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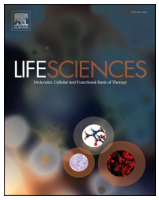



Metabolomics and mitochondrial dysfunction in cardiometabolic disease

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ABSTRACT

Circulating metabolites are indicators of systemic metabolic dysfunction and can be detected through contemporary techniques in metabolomics. These metabolites are involved in numerous mitochondrial metabolic processes including glycolysis, fatty acid β-oxidation, and amino acid catabolism, and changes in the abundance of these metabolites is implicated in the pathogenesis of cardiometabolic diseases (CMDs). Epigenetic regulation and direct metabolite-protein interactions modulate metabolism, both within cells and in the circulation. Dysfunction of multiple mitochondrial components stemming from mitochondrial DNA mutations are implicated in disease pathogenesis. This review will summarize the current state of knowledge regarding: i) the interactions between metabolites found within the mitochondrial environment during CMDs, ii) various metabolites' effects on cellular and systemic function, iii) how harnessing the power of metabolomic analyses represents the next frontier of precision medicine, and iv) how these concepts integrate to expand the clinical potential for translational cardiometabolic medicine.

1. Introduction

Metabolomics is the study of the set of small molecules that comprise the structural, signalling, and metabolic units in a biological environment [1]. This environment can range from cells to tissues, circulating and excreted fluids, and intricate biological microenvironments such as atherosclerotic plaques and cancerous tumours. The analysis of metabolites has recently become a field of intense focus, both to provide mechanistic insights to disease pathogenesis, and understand the effects of pharmacological interventions on various physiological environments [2]. To analyze the composition and abundance of metabolites, several techniques have been developed that exploit the size, electric charge of an ionized molecule (in the case of mass spectrometric techniques), structural chemistry, and magnetic nuclear spin (in the case of nuclear magnetic resonance spectroscopy) of molecules. Using a variety of separation techniques, these molecules can be analyzed repeatedly to both identify and describe relative metabolite concentrations within and between samples [3,4]. Metabolomics has been particularly useful in identifying the relative abundance of metabolites in various physiological systems that are implicated in disease states such as cardiometabolic diseases (CMDs) [5–7]. Historically, there has been significant interest in the analysis of *circulating* metabolites (those found in total blood or blood components), as they provide a snapshot of irregular metabolic perturbations that characterize various systemic diseases, help identify corollary and causative markers for disease, and are relatively easy to access clinically (accessible from a single blood draw) [8]. The blood carries both the inputs and regulators of cellular metabolism, and functions as a transport mechanism for signalling between organ and tissue systems; fluctuations in metabolite ratios can alter metabolism and downstream regulation of gene expression [9–12]. Epigenetic modifications and changes in gene expression are known to be caused by alterations in circulating metabolites, particularly in tissues and cells sensitive to changes in metabolite levels [13]. Additionally, on a cellular level, the metabolites generated from oxidative and glycolytic metabolism within mitochondria have gene regulatory roles in both the nuclear and mitochondrial genomes [13–15]. Mitochondria are hubs for cellular metabolism and can mediate the generation of metabolic intermediates involved in metabolic reprogramming in CMDs. This review will synthesize current knowledge of well-characterized and novel metabolites that influence cellular metabolism and redox balance in skeletal muscle as a model organ and opportunities in integrating metabolomics for use within clinical metabolite profiling and biomarker discovery. As metabolomic analyses often produce large sets of differentially regulated metabolites, it is important to outline that this review

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will discuss key bioenergetic metabolites derived from metabolomic analyses of the circulation. Due to the length and scope of this review, only energy-related metabolites outlined as biomarkers from relevant studies of CMDs will be explored, but various other types of metabolites are also important to explore in understanding the full metabolomic landscape in CMD.

2. Metabolomics - outline of the field and contemporary techniques

2.1. Omics and assay-based metabolite analysis

'Omics' refers to a group of related analytical tools that help understand 'omes', a (typically large) set of biochemical objects found within a biological system [16]. The now-familiar term 'genome', which refers to the total set of genes in an organism, was introduced by Hans Winkler in 1920 as a portmanteau of the words ***gen****e* and *chromo****some*** [17]. The genome is a 'static' omics set in that the set of genes comprising the genome are unchanged over time unless they are directly manipulated [16]. Since the establishment of the genome concept, numerous 'dynamic' omics sets and their respective omics disciplines have emerged. Dynamic omics sets are susceptible to change through the influence of numerous physiological, pathological, and environmental variables [16]. These sets include the total set of transcriptional (gene) products (transcriptomics) [18], protein products (proteomics) [19], and metabolites (metabolomics) [20]. Historically, characterizing cellular physiology through the techniques of molecular biology has relied on experiments that manipulate one or several cellular components, including proteins, enzymes, lipids, signalling molecules, and transcription factors. Through the accumulation of this relational data through assay-based experiments, the function, activity, and regulation of these cellular components and their role in maintaining cellular homeostasis have been elucidated and validated [21]. Further, dysregulation of the production, elimination, or activity of these components during disease states has been used to characterize and propagate the understanding of disease mechanisms. A prominent example of such an experiment is the DiaSorin Liaison® Platform Assay (https://www.diasorin.com/en/node/8476). This assay reports the concentration of circulating 25-hydroxyvitamin D (25(OH)D), a bioactive by-product of vitamin D metabolism, using a chemiluminescent signal. 25(OH)D is crucial for the maintenance of stable health and the prevention of numerous diseases including cancer, autoimmune, cardiovascular, and metabolic disorders; therefore the quantification of circulating 25(OH)D is vital in providing the justification for any therapeutic interventions [22]. However, biochemical assays such as this can suffer from a lack of reproducibility [23], the inability to multiplex (i.e., detect the levels of multiple related metabolites simultaneously), and tend to be relatively more expensive for repeated use versus high throughput analysis techniques [24].

Many assay-based studies have helped elucidate molecules involved in the development of metabolic disorders, such as the elevation of blood and urine glucose levels in diabetics [25]. These assays detect molecular abundance using a variety of indicators, including by colourimetric means (colour changes due to chemical reactions with assay reagents or by reporter molecules), and chemiluminescence (upon stimulation or binding to a light-emitting antibody). Quantification of molecules of interest relies on the comparison of emission/absorbance/relative colour change to that of standards of known concentration. However, these studies typically cannot quantify the abundance of multiple related and unrelated metabolites adequately and simultaneously, which leads often to unreproducible results. For example, a well-documented issue with 25(OH)D detection is that this molecule exists in multiple isoforms, namely 25(OH)D_2 and 25(OH)D_3. The former arises from Vitamin D_2, a plant-derived version of Vitamin D, and the latter from Vitamin D_3, a version produced de novo by epithelial cells upon UV exposure. Numerous assays that measure 25(OH)D are unable to differentiate between these two byproducts, overestimate levels of 25(OH)D_3, and cannot accurately measure low levels of 25(OH)D_2 [26,27].

2.2. Emergence of metabolomics

In 1971, Evan and Majorie Horning outlined a method for 'metabolic profiling' in urine using a gas chromatography technique to analyze multiple metabolites within a sample at once; one of the first documented uses of 'metabolomics' [28]. Since then, numerous metabolic profiling techniques have been implemented, several of which incorporate the basic tenets of chromatographic separation [4]. After the recognition of these tools in characterizing the metabolite 'environment', the metabolome had been increasingly appreciated as an incredibly descriptive means of understanding physiological dynamics. Since the suite of human metabolites has largely been defined, current techniques allow for robust, simultaneous analyses of a consistent number of known metabolites [29]. Metabolomics has the power of identifying the abundance of all metabolites detected within a sample (untargeted metabolomics), analyzing predetermined metabolites of interest using internal standards (targeted metabolomics), and characterizing the metabolome typical of the biological specimen, system, or pathological state in question (metabolomic fingerprinting) [4]. This 'fingerprint' allows for robust comparisons between metabolomic screens across various independent experimental variables – diet, age, sex, genetic variation, etc., which may be useful for the diagnosis of disease, generation of predictive biomarkers for disease onset, and to gauge the systemic effects of drug interventions [30,31].

2.3. Metabolomic analysis techniques

Metabolomic methods consist of a variety of technologies to analyze the total or partial metabolite content within a sample. Traditional metabolomic studies have frequently utilized nuclear magnetic resonance (NMR) spectroscopy, a technique of structural chemistry that detects the relative native resonance profile of metabolites in a magnetic field. These measurements provide insight into the structures of individual metabolites based on the resonance of individual atoms and their spatial relationship to others within a molecule [32]. NMR characterizes the atomic spin of atoms that have a magnetic moment including ^{1}H and ^{13}C (H-NMR and C-NMR, respectively). Using H-NMR as an example, in the presence of a magnetic field and the emission of radio waves (of a frequency typically between 60 and 100 MHz), hydrogen atoms will reach a high-energy state by absorbing wave energy. The release of this absorbed energy after atoms are returned to the low-energy state is detected by a radio wave receiver. The distribution of recorded peaks in an NMR spectrum (whose distance from each other is measured in parts per million (ppm)) is determined by the dividing the radio frequency value needed to create the high-energy state above that of a known chemical reference (tetramethylsilane) by the operating frequency of the radio-wave transmitter. The relationship between the areas underneath each peak helps elucidate the structure of the molecule through an understanding of intramolecular electronic and atomic dynamics [32]. Emwas and colleagues have presented guidelines and recommendations of the processing of this spectral data and subsequent data analysis [33]. Analysis of plasma or serum samples through NMR is beneficial as samples are not destroyed or wasted during NMR analysis. Therefore, it is useful for the analysis of samples to be kept for long term use [34]. Additionally, NMR provides highly accurate data regarding the structural chemistry of metabolites. However, NMR suffers from both a lack of sensitivity and low resolution in samples that are highly complex, such as biological fluids. Therefore, the amount of features detected is relatively lower compared to other metabolomic techniques [35,36].

Mass Spectrometry (MS) technologies are now more commonly used to separate and quantify metabolites found within a biological sample. MS utilizes two structural aspects that differentiate molecules from one

another: size and ionic charge. In MS, molecules are ionized (which creates intact ions through 'soft ionization' or charged ionic fragments through 'hard ionization'). Numerous soft ionization methods are currently employed, the most common of which include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). ESI is the most common soft ionization technique and uses a nebulizing fluid to carry the sample solution. The solution is exposed to high heat and an electric field of 3–5 kV, where the surface of the liquid droplets become charged. Through solvent evaporation, the droplets become increasingly smaller and multiple-charged molecular ions are left over. This method is suitable for large molecular mass, thermally unstable, moderately polar metabolites including major biomolecules such as nucleic acids, sugars, and amino acids. APCI utilizes a corona discharge electrode that heats up and ionizes the analyte solution directly in the gas phase, rather than using a nebulizing fluid. Molecules that are more non-polar and lower molecular mass are preferred in APCI and can be conducted in atmospheric pressure conditions. In APPI, photon emission by a discharge lamp (typically emitting Krypton at 10 eV) ionizes analyte molecules without the need for a nebulizing solution, similar to APCI. Largely non-polar and small molecules such as fatty acids can be detected using APPI. The use of soft ionization for ESI, APCI, and APPI ensures that ions are not heavily fragmented and these methods can be used with a wide variety of mass analyzers [37]. Compared to these soft ionization techniques, electron ionization (EI) is a traditional, yet highly effective and reproducible method used for volatile samples with low molecular weight. EI utilizes a beam of electrons which collide with analyte solution in the gas phase to produce positively charged ions and ion fragments. The mass spectra of EI are relatively reliable compared to soft ionization techniques, however, the formation of ion fragments may complicate analysis due to the mixture of molecules of different charges and sizes entering the mass analyzer [37]. The uses, benefits, and drawbacks of these ionization methods as well as NMR methods are outlined in Table 1.

