been confirmed by the generation of a mouse lacking the *Ant1* gene which also developes mitochondrial myopathy and hypertrophic cardiomyopathy. The *Ant1*-deficient mouse has a partial defect in cardiac mitochondrial ATP production, since the heart also expresses a second ANT isoform, Ant2. The *Ant1*-deficient mice develop lactic acidosis, mitochondrial ROS production, and a striking increase in the accumulation of cardiac mtDNA somatic mutations [209, 357]. The hypertrophic cardiomyopathy of these mice progresses to dilated cardiomyopathy as the mice age [358]. Presumably this is because of the age-related accumulation of somatic mutations which exacerbate the inherited nuclear *Ant1* mutation.

Patients that develop dilated cardiomyopathy associated with myocarditis develop antibodies to ANT [359, 360]. These antibodies may be either the initiation and/or promoting factor in developing the cardiomyopathy. In either case, a mitochondrial bioenergetic defect cause by viral infection or preexisting mitochondrial genetic defect which impairs mitochondrial energetics could inhibit the energy demanding apoptotic process permitting the release of mitochondrial antigens (DAMPs)[cardiolipin, mtDNA, N-formyl methionine initiated polypeptides, ANT1, etc.] [311, 314, 361, 362] into the blood stream where the can initiate a cardiac inflammatory response.

Mutations in many other mitochondrial encoded genes have been reported to cardiovascular disease. For example, defects in the mitochondrial matrix protein frataxin are involed in Friedreichs ataxia [363]. Defects in the mitochondrial phosphate transporter have also been associated with cardiac defects [364].

nDNA-mtDNA Interactions and Cardiomyopathy: The genetic complexity of cardiac diseases is further enhanced by the potential of the deleterious interaction of nDNA and mtDNA genetic variants. This pathogenic nuclear-mitochondrial interaction was first demonstrated by the discovery that mutations in the nDNA-coded mtDNA polymerase γ gene can resulted in autosomal dominant multiple mtDNA deletion syndrome, the mtDNA damage causing CPEO and potentially cardiac symptoms [322, 337]. Mitochondrial disease has also been shown to result from the interaction nDNA-coded partial mitochondrial genetic defects and homoplasmic mtDNA mutations. This was exemplified by in a family segregating a missense mutation (G32R) in the X-linked complex I *NDUFA1* gene resulting in an approximately 30%reduction in complex I plus a pair of non-haplogroup-associated homoplasmic missense mutations in the mtDNA complex I genes *ND1* 3308T>C (M21T) and *ND5* 12599T>C (M88T). Both the X-chromosomel and the mtDNA in boys are inherited form the mother resulting in affected boys along the maternal lineage [365].

The severity of cardiac disease resulting from a nDNA mitochondrial gene defect can also be modulated by the inheritance of otherwise normal mtDNA haplogroup. In a 13 generation pedigree segregating a frameshift mutation in the *ANT1* gene, multiple homozygous patients were identified with mitochondrial myopathy and cardiomyopathy. However, the severity of the cardiomyopathy varied strikingly with some individuals progressing to dilated cardiomyopathy necessitating heart transplantation while others maintained a relatively stable hypertrophic cardiomyopathy. Sequencing of the mtDNAs from the homozygous *Ant1* frameshift patients revealed that those that progressed to heart transplant had mtDNA haplogroup U2 while those that manifest stable hypertrophic cardiomyopathy were haplogroup H [356].

This augmentation of deleterious nDNSA mutations by mtDNA variants may be a more general phenomenon. Analysis of the mtDNAs of cardiomyopathy patients with mutations in the nDNA-coded cardiac contractile apparatus proteins have been found to contain potentially contributory mtDNA mutations [366, 367].