These ions are then accelerated through a mass analyzer. Mass analyzers employ different physicochemical aspects of input ions to separate them for detection. These mass analyzers are summarized and evaluated in Table 1.

2.3.1. Magnetic sector

In Magnetic Sector mass analysis, ions are injected into a magnetic sector mass analyzer and follow a path that is perpendicular to a magnetic field of strength B, which itself is perpendicular to the motion of the ion. Because of this, the ion follows an arched circular path whose radius r is inversely proportional to the charge of the ion (q), the ion's mass (m), and the magnetic field strength (B). The ions are separated from one another by their momentum through the field [62]. A magnetic field scan is performed by varying the field strength which allows ions of various sizes to separate continuously. Magnetic sector instruments are used in inorganic analysis to determine levels of isotopes and broadly in organic mixtures. Increasing the mass range (which is traditionally relatively narrow for magnetic sector instruments) is accomplished by increasing field strength and the radius of the magnet, but fringing effects and transmission limitations are often found in magnetic sector instruments due to the shape of the collection slit where the ions exit. Magnetic sector instruments can accept hard or soft ionization sources but the scanning speed and cost make it unsuitable for modern use [38].

2.3.2. Time-of-flight

Time-of-Flight (TOF) mass analyzers exploit the inverse relationship between mass and velocity in a stable electric field or no field [48]. Ions of different mass and velocity that are launched at the same time will disperse in space and take varying amounts of time to finish their travel; the lower mass ions will approach the detector in a shorter time than heavier ions. TOF provides rapid scanning speed, and it is often paired with Matrix-Assisted Laser Desorption Ionization (MALDI) methods [46]. MALDI utilizes a solid organic compound called the 'matrix'. The specimen (typically peptide or protein mixtures) is extracted, purified, and then dried after being mixed with the solid matrix compound. A UV laser irradiates the specimen-matrix crystals, stimulating proton transfer and resulting in singly-charged ions. The ions are vaporized and then accelerated through a constant electric field so that they acquire the same kinetic energy necessary to enter the time-of-flight tube which lacks an electric field. TOF uses the mass (and momentum) of the ion in determining its flight time and the use of vacuum conditions improves resolving power drastically [38]. This method is widely employed and is often paired with quadrupoles for mass selectivity.

2.3.3. Quadrupole

Linear Quadrupoles utilize four hyperbolically shaped rod electrodes; pairs of opposite rods hold DC or AC components. When ions are launched into the quadrupole chamber, an attractive force is enacted upon the ion by the DC current and radiofrequency (RF) potentials on the rods. By selecting for specific DC and RF potentials, the rods can be used to select for specific mass-to-charge (m/z) ratios of the incoming ions and trap these ions when desired, so accurate quantitation can be performed [39,63].

2.3.4. Linear ion traps

Linear ion traps: (also called Paul traps) are highly popular due to their increased sensitivity to low abundance molecules, while maintaining a strong ability to resolve low abundance metabolites. Ion trapping involves two end-cap electrodes which provide a route for ions to enter and exit, as well as two hyperboloidal ring electrodes to which voltage is applied. Ions that are injected into the ion trap orbit the chamber in a periodic motion that is induced by the electric field maintained by a constant voltage applied to the ring electrodes. This is typically an alternating current (AC), however optional direct current (DC) can be added to influence ion trajectory if needed. The AC voltage is called the radiofrequency (RF) voltage as it is in the radiofrequency range, and this is raised in order to influence the trajectory of ions based on their mass and charge properties [55]. When the frequency of the ions' motion matches the frequency introduced by the supplemental AC source, the ions are ejected in the axial direction through gaining kinetic energy. The entering ions are introduced in a pulsed mode, unlike magnetic sector and quadrupole systems however, which utilize continuous introduction of ions into the mass analyzer in scanning mode. The pulsing of ions allows for ions of specific m/z to be selected and analyzed at a given time but considers the fixed volume of the ion trap that can only be filled with an estimated 10^5 ions. Ion injection is done in a way to eject packets of ions of specific m/z while considering that an accumulation of ions in the trap space can cause electric repulsions that lower the overall resolution of detected clouds of ions over a large period of time [52]. The RF voltage is raised at a fixed rate which ensures all ejected ions are detected through an external detector based on their resonant RF value. Early implementations of ion traps utilized the mass selective instability technique, where the frequency of the end-cap electrodes is kept constant but the main RF amplitude increases; when this happens, ions with the lowest m/z value are ejected when they match the auxiliary frequency. This scanning continues to higher m/z molecules [51,65]. The mass cut-off in this mode is inversely proportional to the RF amplitude, so applying higher RF voltages lowers the mass cut-off limit, theoretically increasing the possibility that low mass metabolites can be detected [54]. Additionally, collision-induced dissociation (CID) was introduced as a means to further fragment excited precursor ions using inert gases such as helium to drive increased collisions. Increasing the partial pressure of helium has been shown to increase resolution and sensitivity of detected metabolites. CID creates product ions that can be detected [50]. Si and colleagues (2017) have demonstrated that the use of 25 consecutive cycles of linear ion traps – called 'repeated ion accumulation' – aided in the detection of low-abundance ions such as ATP, whose detection sensitivity was

Table 1
A summary of the uses, advantages, and disadvantages of common ionization sources, mass analyzers, and chromatographic separation techniques in mass spectrometry, and variations of nuclear magnetic resonance spectroscopy, used in metabolomic analyses.

		Uses	Advantages	Disadvantages
Chromatography – Mass Spectrometry				
Separation techniques	LC-MS	Thermally stable, non-volatile, large molecular mass, moderately polar molecules, broad applications in metabolomics [38]	• Adequate separation of metabolites in biofluids • Simpler sample preparation than GC–MS • Can be used with various mass analyzers • Broad detection of metabolites with good sensitivity [38,39]	• Ion suppression effects • Presence of adduct ions • Not as good as GC–MS for low molecular mass and non-polar metabolites • Results not as reliable as NMR [36,40]
	GC–MS	Thermally unstable, volatile molecules of low molecular mass, used in lipidomics and chemical analysis [38,39]	• Very sensitive compared to LC-MS • Good resolution for volatile molecules • Can be used with multiple ionization sources • Good for analysis of complex lipid mixtures and chemical identification [38,39]	• Derivatization is required • Not suited for non-volatile and polar metabolites • Lower dynamic range [38,39]
Ionization methods	ESI	Commonly used in LC-MS, used for broad ionization of non-volatile, thermally stable molecules [41]	• Most common mode of soft-ionization • Creates multiply charged ions – can increase resolution • Good for moderately-highly polar metabolites that are easily ionized[41]	• Lower ionization efficiency than APCI and APPI • Not suitable for non-polar and low mass molecules • Collision of gases and overcrowding further reduces ionization efficiency [42,43]
	APCI	Lipidomic and environmental chemical analysis, analysis of low polarity molecules and those below 1500 da [42,44]	• Good coverage of small and more sensitive for non-polar molecules (fatty acids, lipids) • Greater ionization efficiency than ESI • Can accommodate more ions compared to ESI • Use of dopant increases conductivity and ionization in non-polar molecules [38,42,45]	• Not suitable for large, polar metabolites (carbohydrates, large sugars) • Less common than ESI for LC-MS • Smaller mass and polarity range • Ionizes LC solvents which (reduces selectivity) [42,45]
	APPI	Used in analysis of non-polar metabolites containing ring structures, lipid species, used in food, natural product, and pharmaceutical analysis [42–45]	• Covers lower polarity range than APPI and ESI • Reduced noise • Great ionization efficiency due to direct ionization and use of dopant molecules • Pairs well GC–MS both need analytes to be in gas phase [42,45]	• Not suitable for polar and very low molecular weight molecule • Not as common in practical use • Type of dopant molecules effects ionization efficiency and sensitivity • Not as well suited for LC-MS [43,45]
	EI	Commonly used and early form of ionization, good for volatile and very small molecules [38]	• Reliable and reproducible mass spectra • Good for electrically conductive and volatile compound analysis • Fragments ions which may increase structural information • Complex mass spectra [38]	• High fragmentation makes identification difficult • Crowding of large molecules make identification difficult • Thermally stable ions not analyzed[39]
	MALDI	Identification of molecules in tissue and solid mixtures, low molecular weight and in microbial identification [41,46]	• Laser ionization produces singly charged ions • Reduces loss of sample integrity • High throughput speed • Can be run on solid samples [41,46]	• Lower sensitivity for less abundant metabolites and proteins • Poorer resolution compared to other ionization methods • Typically only paired with TOF mass analyzer • Affected by matrix adduct formation [41,46,47]
Mass analyzers	Magnetic sector	Early form of mass analyzer, used in inorganic chemical analysis, broad applications for chemical separations [38]	• Can be used with EI and ESI • Generally accurate mass measurement • Use of double-focusing magnetic fields can improve resolution [38]	• Not as common for organic and biomolecule analysis • Slow scanning speed and moderate resolution • Requires internal calibration [38]
	Time-of-flight	Analysis of complex mixtures of protein, lipids, small molecules from biofluids and tissues [48]	• Widely used in metabolomics • Used in diagnostics to identification of low abundance and molecular weight metabolites • Pairs well with MALDI and other mass analyzers (i.e. Quadrupole) for mass selectivity • Use of internal and electronic mirrors increases range and resolution [48,49]	• Worse sensitivity than other mass analyzers including quadrupoles • Depends on space allocation within time of flight tube (aided by use of internal mirrors) [38]
	Quadrupole	Analysis of complex mixtures of generally polar metabolites, commonly used with TOF analyzer in tandem [50,51]	• Useful for identification of molecular ions and ion fragments • Very good sensitivity • Can be paired with numerous ionization sources • Use of multiple quadrupoles increases yield of low signal ions and detection range [38]	• Lower resolution • Limited by instrumentation and electronics used [50,51]

(*continued on next page*)

Table 1 (*continued*)