Conclusion: The high energetic demands of the heart are primarily met by mitochondrial OXPHOS. Hence, it follows that defects in mitochondrial bioenergetics should preferentially affect the heart. This is proven by the fact that cardiomyopathy is the most obvious phenotypic manifestation in both humans and mice harboring null mutations in the heart-muscle-brain isoform of the ANT. If mutations in nDNA-coded mitochondrial genes can generate cardiomyopathy, it follows that mtDNA mutations causing partial OXPHOS defects should also contribute to cardiomyopathy. This is supported by the observations that mtDNA haplogroups and *de novo* mtDNA mutations can augment the deleterious consequences of nDNA mutations. If inherited homoplasmic or heteroplasmic mtDNA mutations can cause cardiomyopathy, the mitochondrial dysfunction resulting from the accumulation of somatic mtDNA mutations could also cause cardiomyopathy. This leads to the conclusion that the age-related accumulation of mtDNA mutations which augment inherited or acquired mitochondrial defects, in addition to the effects of secondary inflammation from the release of mitochondrial DAMPs, may provide a coherent conceptual framework for understanding the etiology of many forms of mtDNA mutations which augment inherited or acquired mitochondrial defects, in addition to the effects of secondary inflammation from the release

of mitochondrial DAMPs (see Section I.G), may provide a coherent conceptual framework for understanding the etiology of many forms of cardiomyopathy.

B) Cardiotoxicity

Anticancer treatments have improved significantly over the past few years. However, despite the improvement in their target effects on cancer cells, anticancer treatments have also been associated with an increase in the incidence of side effects. One of the major side effects of anti-cancer drugs is their toxicity towards cardiac muscle cells. This cardiotoxicity can manifest at an early stage of therapy (within days) or many years after treatment. Thus, patients undergoing these treatments should be monitored closely. More importantly, patients at high risk should be identified before treatment is started in order to reduce morbidity from cardiotoxicity. This would require close collaboration between cardiologists and oncologists.

Two forms of cardiac damage have been characterized. One form, typically seen with anthracyclines, is associated with irreversible damage and death of cardiac cells, while the second form generally occurs with protein kinase inhibitors, and is associated with reversible myocardial dysfunction. These two forms are discussed below.

Anthracycline-related Cardiotoxicity (Type 1): Anthracyclines have been used for the treatment of various forms of cancer for several decades. They constitute one of the major successes in the field of cancer. For example, in pediatric oncology, the 5-year survival rate has increased from ~30% in the 1960s to 70–80% today [368, 369], and >50% of childhood cancer patients have received anthracyclines [370]. Different statistics have been published on the incidence of anthracycline -mediated cardiotoxicity, ranging from ~1% to up to 48% [371]. However, a strong correlation exists between the incidence of cardiotoxicity and the dosage of the drug [372].

Although the effects of anthracyclines on cancer prevention is thought to be mainly through inhibition of DNA replication, RNA replication, DNA cross linking and topoisomearses [373], their cardiotoxic effects appear to be through distinct mechanisms. The pathophysiology of anthracycline-mediated cardiotoxicity is likely multi-factorial and multiple mechanisms have been proposed. It has been suggested that anthracyclines induces an increase in reactive oxygen species (ROS) production [374, 375]. According to this model, oxidation of the aglycone portion of doxorubicin (DOX) results in the formation of a semiquinone radical, which can rapidly revert to its parent compound by using O_2 as an electron acceptor [376]. This futile redox cycle leads to the formation of superoxide, which is converted to H_2O_2 spontaneously or by superoxide dismutase. Subsequently, H_2O_2 may be converted to highly toxic hydroxyl radicals in the presence of heavy metals, such as iron, through the Fenton reaction. In addition, DOX can interact with iron directly to form a DOX-Fe complex [376, 377]. In addition to production of ROS, other mechanisms including mitochondrial dysfunction and depletion of energy, induction of apoptosis and changes in topoisomerase IIb activity have been proposed [378, 379].