		Uses	Advantages	Disadvantages
	Ion traps (Fourier Transform)	Separation by time of ions compared to separation by space methods, broad range of ions can be resolved in complex mixtures [50,52–54]	• Excellent resolution and sensitivity of ions and broad ranges of molecular masses • Can be used with multiple ionization sources and other separation-in-space mass analyzers (i.e. TOF, quadrupole) • Helpful in structure elucidation • Potential for tandem MS in-time [52,53]	• 1/3 cutoff rule (1/3 cut-off rule): failure to trap ions at low end of *m/z* range, below 1/3 of *m/z* value of precursor ion • Space charge effects negatively impact mass accuracy, dynamic range, resolving power [52,53,55]
Nuclear Magnetic Resonance Spectroscopy				
NMR modalities	^{1}H NMR	Used in numerous applications in inorganic and organic analysis (due to almost universal presence of H atom), early and popular form of NMR [56]	• Sample preparation is absent or minimal compared to Chromatography-MS techniques • Excellent structural information in less complex mixtures • Best signal intensity compared to other NMR types • No sample destruction [57]	• Lower sensitivity than MS • Overlapping peaks complicate identification • Low resolution and ambiguity in complex mixtures [36,57]
	^{13}C NMR	Useful for metabolite tracing (e.g. ^{13}C-enriched glucose, acetylation tags), fluxomics work, quantifying metabolic activity [57–59]	• Greater resolution than ^{1}H NMR • Useful in isotope tracking in cell-based experiments [57]	• ^{13}C atom is low abundance • Lower sensitivity than ^{1}H NMR and MS • Less common in metabolomics [60]
	2D-NMR	Identification of unknown metabolites, use in complex biological mixtures when overlapping in 1D is an issue, metabolite quantification [57]	• Increased spectral resolution • Better identification of low abundance metabolites • Increased sensitivity • Correlation between two spectra using same or different atoms (e.g., ^{1}H with ^{1}H, ^{1}H with ^{13}C, etc.) [36,57]	• Recording of two dimensions requires long experiment times [36,57,61]

increased 22-fold. These consecutive cycles created a tandem-in-time mass spectrometer capable of scanning a larger amount of ions with accurate mass spectra and enhanced spectral resolution [66]. Further fragmentation using CID also avoids the issue of overlapping peaks in the detection of similar metabolites. Its use in tandem MS is indispensable in the detection of low abundance metabolites such as ATP which has broad applications such as in single-cell analysis where metabolite abundance is necessarily limited [55,66].

2.3.5. Penning trap – fourier transform-ion cyclotron resonance

Another broad type of ion trap is the Penning trap, which utilizes a magnetic field to manipulate the trajectory of ionic molecules perpendicular to the field. In Fourier Transform-Ion Cyclotron Resonance (FT-ICR) mass spectrometry, the ions orbit the cell in a manner reflective of their unique *m/z* ratio; the angular frequency is inversely proportional to mass and directly proportional on the electric change and magnetic field strength. An image current is induced by the ion cloud movement which is detected on the excitation plate as an oscillating voltage, digitized, and stored as a time domain signal on a file. This signal, also called the transient, denotes the oscillations of all ions over the trapping time, which is usually a maximum of several seconds. The amplitudes of these frequencies correspond to the abundance of the particular ion [67]. This time domain signal is then Fourier transformed, which generates a complex function that represents a series of sine waves which are represented in a frequency function as intensity signals. This is analogous to taking a musical note (the time function) and breaking down the note into its individual pitches and noting these pitches as intensities. High resolution is maintained by increasing the transient duration to several seconds. Increasing the transient time has the effect of increasing frequency measurement precisions lower than 1 Hz and lowers the amplitude of the transient frequencies over time [55]. Ultra-low vacuum conditions are needed here to help maintain this amplitude and therefore overall resolution, but also reduced unnecessary collision of these ions with neutral gas molecules helps maintain kinetic energy, which maintains the orbit frequency and therefore the transient intensity. The transient duration is also determined by the abundance of the ion. The magnetic field strength, transient duration, and internal ICR cell pressure, among other factors, are primary determinants of the excellent resolving power of FT-ICR. *m/z* ratios can be approximated from the frequency values (which are themselves governed by the magnetic field), and the resolving power decreases with increasing m/z value [53]. Therefore, FT-ICR is highly useful in capturing the spectra of small molecules such as peptides, lipids, and others.

2.4. Tandem MS and separation techniques

The abundance of molecules with the same molecular weight within a single biological sample can complicate analysis; pairing MS with other analytical methods typically increases sensitivity and detection accuracy and permits the distinguishing of molecules of similar weight and charge. A method of increasing sensitivity is the use of tandem MS (MS/MS), which sends the ionized molecules through two mass analyzers. The use of a second mass analyzer helps further fragment the precursor ion selected in the first mass analyzer, which increases the resolution of the resulting spectra and differentiates molecules with similar *m/z* ratios [68]. Chromatographic techniques can also be paired to MS to increase detection resolution and sensitivity. Using chromatographic techniques before subjecting the metabolite mixture to MS has the advantage of separating the mixture into individual molecular components based on their degree of attraction to the *stationary phase*, a fixed solid or liquid that coats the walls of the chromatographic column. The molecules are carried through the column by the *mobile phase*, which has the same physical properties of the molecules in question; typically, multiple mobile phases of different hydrophilicity and acidity are used to ensure all molecules pass through the column [69]. Stationary phases use biochemical properties including polarity, ion charge, and binding affinity to a conjugated antibody; molecules that are attracted to the stationary phase move more slowly through the column and therefore elute later. This method separates molecules by elution time. An overview of NMR, MS, and Chromatographic techniques and workflow is summarized in Fig. 1.

Gas chromatography (GC) uses an inert gas (e.g., helium) as the mobile phase, whereas liquid chromatography (LC) uses a liquid such as formic acid. The mode of chromatography used depends on the characteristics of the metabolites in the mixture; if the mixture is known to possess smaller, more thermally unstable (volatile) molecules (i.e.

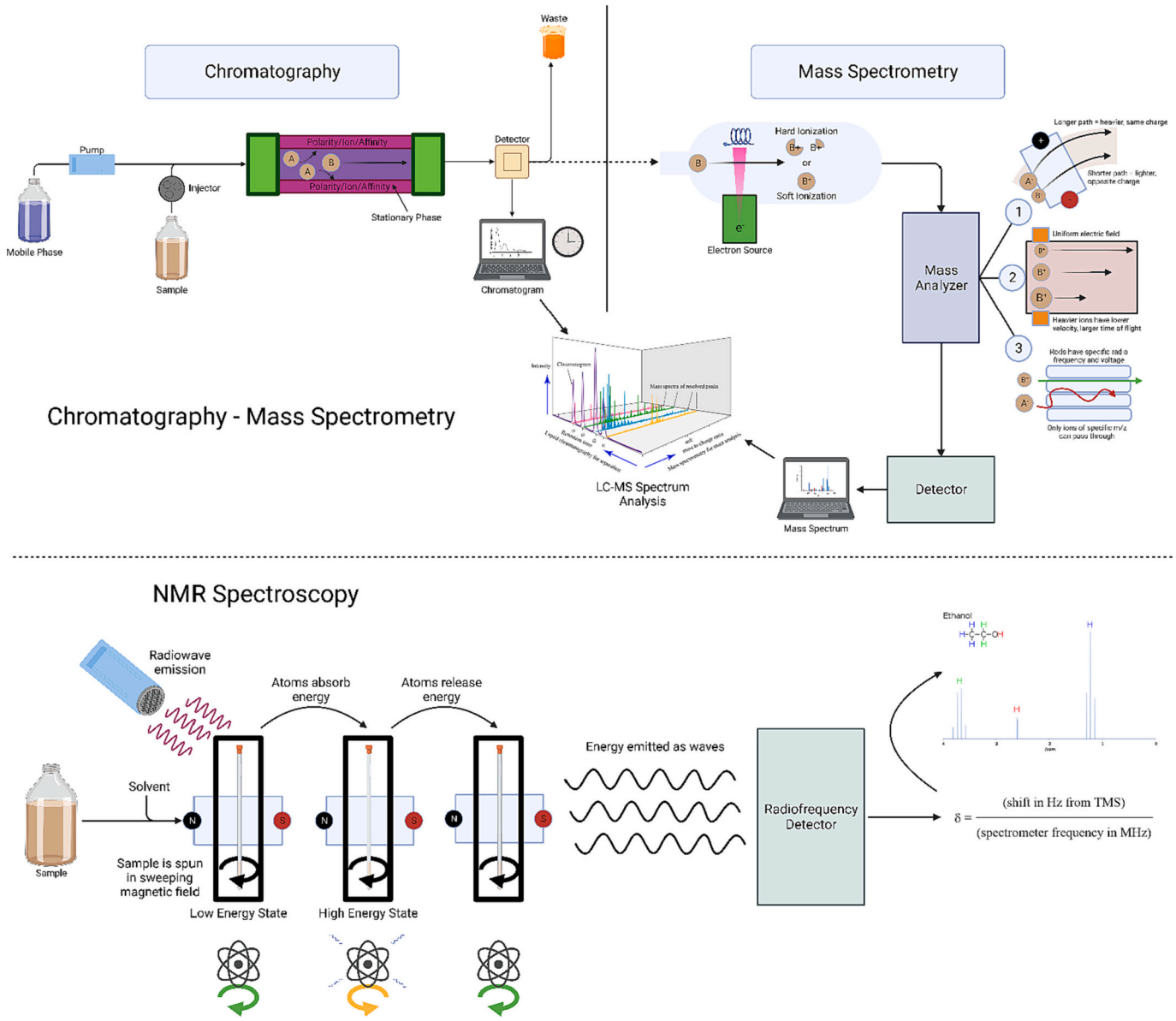

Fig. 1. Overview of the most common metabolomic techniques. Workflow for Chromatography paired with Mass Spectrometry (Top): The liquid or gaseous mobile phase is pumped into the chromatographic column and carries the sample. Molecules that have greater affinity to the stationary phase due to polarity, ionic charge, or antibody binding will have a longer elution time and are then read by the Detector to produce a chromatogram. These molecules can be eluted directly into a ionization source, which either ionizes whole molecules or generates charged fragments. These ions travel through a Mass Analyzer. Various types of Mass Analyzers are currently utilized, including (1) Magnetic Sector, (2) Time-of-Flight, and (3) Quadrupole. These ions are sent into a detector to produce a mass spectrum. NMR Spectroscopy (Bottom): Samples are energized using radio waves and energy emission from the sample is read and translated into shift in Hz from the chemical standard (TMS).

terpenes, hydrocarbon, ketones, alcohols), GC is more appropriate whereas LC is more appropriate for the analysis of mixtures possessing larger, more thermo-labile molecules (i.e. peptides, lipids, sugars, nucleosides) [69]. More recently, the use of High Performance Liquid Chromatography (HPLC) has emerged as the technique of choice, as it uses high pressures (>6000 psi) to elute the mobile phase, generates a larger flow rate and subsequently, a more resolved chromatograph than traditional LC [70]. The time that the molecules take to reach the spectrophotometer, which measures the absorbance value and therefore the identity of the molecule, is known as *retention time*. Combining LC with MS (LC-MS) alleviates the challenge of having multiple overlapping peaks which may occur when using either LC or MS alone - combining techniques allows recording of peaks at various retention times as well as *m/z* ratios at various intensities, which produces a 3-dimensional spectrum that can better allow for differentiation of metabolites with equal mass and structural properties [69]. Returning to 25(OH)D, it has been reported that measurements of serum 25(OH)D obtained through biochemical assays is typically inconsistent and inaccurate compared to LC/MS-MS. This discrepancy can result in clinical consequences; in one study, the number of participants that were identified as eligible for vitamin D treatment varied significantly depending on detection technique [24,71].

LC-MS is used for both untargeted and targeted metabolomics as it allows for technical consistency between studies, and for ease of use between different biological standards. However, in targeted metabolomics, the mass analyzer can be programmed to only select fragments that fit within certain *m/z* and intensity parameters that resemble those in the metabolites of interest, which helps filter for only those select metabolites [64]. Roberts and colleagues have published numerous protocols regarding the targeted extraction of various classes of

metabolites including hydrophilic metabolites and amino acids and processing of LC-MS outputs. [72]. Therefore, investigators performing metabolomic analyses of circulating metabolites must evaluate the benefits and limitations to each technique, which are outlined in Table 1. An outline of untargeted and targeted metabolomics workflows is found in Section 2.5.

2.5. Metabolomic analysis workflow

An untargeted metabolomic analysis is the simultaneous measurement of the abundance of multiple metabolites without the need for a specific testable hypothesis prior to analysis; rather, this sort of analysis provides a snapshot of the general metabolite landscape between samples in a given experiment. Untargeted metabolomics also provides relative quantification of the levels of metabolites between conditions and results are starting points for the generation of specific, targeted experiments to measure metabolites of interest. On the contrary, targeted metabolomics requires a predetermined hypothesis that a metabolite or predetermined set of metabolites will change in abundance given an experimental intervention or condition. The level of metabolites in this analysis are compared to reference standards, whereas in untargeted metabolomics, the results of MS analysis are compared within biological samples using annotation from spectral databases and libraries [29].

Targeted analyses begin with an identification of a metabolite or group of metabolites of interest. These are experiments done to address a biochemical question or further evaluate a hypothesis about changes in metabolite levels given particular experimental or biological conditions. In LC-MS methods, the metabolite standard is injected into the system in pure form and optimization of various electronic, flow speed, and other settings is performed to evaluate the best conditions for adequate resolution and intensity. A standard curve is created using isotopic metabolite standards as baseline, and the abundance level of the metabolite of interest within the experimental sample is compared to this reference. In this way, targeted metabolomics provides an absolute value of metabolite changes within the sample. An important aspect of reading experimental mass spectra is deconvolution, which involves the decomposition of overlapping peaks into their individual component peaks. The area under the curve (AUC) of these individual peaks can then be ascertained within the same region of the spectrum. After deconvolution, the comparison of peak intensities and fitting the linear curve according to exact tests can be done in a similar manner to the untargeted workflow (mentioned below). Targeted metabolomics largely employs three formats within MS, namely GC–MS, forms of MS/MS, and HPLC-MS or Ultra-Performance Liquid Chromatography-MS (UPLC-MS), primarily due to the wide extent of polarities and molecular masses covered by these techniques, paired with their high sensitivity and mass resolution across broad dynamic ranges. Sample preparation associated with targeted metabolomics is necessary when including internal standards (added to the sample before extraction) or external standards (added to sample after extraction). These additions help alleviate concerns about data comparability between experiments and from the use of different instruments and platforms [29,75].

Untargeted analysis begins with collection of spectral data from specific analytical sources (e.g., NMR, LC-MS, GC–MS, etc.) which are highlighted in Table 1. This spectral data is pre-processed to remove experimental artifacts and noise arising from variation in the instrument used or experimental design. This baseline correction is performed to improve signal quality for downstream processing.

Next, feature detection is performed which involves peak detection and spectral alignment. Peak detection methods can be further split into those used in MS studies – Peak-based methods – and those used in NMR studies – binning-based methods. In peak-based methods, peaks are detected across the spectrum and the area under the peak is integrated to provide a quantification level; numerous algorithms have been utilized to identify notable peaks within a spectrum but generally these algorithms take into account the relative intensity of the peak compared to its neighbours, if the peak crosses a pre-set threshold, and if the peak matches a set minimum signal-to-noise threshold [76,77]. NMR binning methods involve the division of small regions of the NMR spectrum (typically under 0.05 ppm) which include at least one NMR peak. These bins are then used to find the area under the peaks within each bin; the most significant bins are then selected and assigned to metabolites. Numerous binning techniques have been described and can be done using prior knowledge or computer algorithms. Through both methods, notable peaks are classified as ‘features’ which are further analyzed downstream [33].

Spectral alignment accounts for instrument- and experiment-based non-linear shifts in the position of mass spectra or NMR spectra. Peaks corresponding to the same feature/metabolite between multiple spectra may be misaligned on the retention time axis for chromatographic-MS analyses and on the ppm axis for NMR-based studies, and broadly two algorithmic methods have been utilized to correct for these shifts. Whole spectrum alignment methods, where spectral data is aligned before peak detection is performed, use warping (stretching and shrinking) and segmenting (division of spectra into parts which are equally shifted) techniques. Alternatively, in the case of high resolution chromatographic-MS, peak alignment occurs after peak detection. Various algorithms have been utilized for peak-alignment, all of which evaluate the alignment of peaks within or across retention times and bin *m/z* data to perform small alignments. Spectral alignment is crucial for the reliable comparison and identification of peaks across mass spectra from different samples [29][64]. Numerous algorithms have been proposed to correct for differences in spectral alignment between mass spectra while accounting for confounding factors such as batch effects [78–81]. After features are detected and aligned between spectra, feature normalization is performed either before or after metabolomic analysis. In pre-analysis normalization, internal standards comprising of endogenous metabolites are used to gauge the relative concentration of the total sample and the metabolite concentrations within the sample. This is crucial for biofluids such as plasma, serum, or urine, where metabolite concentrations can vary drastically for a variety of reasons [82]. However, there are limitations to the use of internal standards as the proper quantity needed between experiments even using the same sample types can vary and need to be optimized. In post-analysis normalization, signal intensities are evaluated and adjusted according to particular assumptions about the characteristics of spectral data. Popular approaches include total useful signal (assuming that all samples have the same total peak intensity), quantile normalization (assuming peak intensities are distributed equally across samples), and the probabilistic quotient normalization (assuming that the fold-change, determined by the median of quotients, between samples relates to their concentration differences) [29,64]. Wu & Li (2016) provide an excellent review of sample normalization methods in MS-based metabolomics, detailing both pre- and post-analytical methods currently in use [83]. Recently Yu & Huan (2022) proposed MAFFIN, a normalization method that utilizes the maximal density fold change between samples [84]. MAFFIN uses the sample with the most high-quality features as a reference to which corresponding features from other samples are compared; the fold-change ratios are then derived which are then transformed to produce a normalization factor that can be applied to all samples within the feature-abundance matrix. This method provides a reliable normalization method between groups and has been demonstrated to outperform other more common methods.

2.6. Metabolite annotation and data analysis

From here, univariate and multivariate analyses can be performed directly onto the detected features themselves before any metabolite annotation, or metabolite identification can be performed first. By performing analyses first, potential selection bias can be eliminated and further processing and filtering methods can be applied to peaks to

remove peaks of low intensity which represent instrumental noise, artifacts, or molecules introduced through chromatographic-MS. Metabolite identification involves the comparison of NMR or mass spectra to neutral databases using a tolerance threshold. Numerous spectral databases have been popularly used to match one or multiple peaks to known peaks of neutral, pure metabolites in standard conditions. However, due to the rate of false positives using a manual identification method, multiple parameters are included by programs to make metabolite identification more accurate, including ppm position, peak intensities and inter-sample peak intensity correlations for NMR, and the identification of isotopic and adduct products formed in MS experiments to identify multiple peaks corresponding to the daughter ions of a parent compound [64]. Multiple databases for the deposition and annotation of mass spectra exist including MassBank, Human Metabolome Database, METLIN, and NIST14. An outline of the benefits and limitations of the use of each of these popular databases and libraries is present in Table 2.

Numerous programs exist for automated metabolite identification and annotation of the mass spectra and NMR spectra of complex mixtures; popular among these are MetaboAnalyst [77], XCMS [78], mzMine [79], OpenMS [80], BATMAN [81]and Camera [83], although others exist [64,84].

The variability and reliability of records within MS databases are a major concern which persists in regards to the accurate annotation of metabolites and the variety of MS instruments that themselves produce varying number of mass spectra, average numbers of features, and ion products. MassBank is a collection of repositories of mass spectral data and to date holds >2,000,000 spectral records and >650,000 unique compounds. However, the overwhelming majority of MassBank data is from MS2 analyses (results of tandem MS) whereas MS1 data presents a represents minority. The curation and filtering of this data varies across MassBank databases [73]. The Human Metabolome Database (HMDB) contains a highly curated collection of known metabolite standards and submitted mass spectra that have increased across HMDB versions. HMDB is frequently updated with new features and presents visualization tools and provides biological contextualization; a major benefit to the use of HMDB is goal of integrating metabolite identification within biological contexts including modern understanding of pathways and disease mechanisms. HMDB is constantly updated and the current version, HMDB 5.0 has included numerous improvements including enhancement of metabolite descriptions, search functions, more accurate predicted spectral datasets (including NMR and MS). HMDB has the benefit of curating records from a variety of hyphenated mass spectral (e.g. GC-MS, LC-MS) and NMR sources [29]. METLIN (current version called 'METLIN Gen2') is an expansive database for MS/MS spectra with nearly 1 million compounds identified across major classes including lipids, small peptides, steroids, plant and microbial metabolites, and more. METLIN is a database used by XCMS to annotate and identify metabolites as well as generate metabolite networks [75]. Wiley Registry/NIST Mass Spectral Library is a combination of two major libraries (Wiley Registry and NIST Library) that incorporates a wide range of spectra such as those generated using electron ionization, MS/MS, and GC/MS, and has the benefit of being curated from a variety of experts and is widely compatible with common instrument manufacturers. It contains over 1 million chemical structures and >950,000 unique compounds; however, as it is a Wiley product, it is not publicly accessible nor free of charge [76].

The validation of metabolite annotations across multiple databases may provide greater assurances of accuracy but is often time-consuming to perform manually, especially as some programs limit the use of batch inputs in searching mass spectra. Zhou and colleagues (2012) generated a web-based tool called *MetaboSearch* for the identification of metabolites in multiple databases simultaneously and the cross-referencing of metabolite results from these databases to produced integrated labelling results [85]. They purported better metabolome coverage than the use of individual databases alone, which contain variable amounts of mass spectra and variable filtering methods based on instrumentation settings (e.g. ionization modes, ionization sources, mass analyzer types). However, the accuracy of metabolite identification across even a single database can be variable. Hoffmann and colleagues (2022) developed *COSMIC*, a method to assign confidence scores to metabolite annotations found through metabolomic databases by utilizing structure database generation, in silico annotation of features, and a machine learning method that determines the confidence score of putative identifications so that the user can select from a variety of potential metabolites for downstream analysis [86].