Although different mechanisms have been proposed for the cardiotoxicity of anthracycline, the mitochondrial effects are likely the major contributor to this disorder. Mitochondrial

dysfunction, including mitochondrial swelling, mitochondrial cristae disruption and accumulation of myelin figures have been observed after treatment with anthracyclines. There is also evidence that anthracyclines penetrates into the mitochondria (although the mechanism for this transport is not clear). After translocation of anthracyclines into the mitochondria, it can then interact with a number of molecules, including: 1) mitochondrial DNA (mtDNA). Anthracyclines can cause damage by inducing large scale deletions within mtDNA, form covalent bonds with mtDNA after transformation of the quinone ring to quinone methide, and cause damage to mtDNA indirectly by producing ROS [380]. 2) Electron transport chain (ETC)/oxidative phosphorylation (OXPHOS). Anthracyclines can cause damage to ETC and OXPHOS proteins through ROS production or by forming a complex with cardiolipins present in the mitochondrial membrane [381, 382]. The latter can also cause an increase in ROS or may lead to cardiolipin dysfunction, which is needed for normal activity of several enzymes in the ETC/OXPHOS pathway. 3) Mitochondrial permeability transition pore (mPTP). mPTP plays a major role in the regulation of cell death [383]. Anthracyclines have been shown to induce mPTP opening and cyclosporine A (which inhibits mPTP by binding to cyclophilin D) prevents mitochondrial failure and cell killing [384]. 4) Mitochondrial iron. Iron has been known to mediate some of the cardiotoxic effects of anthracyclines, however, iron chelators were shown not to be effective against anthracycline-mediated cardiotoxicity. It is now demonstrated that anthracyclines cause mitochondrial iron accumulation and that a reduction in mitochondrial iron is protective against the cardiotoxic effects of anthracyclines [385].

Drugs for other disorders: Several drugs that are currently routinely used for common diseases also have cardiac side effects. For example, it is now known that glitazones can worsen heart failure, and their mechanism of action may be through modulation of the activity of peroxisome proliferator-activated receptor (PPAR) proteins in the heart [386]. Although PPARs regulate metabolic processes, they may also have an effect on the mitochondria through indirect mechanisms.

C) Biomarkers

Primary mitochondrial diseases constitute a broad spectrum of disorders affecting multiple tissues and organ systems. It is estimated that mitochondrial diseases affect 1 in 5000 individuals [387], however it is suspected that 1 in 250 may be more accurate [388], as many cases likely go undiagnosed. Clinical diagnosis of mitochondrial diseases is complicated by diverse phenotypical manifestations, in part due to variably affected genomes (i.e., mitochondrial or nuclear), organs (e.g., muscle, liver) and age of onset. Thus, clinical protocols required for definitive diagnosis are labor-intensive and routinely involve invasive procedures with variable success. It would therefore be of great clinical use to discover biomarkers in accessible biofluids (e.g., blood, urine) that specifically inform on primary mitochondrial diseases. Mitochondrial-derived biomarkers, including mitochondrial DNA (mtDNA) (see Section I.G), proteins and metabolites, have been explored, however these have exhibited limited specificity, sensitivity and diagnostic/prognostic accuracy. Current markers can only add weight to the likelihood that a patient may have a primary mitochondrial disorder, and at best may only substantiate a more invasive diagnostic testing regimen.

MtDNA sequencing from intact cells acquired through tissue biopsy (most commonly skeletal muscle) in symptomatic patients constitutes the most definitive current measure for diagnosing mitochondrial disease. However, this method is invasive and is not amenable to routine screening or suitable for high-risk populations. The non-invasive quantification of cell-free mtDNA in plasma has gained substantial interest for diagnosing certain cancers [389, 390] and for other clinical scenarios such as predicting ICU mortality [391], but has not yet been successfully interrogated for the diagnosis of primary mitochondrial disorders. Mitochondrial proteins and metabolites quantified in accessible biofluids have received limited interest; however show great promise as biomarkers of mitochondrial diseases in that they are highly specific and sensitive indicators of underlying metabolic changes.

Improved strategies are warranted for the specific diagnosis of mitochondrial disorders, and biomarkers are most promising to this effort, as disease-specific mitochondrial-derived proteins, metabolites, or mtDNA would likely offer superior sensitivity and specificity in diagnosis/risk assessment and could be accurately and consistently measured across all clinical settings. Below is an overview of mitochondrial-derived markers (mtDNA, proteins and metabolites) that have been the subject of intense investigation.

Blood and urine are readily accessible biofluids acquired with minimal invasiveness. Blood can be centrifuged to separate aqueous plasma or serum from blood cells. Mitochondrial-derived biomarkers found in plasma/serum are cell-free circulating (cfc)-mtDNA, metabolites and proteins. Thrombocytes and leukocytes, but not erythrocytes, contain mitochondria, thus mtDNA and proteins can be obtained from intact blood cells in the more dense fraction. However, since mitochondrial genomes are heteroplasmic and primary mitochondrial diseases can affect various organs, disease markers will likely be tissue-derived, rather than blood cell-derived; thus, cell-free markers in plasma likely represent a more relevant and unbiased assessment of mitochondrial disorders. Urine would also provide an unbiased source of metabolites. Cfc-mtDNA in plasma and serum has been a subject of intense interest as a biomarker of disease since the first published study showing a mutation in cfc-mtDNA in patients with type-2 diabetes mellitus [392]. Mt-DNA is a 16.5 kbp circular strand of DNA, and hundreds to thousands of copies of mtDNA can be found in a single cell. Though the precise physiology is unclear, it is thought that cfc-mtDNA enters the bloodstream through cell necrosis, apoptosis or active secretion[393]. Cfc-mtDNA in plasma exists in both particle-associated and free forms; and methodology to measure amounts in whole blood are exquisitely dependent upon preparatory protocols used (e.g., size exclusion filtration), indicating that cfc-mtDNA exists in various conformations [394]. Mt-DNA can be reliably assayed using a quantitative real-time PCR approach, which exhibits a dynamic range of 5 orders of magnitude and a sensitivity of detection down to one copy of mtDNA [394]. Thus, mtDNA possess favorable physical characteristics for a good candidate biomarker of various physiological states. However, while cfc-mtDNA has shown promise for early detection and tumor classification in oncology patients [389, 390], its role in diagnosis and prognosis of mitochondrial diseases remains to be determined.

Despite the sophisticated and sensitive technologies we have available to measure mitochondrial-derived biomarkers, no non-invasive biomarkers for mitochondrial disorders have materialized from the discovery phase to the clinic. One of the most prominent reasons

for this is that studies aimed at biomarker discovery have been poorly designed, having little clinical translational potential [395]. Plasma biomarker studies should ideally proceed through an evolution of systematic tests that appropriately credential markers for clinical use, [see [396]]. A gap exists between biomarker discovery studies, where thousands of biomarkers exhibit differential profiles in case versus controls, and clinical tests, where assays are optimized and standardized for rapid and efficient measurement in the clinic. Within this gap is the narrowing and prioritizing of likely biomarker candidates, followed by rigorous steps of validation. Despite the overall lack of clinically available biomarkers, there are promising candidates that have surfaced in recent studies that, with appropriate validation strategies in large clinical cohorts, may add value or replace current clinical markers and diagnostic/prognostic regimens. A study by Suomalainen et al.[397] found that serum fibroblast growth factor 21 (FGF-21) was a sensitive and specific marker for primary muscle-manifesting respiratory chain deficiencies in adults and children. This study employed 67 patients with mitochondrial diseases diagnosed by muscular biopsy and DNA analysis, 34 non-mitochondrial neurological disease controls and 74 healthy controls. FGF-21 had superior diagnostic accuracy to conventionally-used, more non-specific mitochondrial disease indicators, including lactate, pyruvate, lactate-to-pyruvate ratio and creatine kinase. A later prospective study supported these results and indicated that FGF-21 is a superiorly sensitive biomarker for diagnosing both mtDNA and nuclear DNA encoded mitochondrial diseases [398]. Taken together, these results showed that FGF-21 may provide a first-tier, non-invasive biomarker for diagnosing mitochondrial diseases that may diminish the need for invasive muscular biopsies. Rigorous validation using large, well-characterized patient cohorts is warranted to verify the utility of FGF-21 in the clinic. Another recent study by Enns et al.[399] examined GSH and GSSH protein levels in whole blood from 58 control and 59 patients with primary mitochondrial disease of varying etiologies, including Leigh syndrome, electron transport chain abnormalities, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes, mtDNA deletion syndrome, and mtDNA depletion syndrome. Quantitative mass spectrometric analysis determined that in patients with mitochondrial disease, redox status was significantly more oxidized (lower GSH and higher GSSH), showing the greatest change in patients that were hospitalized with metabolic crisis. Hence, GSH and GSSH may be clinically useful biomarkers for all primary mitochondrial disease subtypes, and should be rigorously evaluated in clinical trials for demarcating clinical prognosis and patient response to redox-modulating treatments.