Univariate data analysis in metabolomics provides a simple, interpretable analysis of data patterns based on independent changes in metabolite level, and comparisons are made across a single variable. Univariate models require multiple-test correction as the number of false positives increases with greater sample size unless one adjusts the false discovery rate according to the chosen significance value and sample size. Multivariate analyses such as principal component analysis (PCA) are used to understand the contribution of various variables within an experiment to the variance in the levels of metabolites and their complementary behaviour in biological pathways. PCA and Partial Least Squares (PLS) methods are commonly used in multivariate analyses [87]. PCA is an unsupervised technique (no information about the relations between samples) and creates a summary matrix that captures the variation of the original data across multiple variables. Through further linear transformations, PCA allows one to see how much of the

Table 2
Popular databases for metabolite identification information from MS or NMR analysis.

Database/library	Size (# of unique compounds)	Date created	Type of data	Strengths	Limitations
MassBank [73]	>650,000	2006	Mass spectra only	Public, Large Dataset, Downloadable Spectral Data, spectra from different MS analyzers and ionization techniques, use of tags for filtering	Mis-annotation and poor curation for some records, reduced usability [74]
Human Metabolome Database [29]	>250,000	2007	Mass spectra and NMR spectra	Supports precursor ion-based searching, metabolite reaction mechanisms, provides spectra from different chromatography-MS setups, can search in large batches, downloadable results, includes various types of NMR analysis, numerous entries	In MS/MS search -missing instrumentation type
METLIN [75]	>960,000	2003	Mass spectra	High resolution MS/MS data, batch searching of precursor ions, fragments are searchable, spectra of known, reliable standards from different MS instruments	Data not downloadable, proprietary software of Agilent MS instruments, missing adduct ions for many metabolites, not suitable for MS1 or non-MS/MS spectra, lack of NMR data [74]
Wiley/NIST14 [76]	>950,000	2023	Mass spectra and NMR spectra	Strong electron ionization, GC–MS, and MS/MS libraries along with traditional LC-MS, multiple specific and varied libraries for numerous applications (pharmaceutical, forensics, etc.), well-curated	Connected to Wiley vendor software, not freely available, no link to other databases/libraries for metabolite information [74]

variation between samples is explained by a particular 'principal component'. PLS is similar to PCA but captures this variation within groups based off a linear regression since data about sample groupings is known (this is a supervised method) [88]. Typically, variations of PLS are applied to metabolomics analyses but this technique may lead to over-fitting where associations derived from analysis are not generalizable and therefore the results of multivariate analysis need to be validated [64]. A mix of univariate and multivariate analyses is recommended to gather a full picture of metabolite changes and interactions [89].

Finally, pathway and network analyses are performed to evaluate the relationships between metabolites through participation in common biological processes and shared enzymatic reactions. These pathways have been well-documented in multiple large databases. Over-representation analysis is a common method that takes a list of metabolites of interest as input (along with their relative abundance values and false discovery rate) and statistical tests (e.g. Fisher's exact test) evaluates whether metabolites in these groups fit within a biological pathway more than expected than chance alone [64]. The results of ORA are a list of biological pathways that are likely to be altered between experimental conditions. Numerous tools have been generated for pathway analysis that have also been useful in metabolite identification, including MetaboAnalyst and XCMS, which vary in their use of pathway databases including KEGG, Reactome, and HumanCyc, among others. The performance of these tools created to perform pathway analysis on metabolite sets was extensively examined by Marco-Ramell et al. (2018). Although the sensitivity and performance of popular tools and their associated databases are relatively similar, authors noted that a lack of regular updates to functional information about metabolites and metabolite networks, redundant identifiers, and the use of chemical class identifiers instead of specific metabolite names all contribute to inaccuracies in the number of metabolites identified and the results of enrichment analysis [90].

Correlation/network analysis, as differentiated from pathway analysis, displays the nodal connections between metabolites according to the relationship patterns observed within the data itself, and not necessarily by mapping to known biological pathways/databases. Therefore, the co-regulation of metabolites that are known or unknown to be related can be ascertained [91].

3. Using metabolomics to investigate circulating mitochondrial metabolites.

3.1. Classical metabolites associated with CMD

Metabolomics allows us to understand the dynamic landscape of the metabolome within a set biological environment at any moment. The use of these methods to study the circulating metabolome is of particular interest, as blood not only acts as a transporter of various molecules, but blood metabolites are an easily accessible indicator of systemic metabolic changes for clinicians and researchers. Analysis of these changes between pathophysiological states (e.g., hyperglycemic vs normoglycemic, obese vs lean, etc.), a list of critical biomarkers that are characteristic of the disease state can be elucidated.

It is important to differentiate between components of blood in the context of characterizing the circulating metabolome. Studies in this area largely investigate two components of blood – serum and plasma. Serum is derived from blood that has been coagulated, whereas plasma isolation involves the addition of anticoagulants such as citrate or ethylenediaminetetraacetic acid (EDTA). It has been previously shown that levels of metabolites derived from serum and plasma from the same individual can differ significantly, with a serum matrix possessing increased detection sensitivity, whereas plasma matrices have greater reproducibility between tests [92]. Additionally, blood collection methods also create variation in metabolomic profiles in the same individual, and therefore consistency in serum or plasma collection protocols is important for data reliability [93]. Overall, the choice between serum or plasma analysis is dependent on the technical question being asked to ensure consistency between studies. However, for the purposes of this review, metabolomics screens of both serum and plasma will be analyzed to evaluate circulating metabolites whose abundance is differentially regulated in CMDs. Noteworthy classical and novel metabolites representative of CMD-related pathologies are outlined in Table 3. We will also focus on the interactions these metabolites have within skeletal muscle primarily as it is a highly metabolically active and insulin-sensitive tissue [94,95]. Skeletal muscle accounts for 80 % of postprandial glucose disposal and relies on proper insulin regulation [96]. Skeletal muscle is also noted to be an indicator of early CMD progression (compared to the liver or adipose tissue) as pathological changes to systemic insulin regulation are primarily noted in the skeletal muscle response to insulin: decreased glucose uptake, decreased lipolysis, decreased gluconeogenesis, and the promotion of hepatic glucose output. Therefore, the detection of perturbations to the innate insulin sensitivity of skeletal muscle provides us a window from which to view the pathogenic contributions of abnormal metabolite levels, detectable in circulation (e.g., triglycerides, fatty acids, amino acids, etc.) [97]. Prolonged systemic insulin resistance leads to further complications such as Type 2 Diabetes and cardiovascular diseases; therefore, understanding the mechanisms and metabolites that drive perturbations in the normal insulin sensitive state in muscle may provide us with strategies to earlier prevent the consequences of insulin resistance [96,98].

CMDs are multi-organ pathologies that include insulin resistance, abdominal obesity, hypertension, dyslipidemia, and chronic inflammation [99]. CMDs are early risk factors for cardiovascular disease and diabetes, which are both among the heaviest contributors to mortality globally. Thus, metabolites modulating CMD phenotypes will be examined in this review [100–102]. Significant metabolite interactions between muscle and adipose tissue, within particular cell types, and between proteins, enzymes, and other metabolite intermediates are noted here. Table 3 contains circulating metabolites that are differentially regulated during CMDs in human and animal models.

3.1.1. Branched-chain amino acids

Branched-chain amino acids (BCAAs; coloured dark blue in Fig. 2) are increasingly recognized as a clinical diagnostic marker of early insulin resistance and hyperglycemia [125]. However, as insulin resistance is associated with obesity and the metabolic syndrome [126,127], several studies investigated the use of BCAAs as a more general marker of CMD as a systemic disease [103,128,129]. The accumulation of BCAAs in highly-metabolically active organs, such as skeletal muscle, has been directly linked to insulin resistance. Several studies have

Table 3
Circulating biomarkers of various CMDs detected through metabolomics.

Marker	Species	Sample type	Study and expression
Branched-Chain Amino Acids (BCAAs): Valine, Leucine, Isoleucine	Human	Serum	Elevated: [103]
	Human	Plasma	Elevated: [104–110] Reduced: [111]
	Rat	Plasma	Elevated: [112]
Acylcarnitines	Human	Serum	Elevated: [103]
	Human	Plasma	Elevated: [104,105,109,113,114]
3-Hydroxyisobutyrate (3-HIB)	Human	Plasma	Elevated: [107,108,115,116]
	Rat	Plasma	Elevated: [117]
α-Hydroxybutyric Acid (α-HB)	Human	Serum	Elevated: [118]
	Human	Plasma	Elevated: [115,119–121]
Succinate	Human	Plasma	Elevated: [109,117]
Lactate	Rat	Plasma	Elevated: [122]
	Human	Plasma	Elevated: [109,110,122]
S-adenosylmethionine (SAM)	Human	Plasma	Reduced: [111]
S-adenosylhomocysteine (SAH)	Human	Plasma	Reduced: [111]
Glycine	Human	Plasma	Reduced: [123,124]