In conclusion, the low diagnostic/prognostic accuracy of primary mitochondrial diseases warrants intense research on mitochondrial biomarkers. The proper experimental design utilizing cutting edge omics technologies will undoubtedly propel this area of research in fields of study is the best response to the challenge of mitochondrial biomarker realization.

III. Summary and Recommendations:

The data in the literature show that mitochondria play a key role in cardiovascular disease, specifically in the response to myocardial ischemia and the transition to heart failure. The accumulation of defects in mitochondrial electron transport, ion transport, metabolism, redox regulation, and mitochondrial quality control lead to a feed forward cycle of further acquired defects. Ultimately the mitochondria can no longer meet the high energetic

demands of the cardiac cell and that coupled with an increase activation of cell death pathways leads to the death of the myocytes. The role of mitochondrial defects in heart failure and other cardiovascular diseases needs to be considered and evaluated.

There are a number of gaps in our understanding that need to be addressed in future studies. We need a better understanding of the effects of post-translational modifications on protein function. In recent years we have made great strides in defining mitochondrial post-translational modification, but the consequences of the modification is in many cases poorly understood. We need additional information on the mechanisms by which mitochondria regulate apoptosis and necroptosis. We need to define the mitochondrial permeability transition pore. The F1F0-ATPase has been proposed as the pore; this need to be verified by other groups and the mechanism of its regulation needs to be defined. We need to define the redox sensitive component of this pore. We need to more completely define the mechanisms that regulate mitochondrial dynamics and elucidate how this regulates cell function and metabolism. We also need additional information on the mechanisms that regulate mitochondrial turnover at the level of mitochondrial proteins and the organelle. What are the signals for turnover of mitochondrial proteins and how is this accomplished?

Additionally, the interrelationship between mitochondria and other intracellular compartments (endoplasmic reticulum, lysosomes etc.) and intracellular structures (mitochondria associated membranes) to regulate mitochondrial function and overall cellular homeostasis, are increasingly being recognized [400–402]. Our understanding of how mitochondrial function and pathophysiology integrate within this more complex intracellular environment will also be necessary to enable the modulation of quality-control programs to sustain cardiomyocyte homeostasis and stress resistance.

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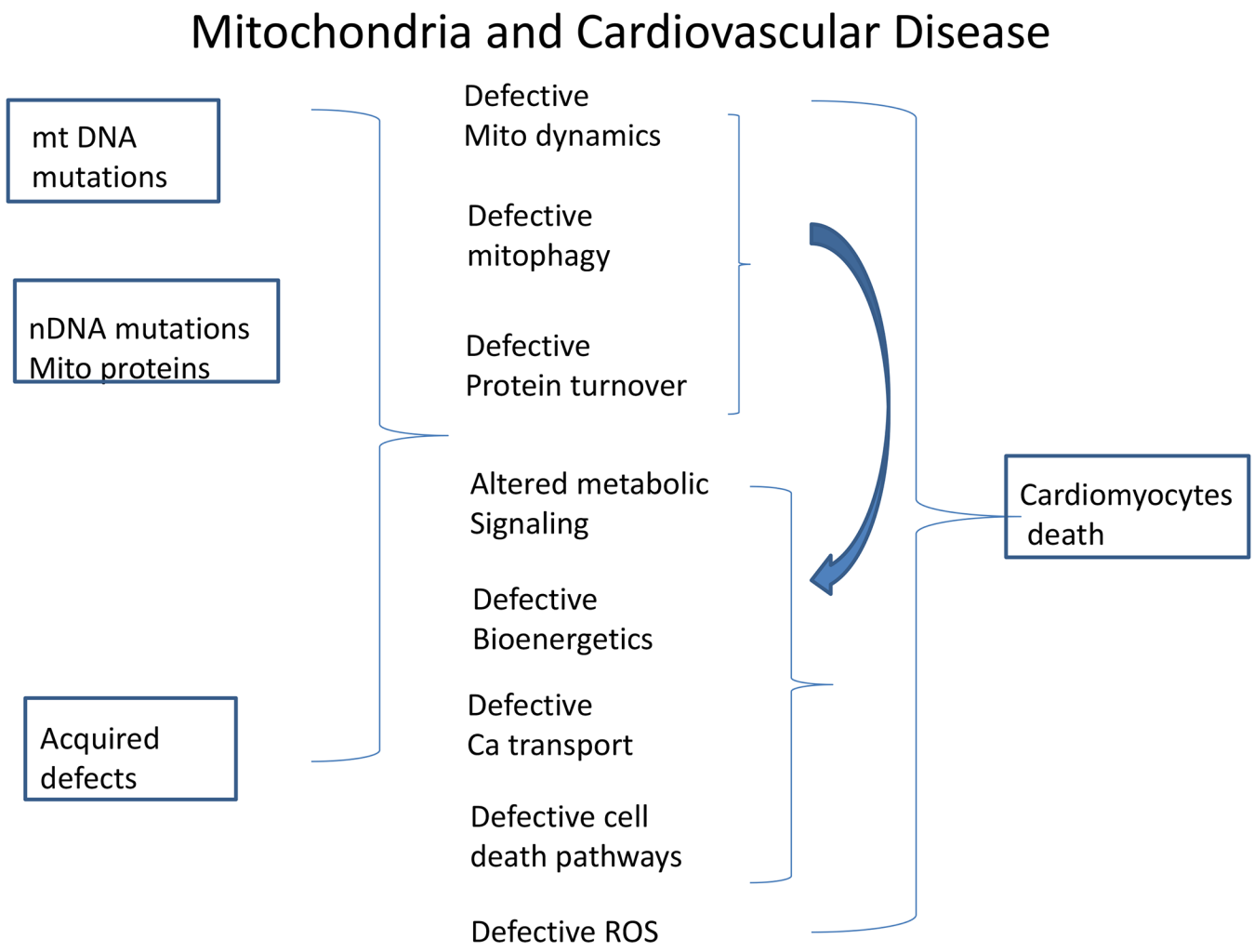

Figure 1.

Mutations in mitochondrial proteins (either from mutation in mitochondrial DNA or nuclear DNA) or acquired defects can lead to defects in mitochondrial quality control which leads to a vicious cycle of more acquired mitochondrial defects and defects in metabolic signaling, bioenergetics, calcium transport, ROS generation and activation of cell death pathways. This leads to a vicious feed-forward cycle leading to cardiomyocyte cell death.