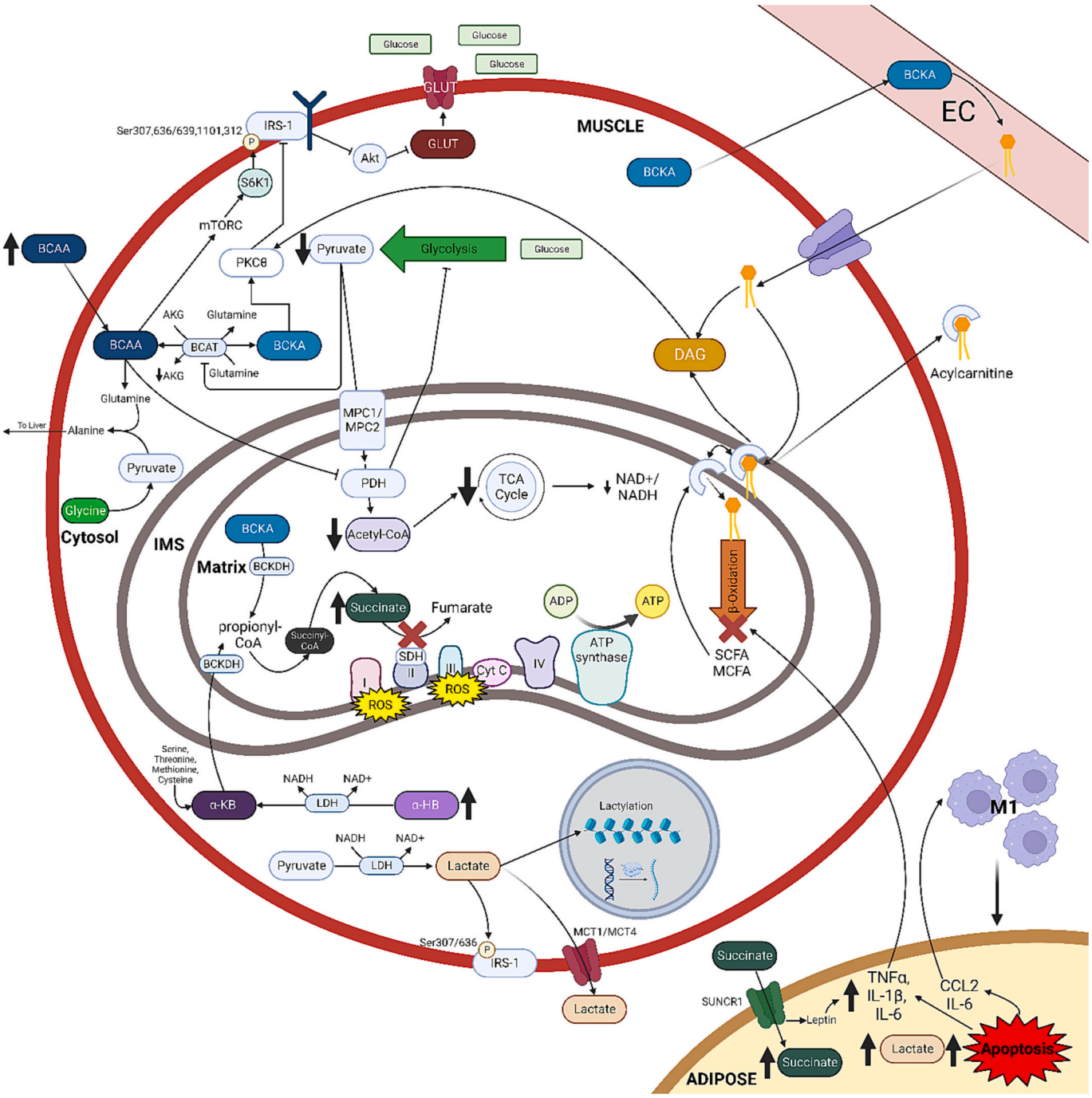

Fig. 2. Circulating metabolites identified in CMD pathogenesis contextualized in skeletal muscle.
BCAAs directly inhibit insulin signalling through activation of MTOR, leading to IRS-1 phosphorylation and inhibition of GLUT mobilization. BCKAs inhibit insulin resistance through PKC-θ activation, can be used to generate succinate (along with α-KB), and promote the transport of fatty acids into muscle promoting lipid accumulation and insulin resistance. Lactate is associated with IRS-1 phosphorylation and enhanced lactylation of histone proteins, leading to altered gene expression inhibiting lipid oxidation. Succinate-receptor interactions and lactate accumulation in adipose tissue to promote the release of pro-inflammatory cytokines which decrease the rate of fatty acid oxidation in skeletal muscle and promote recruitment of M1 macrophages to propagate inflammation in adipose tissues. Decreased complete fatty acid oxidation generates fatty acid and acylcarnitine species of medium and short lengths which are exported out of muscle. ETC and OXPHOS dysfunction inhibits succinate oxidation and lowers the NAD+/NADH ratio, leading to greater oxidative stress and depressed mitochondrial function. Abbreviations: Acetyl-CoA - Acetyl-Coenzyme A; α-HB - α–hydroxybutyrate; α-KB - α–ketobutyrate; AKG - alpha-ketoglutarate; Akt - protein kinase B; BCAA - branched-chain amino acid; BCAT - branched-chain amino acid aminotransferase; BCKA - branched-chain keto acid; BCKDH - branched-chain α-ketoacid dehydrogenase complex; CCL2 - chemokine ligand 2; DAG - diacylglycerol; EC - endothelial cell; GLUT - glucose transporter; IL-1β - interleukin-1β; IL-6 - interleukin-6; IRS-1 - insulin receptor substrate-1; LDH - lactate dehydrogenase; MCFA - medium-chain fatty acid; MCT1/MCT4 - monocarboxylate transporter 1/4; MPC1/MPC2 – monocyte chemo-attractant protein-1/2; mTOR – mammalian target of rapamycin; NAD - nicotinamide adenine dinucleotide (oxidized); NADH - nicotinamide adenine dinucleotide (reduced); PDH - pyruvate dehydrogenase; PKCθ - protein kinase C-theta; Propionyl-CoA - Propionyl-Coenzyme A; ROS - reactive oxygen species.

observed increased activation of mammalian target of rapamycin complex 1 (mTORC1) and subsequent phosphorylation of serine 307,636/639,1101, and 312 on insulin receptor substrate 1 (IRS-1) by S6 kinase (S6K1) as well as Growth Factor Receptor Bound Protein 10 (Grb10) which both inactivate the insulin receptor directly [103,130–133]. IRS-1 phosphorylation prevents Akt signalling, necessary for insulin signal transduction, and leads to impaired mobilization of the GLUT glucose transporters to the plasma membrane [133–139]. Studies investigating the effect of leucine-supplemented diets in animals and humans do not form a consensus regarding its potential role in modulating the insulin signalling pathway. Some studies have demonstrated that Leucine administration in myotubes do not affect insulin signalling in cells resistant or sensitive to insulin, regardless of concentration, but increases muscular lipid content [140]. Similarly in humans, oral leucine supplementation did not change insulin sensitivity or circulating concentrations [141]. However, others have displayed improved insulin sensitivity and glucose control in human patients and mouse models of diabetes upon chronic leucine administration [142,143]. This improvement in insulin signalling has been suggested to manifest through leucine's interactions with mTORC1 and mTORC2 [144]. Contrastingly, numerous studies report an opposing effect, noting impairment of insulin signalling and glucose tolerance upon leucine administration [145–147]; however this effect is attenuated by administration of other amino acids alongside leucine [145]. α-Ketoisocaproic acid (KIC), a product of leucine catabolism, has also been associated with insulin resistance, however deletion of branched chain aminotransferase 2 (BCAT2) which catalyzes the conversion of KIC back to leucine, attenuated KIC-mediated mTORC1 activation [145]. This suggests that leucine directs insulin resistance directly. Another BCAA, valine, has been identified by numerous groups as causing definite glucotoxicity and insulin resistance in muscle due to the activity of its metabolites [128,148,149]. Additionally the reduction of valine and isoleucine but not leucine consumption improves fat accumulation and glucose uptake in multiple tissue types [148,149]. Additionally, BCAAs promote the inhibition of pyruvate dehydrogenase complex (PDH), a vital rate-limiting enzyme involved in glycolysis [150]. This is likely due to feedback inhibition of acetyl-Coenzyme A (acetyl-CoA) through activation of pyruvate dehydrogenase kinase (PDK) [151], as PDH transforms pyruvate into acetyl-CoA; decreased PDH activity reduces the rate of glycolysis and is a marker of metabolic inflexibility in the fed-state [152–154]. Several of these mechanisms are outlined in Fig. 2.

The impaired oxidation of BCAAs into branched-chain keto-acids (BKCAs) causes a buildup of BCAAs in many tissue types, and is caused by depressed activity of the BCAA-BKCA conversion enzyme branched-chain keto-acid dehydrogenase (BCKDH). The mechanisms by which increased fatty acids are necessary to create the obese phenotype contributes to impaired BCAA catabolism function is unclear. However, in skeletal muscle cells where BCKDH activity was promoted (through inhibition of branched-chain ketoacid dehydrogenase kinase (BCKDK) which catalyzes the opposite reaction), BCAA oxidative flux and complete fatty acid oxidation were increased, implicating BCAA catabolism to have a regulatory role on fatty acid oxidation rates [155]. Additionally, rats fed a high-fructose diet were observed to have higher levels of circulating BCAAs and lower levels of skeletal muscle BCKDH and BCAT2 activity [156]. PPARG coactivator 1 alpha (PGC-1α) gene expression in skeletal muscle was increased in mice administered BCAAs and overexpression of PGC-1α induced the expression of BCAT2 and BCKDH [157,158]. Through alterations to the nicotinamide adenine dinucleotide oxidized/reduced (NAD^+/NADH) ratio, BCAA administration induces sirtuin-1 (Sirt1)-mediated expression of PGC-1α, p53 and NF-kB [159]. PGC-1α regulation converges with fatty acid metabolism as palmitate reduces PGC-1α gene expression in muscle cells through a MAPK-mediated mechanism, while PGC-1a overexpression leads to increased trans-endothelial fatty acid transport into muscle cells [160,161]. Therefore, fatty acids and BCAAs work cohesively to modulate PGC-1a levels in order to regulate the concentrations of intracellular fatty acids.

3.1.2. Branched-chain keto acids

Elevated BCAA catabolism products have also been observed during insulin resistance. 3-hydroxyisobutyrate (3-HIB), a BCKA and product of valine catabolism, is elevated proportionally to severity of dysglycemia, promotes the accumulation of diacylglycerols, and is markedly decreased during weight loss in humans [107,108,161,162]. Although the enzymes involved in BCAA breakdown (primarily 3-HIB dehydrogenase) may have reduced activity, the net overproduction of 3-HIB and related toxic catabolites may be related to the elevated initial concentrations of BCAAs intra- and extracellularly. 3-HIB has been noted to decrease oxidative capacity in adipocytes and facilitates the accumulation of fat in white and brown adipocytes as well as skeletal muscle cells [108,161]. The knockdown of the 3-HIB-forming enzyme reduces both accumulation of fat and release of 3-HIB from these cells. 3-HIB is also known to modulate the insulin signalling pathway [108]. 3-HIB acts as a paracrine signalling molecule; increased dietary intake of 3-HIB is observed to increase muscle protein kinase C θ (PKC-θ) localization to the plasma membrane, lower the pAKT/AKT1 ratio and increase blood glucose levels relative to a vehicle control. The presence of 3-HIB in insulin resistance is further confirmed by its increased levels both in the muscle of *db/db* mice and type 2 diabetic individuals compared to their respective controls [161]. Therefore, 3-HIB appears to be a consistent and highly active signalling molecule whose actions mirror that of diacylglycerols (DAGs) in terms of inciting insulin resistance [163]. Fatty acid influx into skeletal muscle is mediated by trans-endothelial fatty acid uptake [161], and this phenomenon has also been replicated in human HUVEC cells and cardiac-derived endothelial cells, but not in adipose tissue-derived endothelial cells [107]. This suggests that 3-HIB acts in a tissue-specific manner, particularly within tissues where abnormal lipid accumulation is a primary driver of the insulin resistance and altered carbohydrate metabolism [161]. See Fig. 2 for BCKA interactions; BCKAs are coloured light blue.

BCAA catabolism, which occurs mostly in extrahepatic tissues such as skeletal muscle, requires the (BCAT)-mediated conversion of BCAA to BCKA and α-ketoglutarate (AKG) to glutamate. Holeček (2023) reviewed major perturbations to the amino acid catabolic pathways caused by CMDs, including decreased total Citric Acid Cycle (CAC) flux and resulting impaired AKG supply to BCAT, reduced levels of glycolytic end-products such as pyruvate which reduces conversion of glutamate to AKG by aspartate aminotransferase and alanine aminotransferase, electron transport chain (ETC) and *β*-oxidation impairments which lower the NAD^+/NADH ratio and increased levels of acylcarnitines leading to feedback inhibition of BCKDH, increased protein catabolism by the liver releasing BCAAs into circulation, and increased transamination of BCKAs back to BCAAs due to an abundance of glutamine and the reverse reaction of BCAA aminotransferase (which may also be an attempt to regenerate intramitochondrial AKG pools due to a depletion of CAC intermediates) [164].