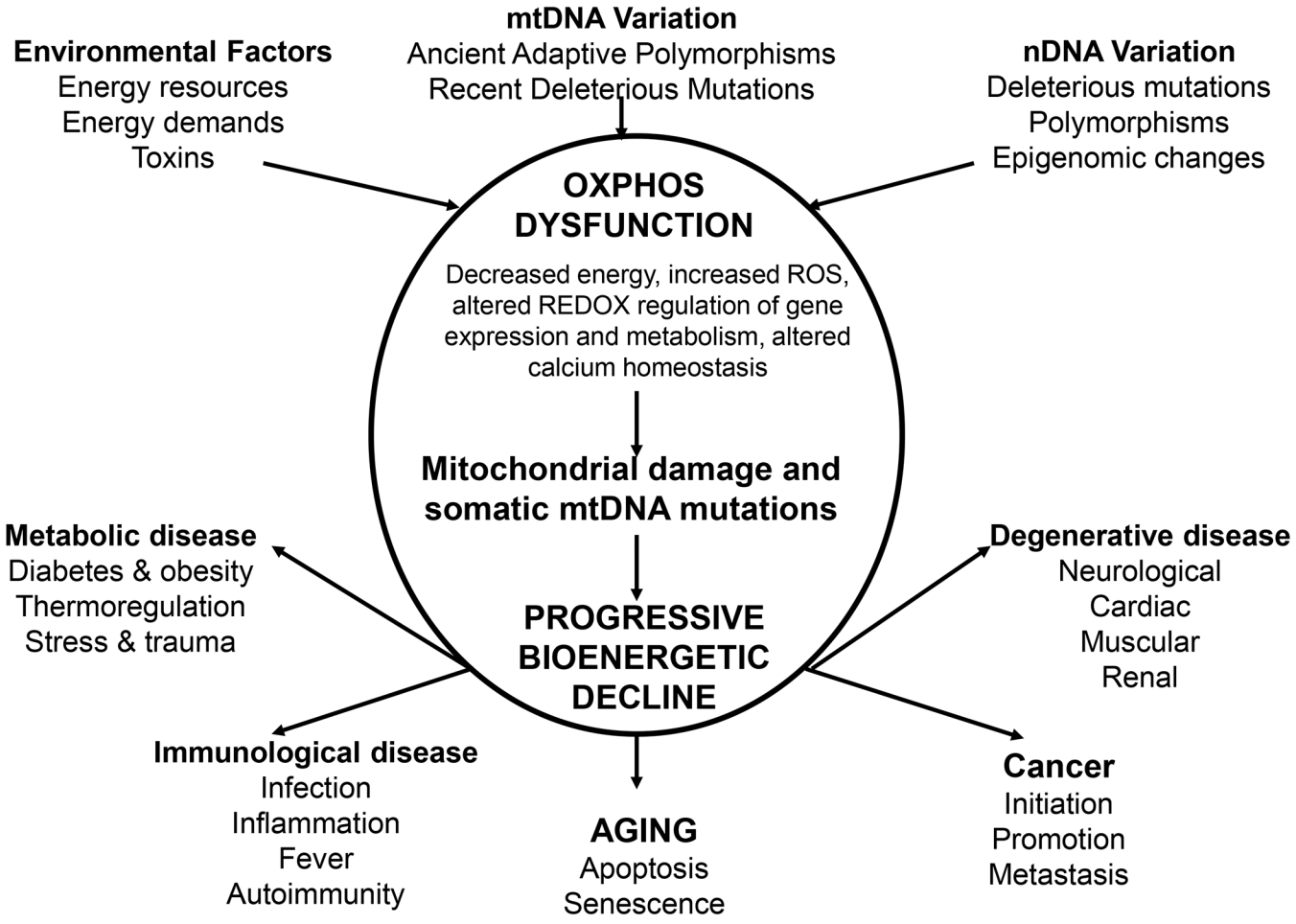

Figure 2:

Bioenergetic paradigm for degenerative and metabolic diseases, cancer, and aging. Mitochondrial OXPHOS can be perturbed by nDNA genetic alterations and/or epigenomic regulation, by mtDNA ancient adaptive of recent deleterious mutations, or by variation in the availability of calories and in caloric demands. Alterations in mitochondrial structure and function can impair OXPHOS, which in turn can reduce energy production, alter cellular redox state, increase ROS production, deregulate Ca^{2+} homeostasis, and ultimately activate the mtPTP, leading to apoptosis. These and other consequences of OXPHOS perturbation can destabilize mtDNA. This results in progressive accumulation of somatic mtDNA mutations and decline of mitochondrial function, which accounts for aging and the delayed-onset and progressive course of degenerative diseases. As energy output declines, the most energetic tissues are preferentially affected, resulting in degenerative diseases of the heart, muscle, nervous system, and kidney. Aberrant mitochondrial caloric metabolism also leads to metabolic deregulation, endocrine dysfunction, and symptoms such as diabetes, obesity, and cardiovascular disease. Energetic failure of apoptosis can result in the release into the blood stream of mitochondrial antigenic cardiolipin, *N*-formylmethionine polypeptides, and mtDNA (mitochondrial damage associated molecular patterns (DAMPs) [311, 314, 361, 362]) can initiate the inflammatory response, contributing to autoimmune diseases (e.g., multiple sclerosis and type I diabetes) and possibly also to the inflammatory component of

late-onset degenerative diseases. Finally, cancer cells must manage energy resources to permit rapid replication. Figure from [2].