3.1.3. Lactate

Lactate (coloured light brown in Fig. 2) is a by-product of anaerobic metabolism in glycolytic tissues, but can occur in all cell types. After it is produced through glycolysis, pyruvate has two fates: 1) import into the mitochondria to be used in aerobic metabolism, through oxidative phosphorylation (OXPHOS); or 2) transformed by lactate dehydrogenase (LDH) into lactate, a process facilitated by the consumption of NADH. It is noted that some lactate production also occurs at rest in aerobic conditions [165]. Although circulating lactate levels are known to rise in individuals undergoing exercise [166,167], patients affected with metabolic syndrome are observed to mirror similar levels of blood lactate, indicating an inappropriate systemic shift to anaerobic respiration instead of oxidative metabolism [168]. Therefore, lactate has been characterized as a predictive marker of mitochondrial substrate utilization, in particular the switching to carbohydrate metabolism instead

of fatty acid oxidation [169]. Altered glucose oxidation and decreased pyruvate consumption caused by hyperglycemia has been shown to increase lactate production in cells [170]. Skeletal muscle PGC-1α overexpression reduces the transcription of LDH A and increases expression of LDH B, serving as a regulatory mechanism to lower systemic lactate concentrations [171]. Lactate can supply the mitochondrion with pyruvate through a lactate oxidation complex located within the inner mitochondrial membrane, which serves to promote CAC-mediated generation of cellular ATP in times of stress [172,173]. In addition, excess cellular lactate is shuttled out of glycolytic tissues by monocarboxylate transporters (MCTs) which are activated through increased glycolytic flux and PGC-1α overexpression [171,174].

Several metabolomic studies have confirmed lactate as a useful differentiator of the plasma or serum profiles of obese, diabetes, and metabolic syndrome-affected individuals [109,110,122]. In a study of rodents fed a cafeteria-style high fat diet, levels of serum lactate were elevated in obese rats compared to their lean counterparts, with concordant upregulation of the lactate dehydrogenase B genes in peripheral blood mononuclear cells [175]. A targeted metabolomic analysis of fasting plasma metabolites on 2401 women from the TwinsUK study cohort found that elevated lactate was associated with diabetes, insulin resistance, visceral fat (elevated levels of which are known to be detrimental to metabolic and cardiovascular health), and blood pressure [176]. However, the conceptualization of lactate as a driver of CMDs and the biological mechanisms behind lactate-induced physiological changes is still unclear. Through adipose-specific deletion of the adipose tissue lactate transporter gene Slc16a1 (encoding for MCT1), Lin et al. demonstrated that not only is adipose tissue a significant source of total lactate levels, but that the retention and elevation of lactate levels within adipose tissue is responsible for the release of cytokines and paracrine recruitment of macrophages which generates a robust inflammatory reaction characterized by high levels of circulating TNF-α, IL-1β, and IL-6 [177]. Lactate signalling mechanisms to adipose tissue are outlined in Fig. 2.

Lactylation is the addition of the -lactyl group to histone and non-histone proteins, typically at lysine residues [178]. Increased lactate levels in human skeletal muscle has been shown to increase total protein lactylation proportional to markers of anaerobic metabolism and inversely to oxidative metabolism. Administration of lactate to cultured skeletal muscle cells increased IRS-1 S636 phosphorylation which implies post-translational modifications mediated by lactate can alter the behavioural dynamic of metabolic systems including the insulin pathway and promotes a shift to glycolytic metabolism instead of the more efficient OXPHOS pathway [179]. This state of systemic hyperlactatemia has been associated with increased expression of lactate dehydrogenase enzymes within skeletal muscle and other tissues but interestingly not with changes in MCT1 and MCT4 lactate exporters [180]. Overproduction of lactate has been directly associated with increased histone lactylation, particularly at lysine residues (which are referred to as 'Kla'), which stimulates the transcription of multiple genes in M1-macrophages related to wound healing, inflammation, and homeostasis in a manner resembling transition to the M2 (anti-inflammatory) state [181]. In a high-intensity interval training (HIIT) study of mice, exercise induced widespread lactylation of numerous proteins in muscle, adipose, and liver. Enzymes involved in the regulation of lipogenesis including fatty acid synthetase (Fasn) and ATP-citrate lyase (Acly) were heavily lactylated, inhibiting their functions and promoting lipolysis [182]. Additionally, knockout of the mitochondrial pyruvate carrier 1 (Mpc1) gene induced lactylation of Fasn and lowered fatty acid accumulation in the liver of high fat diet-fed mice [183]. Recently, two mechanisms have been proposed describing the regulation of histone lactylation. The first is an enzymatic mechanism proposed by Zhang et al. where lactate (specifically, L-lactate) is converted to lactyl-CoA through a currently unknown enzyme, followed by the histone acetyltransferase p300- and CREB-binding protein (Cbp)-mediated transfer of the lactyl group from lactyl-CoA to the histone Kla site [181]. A second mechanism has been proposed by Gaffney et al. whereby dihydroxyacetone phosphate (DHAP), a derivative of a glycolysis intermediate, is transformed into methylglyoxal, which subsequently binds with glutathione through the glyoxalase 1 enzyme, forming lactoylglutathione (LGSH). Through a non-enzymatic reaction, a D-lactyl moiety can be transferred from LGSH to histone Kla sites [184]. As lactylation-mediated alterations in histone function present an interesting and novel avenue for which to understand lactate-based changes in gene expression, new techniques have been developed to quantify lactyl-CoA through metabolomics [185]. Protein lactylation presents a novel hallmark of metabolic reprogramming and controls the activity of proteins implicated in lipid accumulation and CMD pathogenesis. Lactylation has only recently emerged as a post-translational modification capable of altering protein function, and may broaden the current perspective of lactate's role intracellularly from a waste product to a master regulator of metabolism through epigenetic and post-translational mechanisms.

Lactate also serves as a marker of the systemic redox state. The circulating lactate:pyruvate ratio has classically been used as a proxy marker of the NAD^+/NADH ratio, as the generation of lactate and its flux into circulation is mediated by high levels of NADH [186]. Therefore, the ratio of lactate:pyruvate is a sign of mitochondrial ETC dysfunction, as the conversion of pyruvate to lactate (instead of entering the CAC as Acetyl Co-A) due to a buildup of NADH displays a lack of proper functioning in the NADH dehydrogenase activities of Complex I [187,188]. When the lactate:pyruvate ratio is reduced through addition of extracellular pyruvate, the NAD^+/NADH ratio is also increased [189]. This relationship is facilitated by the decreased activation of c-Jun N-Terminal Protein Kinase (JNK) and bcl-2-like protein 4 (Bax) signalling, which reduces sensitization to oxidative stress-induced apoptosis by preventing mitochondrial outer membrane permeabilization [190].

3.1.4. Succinate

Succinate (coloured green in Fig. 2) is a core intermediate of the CAC, being generated from succinyl-CoA and transformed into fumarate via succinate dehydrogenase (SDH). Succinate is also utilized by ETC Complex II, an inner membrane-bound multi-unit SDH protein complex [191–193]. SDH subunit A (SDHA) oxidizes succinate to reduce FAD to $FADH_2$. SDH subunit B (SDHB) transfers these high-energy electrons from $FADH_2$to Fe-S clusters found within the protein complex, subsequently funnels these electrons to ubiquinone to form ubiquinol (the bioactive form of Coenzyme Q_{10}) which travels through the inner mitochondrial membrane to Complex III. Therefore, cellular succinate levels are an indicator of oxidative capacity and ETC function [194]. Any disruptions within Complex I and II and the CAC regeneration of succinate would necessarily affect cellular succinate levels [195].

The plasma metabolite profiles of diabetics are known to be enriched with succinate [196]. Elevated succinate levels are implicated in obesity, hypertension, and metabolic disease [197]. Fluctuations in the levels of CAC intermediates are also known to be a marker of the diabetic phenotype due to a decrease in lipid oxidation, which supplies the CAC with the necessary influx of acetyl-CoA. In the visceral adipose tissues of obese individuals, SDH activity in ETC Complex II was decreased and the level of cysteine post-translational modifications in SDH was increased compared to subcutaneous adipose tissue from the same patients. In a surgical weight-loss cohort, reduced visceral adiposity was associated with restored SDH activity, implicating Complex II dysfunction as a key regulator in tissue-specific manifestations of CMD [198]. Circulating succinate also acts as a signalling molecule by binding to tissue-specific receptors such as succinate receptor 1 (SUCNR1). Activation of adipose SUNCR1 induces leptin expression, a modulator of whole-body metabolic homeostasis via an AMP-activated protein kinase(AMPK)/JNK/CCAAT-enhancer-binding protein alpha (C-EBPα) pathway [199]. Hyperleptinemia is therefore a direct effect of overactive succinate/SUNCR1 signalling stemming from elevated levels of circulating succinate and is known to produce a state of chronic inflammation which increases the risk for the development of various conditions including

insulin resistance [200]. Elevated levels of circulating succinate are known to activate the succinate receptor GPR91 which mediates numerous inflammatory responses including the overexpression of TNF-α and IL-1B in myeloid cells [201]. Furthermore, succinate has been suggested to promote the migration of immune precursor cells from their site of generation to the site of maturation [201]. Therefore, circulating succinate is implicated as a modulator of inflammation and whole-body homeostasis through plasma membrane receptor-mediated pathways, which is summarized in Fig. 2.

3.1.5. Acylcarnitines

Acylcarnitines (visualized as orange lipid molecules bound to a blue carnitine shell in Fig. 2) NADH: are compound molecules formed by the carnitine palmitoyltransferase 1 (CPT1)-mediated binding of Acyl-CoA (which contains the hydrocarbon backbone to be utilized for *β*-oxidation) and carnitine on the mitochondrial outer membrane [202]. Acylcarnitines easily pass through the outer membrane but require carnitine-acylcarnitine-translocase to bypass the inner mitochondrial membrane. Carnitines serve as mobile carriers of acyl groups between the cytosol and mitochondrial matrix. Once in the matrix, acylcarnitines are decomposed back into acyl-CoA and carnitine, so that acyl-CoA can utilized for *β*-oxidation. When overproduced, acylcarnitines can be exported from the mitochondria by the same mechanisms used for import. The differentiation between various species of acylcarnitines is based on the length of the acyl hydrocarbon chain. As acylcarnitines can be reformed from the products of *β*-oxidation, studies of cellular stress related to irregular fatty acid oxidation during insulin resistance have noted the increased presence of short chain acylcarnitines in diabetes and early insulin resistance [203,204]. This is due to the presence of incomplete fatty acid oxidation during insulin resistance which creates fatty acid intermediates of variable length (typically long and medium chain) and longer than the final acetyl-CoA product normally formed by healthy *β*-oxidation, necessary for the CAC [114]. However, the specific acylcarnitine species marking and/or driving CMDs is currently unclear, as studies of circulating acylcarnitines during CMD have shown mixed results [205]. Numerous studies have found long-chain acylcarnitines to be representative of incomplete *β*-oxidation and oxidative stress [205,206]. When RAW 264.7 macrophages were treated with physiologically-representative acylcarnitines (C12-C14 length), the secretion of proinflammatory cytokines as well as the phosphorylation of JNK and ERK, common downstream components of many proinflammatory signalling pathways, were significantly increased [207]. It is also contested whether acylcarnitines produced within skeletal muscle (and not imported from circulation) can drive the overproduction of proinflammatory cytokines, however the presence of macrophage infiltration and inflammation within adipose tissue has been noted in the presence of increasing long- and medium-chain acylcarnitines [205,208,209]. As noted previously, this adipose-based inflammatory response can induce insulin resistance; TNF-α is known to activate pathways which suppress fatty acid oxidation and increase DAG levels through an AMPK-mediated pathway (summarized in Fig. 2) [210,211]. DAGs over-activate PKC-θ leading to insulin resistance [163]. Therefore, acylcarnitines may not only be markers of insulin resistance via impaired fatty acid oxidation, but also stimulate inflammatory pathways that contribute to the CMD phenotype globally.

3.1.6. α–Hydroxybutyrate

α–Hydroxybutyrate (α–HB; coloured purple in Fig. 2) is an organic acid that is currently only known to interact with LDH which converts it into α– ketobutyrate (α–KB). α–KB acts as a nexus between the use of various amino acids for substrate synthesis (including methionine, cysteine, threonine, glycine, and methionine intermediates such as SAM and SAH), BCKA and BCAA catabolism and anabolism, and fatty acid oxidation into the CAC. α–KB can be directly used to generate propionyl-CoA which, after conversion to methylmalonyl-CoA and then succinyl-CoA, feeds into the CAC [212](this mechanism is displayed in Fig. 2). This influx of succinyl-CoA may also contribute to the elevation of circulating succinate previously noted in CMD. α–KB also contributes to the generation of BCKA's (such as 3-HIB) and its production may be mediated by PDH [213]. Gall and colleagues (2010) propose two mechanisms by which α–HB is overproduced: i) through increased cysteine anabolism due to the increased demand to generate glutathione in hepatic cells to combat the rise in oxidative stress in the CMD state, and ii) to address the reduction in the NAD^+/NADH ratio resulting from increased incomplete fatty acid oxidation and mitochondrial ETC dysfunction, which reduces the ability of NADH to be oxidized and NAD^+ to be regenerated [119]. α-KB here works to address the balance between the formation of the antioxidant glutathione and reduction of overall oxidative stress caused by a buildup of NADH due to increased fatty acid and amino acid inputs into the CAC. By using up α-KB, there is a drive to further produce more α-KB to replace it and therefore generate increasing amounts of α-KB to act as reserve. Recently, α-HB has been identified as a biomarker for early insulin resistance and *β*-cell dysfunction, even before elevation of other notable metabolites such as acylcarnitines [119,120,214] (reviewed in [215]). Because α-HB contributes to BCAA synthesis, BCAA elevation often co-occurs with α-HB overproduction [216]. Whether α-HB has direct interactions with the insulin signalling pathway or with lipid-mediated insulin resistance mechanisms is currently not known, but as described previously, it may act indirectly through more active metabolites such as BCAAs to propagate insulin resistance through IRS-1 phosphorylation. Regardless, α-HB presents a useful and reliable biomarker to detect early CMDs.

3.1.7. Glycine

Glycine (coloured green in Fig. 2) is a one-carbon, non-essential amino acid that can be generated from threonine, glyoxylate, glucose, and other dietary sources. It has vital roles in the generation of other amino acids such as serine, sarcosine, creatine, glutathione, and more compounds involved in amino acid regulation and energy generation [226]. Glycine levels have been inversely associated with the presence of insulin resistance and obesity in many populations [227–229]. Interestingly, glycine is less abundant in the serum of older adults who possessed greater levels of intramuscular adipose tissue (pathogenic in insulin resistance) and lower levels of subcutaneous adipose tissue (favourable in insulin sensitivity) [230]. It has been noted that individuals with greater circulating markers of oxidative stress and reduced baseline total glutathione benefitted through increased glycine-dependent glutathione production [231]. Therefore, maintenance of normal glycine potentially attenuates systemic oxidative damage. Glycine supplementation has been noted in some small human and rodent studies in improving the insulin response, glucose tolerance, and blood pressure [227,232–234]. Glycine is a co-agonist (along with glutamate or aspartate) for *N*-methyl-D-aspartate (NMDA) receptors found in the dorsal vagal complex (DVC) and pancreatic β-cells. In the DVC, glycine activation of NMDA causes inhibition of glucose production through the triggering of the hepatic vagus nerve, without affecting insulin levels [235,236].

Glycine is also suggested to have anti-inflammatory roles through interactions with Glycine receptors on adipose tissue by limiting the release of pro-inflammatory cytokines and adipokines from adipocytes [237–239]. Glycine may lower the excessive secretion of VLDL-TGs from the liver of high-fat diet fed rats by activating NMDA receptors within DVC resulting in systemic lipid homeostasis [240]. As we have stated earlier, the increased presence of toxic lipid species in circulation and their import into muscle is an infamous driver of insulin resistance and skeletal muscle lipotoxicity. Glycine induces insulin efflux from β-cells. Interestingly, stimulation of overexpressed glycine receptors leads to further release of stored intracellular β-cell glycine; this autocrine feedback loop maintains homeostatic levels of glycine in cases of depletion. Interestingly, insulin itself amplifies glycine-invoked current in β-cells whereas this amplification is missing in cells from T2DM patients. Concurrently, numerous studies have displayed decreased

expression of glycine receptor subunits in patients with T2DM; in this insulin-glycine pairing, insulin and glycine both act to amplify the expression of one another through the invoking of glycine currents and activation of β-cells [241,242]. Therefore, there is at least a partial role of glycine in regulating the release of insulin from β-cells.

Depletion of systemic levels of glycine can be attributed to numerous sources, and several interactions between glycine and host metabolism may contribute. The elevation of BCAAs due to increased consumption of BCAAs in diet and activity of Branched Chain Aminotransferase enzymes are associated with reduced plasma glycine levels. Through an elegant combined genome-wide association study (GWAS) and metabolomic analysis, White et al. (2020) proposed a novel BCAA-mediated mechanism whereby glycine acts as a carbon donor and precursor for the generation of pyruvate, which is a part of the pyruvate alanine-cycle [244]. Through BCAA catabolism into BCKA, glutamate is released [174] and combines with pyruvate through alanine aminotransferase 2 to generate alanine, which is then shuttled to the liver to participate in the urea cycle for NH3 disposal [244]. Glycine also has a vital role as a conjugator of potentially toxic metabolites, which in relevance to the CMD state, includes acylcarnitines and acyl-CoA species. These intermediates are known to interact with glycine through acyl-CoA: glycine-N-acyltransferases to form acylglycines – membrane-permeable molecules that are excreted through urine. This excretion is necessary both as a mechanism to reduce excess fatty acid accumulation but also to regenerate Coenzyme A as it is crucial for multiple metabolic pathways [245]. BCAA-driven glycine depletion reduces the formation of acylglycines and therefore reduces available CoA [246]. Additionally, BCAA restriction alleviates the level of even acyl-CoA intermediates because of the fact that BCAA 'overload' –enhanced BCAA oxidation – induces incomplete fatty acid oxidation [247]. Therefore, glycine acts to maintain relatively stable levels of fatty acids within skeletal muscle and reduce the potential for lipid-induced insulin resistance.

Metabolomic studies have utilized both untargeted and targeted platforms to investigate circulating metabolites reflective of systemic mitochondrial dysfunction as a driver of CMDs. The benefit of using untargeted platforms in this regard to is to discover new biomarkers, including acylcarnitines, BCAA catabolites, CAC metabolites, and other novel molecules. The results of these untargeted screens are often validated or investigated further using a targeted metabolomics run, where known internal standards of the molecules to be targeted are processed on the LC-MS or NMR platform prior to screening experimental samples. Often, the specific species of various metabolites (such as acylcarnitines) cannot be resolved fully using an untargeted screen, where aspects such as chain length need to be differentiated in disease states. Therefore, a targeted screening confirms necessary secondary aspects of molecular targets and is useful for validation and exploration of initial findings from untargeted screens.

4. Using metabolomics to characterize bioenergetic metabolites

Numerous key intracellular metabolites have been quantified using metabolomic techniques. An increased focus on metabolites involved in glycolytic and OXPHOS pathways has been particularly useful in understanding their role in CMDs. This section will focus on three important metabolites that are drastically altered during OXPHOS dysfunction and play a large role in the cellular response to the metabolic alterations underlying CMDs: NAD (NAD^+; deprotonated form, NAD^+ and its reduced form, NADH), acetyl-CoA, and S-adenosylmethionine (SAM).

4.1. NAD

NAD + hydrogen (H+) (NADH) is an energy-dense reducing equivalent generated primarily during glycolysis, the CAC, and during fatty acid β-oxidation from its precursor, NAD^+. NADH aids numerous enzyme-mediated metabolic processes and NAD^+ is regenerated; this recycling of NAD^+ is crucial for maintaining an adequate balance necessary for redox homeostasis. Along with $FADH_2$, NADH represents one of the primary reducing equivalents within the cell, responsible for donating high energy electrons to the protein complexes on the ETC found within the inner mitochondrial membrane, and being recycled to NAD^+ again [236]. In the mitochondria, the oxidation of NADH (donation of its electron to Complexes I and III) and the reduction of oxygen at Complex IV into water is paired with the process of ATP synthesis via ATP synthase (Complex V). This is facilitated by the funneling of high-energy electrons through Complexes I to IV with concomitant proton pumping into the intermembrane space from the matrix. The increase in positive charge within the intermembrane space creates an electrochemical gradient that the mitochondrion exploits to drive ATP synthesis within the mitochondrial matrix by coupling the rapid and natural movement of protons back into the matrix along their concentration gradient with the phosphorylation of ADP to create ATP at Complex V. During glycolysis, only two NADH molecules are produced, whereas during fatty acid β-oxidation, each successive round of β-oxidation produces one NADH, along with one acetyl-CoA and one $FADH_2$. Since fatty acids vary considerably in length, from 2 to 26 carbon atoms, the number of NADH that can be produced can be as high as 12 and as low as 1 [242]. The rate of fatty acid oxidation is a primary determinant of the NAD^+/NADH ratio, and consequently, increased incomplete β-oxidation increases the amount of NADH-mediated reductive stress placed upon the ETC [217].

Due to its critical role as a transporter of high-energy electrons that power OXPHOS, perturbations in the redox state caused by altered NADH oxidation can have profound metabolic consequences. Although there is typically more NAD^+ compared to NADH within the cell at homeostasis, a set NAD^+/NADH ratio must be maintained to reflect a healthy redox state, typically between 7 and 8. A decrease in this ratio is noted during ETC dysfunction; inhibiting specific ETC Complex activity (especially Complexes I, III, and IV) reduces the NAD^+/NADH ratio and proliferation of HeLa cells which are rescued by the expression of an exogenous bacterial NADH oxidase to both the cytosol and mitochondria [187]. When cytosolic pools of NAD^+ are exhausted, mitochondrial NAD^+ acts as a compensatory pool to maintain energy production [218]. The buildup of NADH can be attributed to two main causes: i) dysfunction in the ETC Complexes I and III to oxidize NADH and maintain NAD^+/NADH homeostasis within the cell [187,219–222], and ii) a shift to glycolytic flow due to the presence of increased anaerobic stimuli [223,224].

Moreover, NAD^+ levels are regulated by highly complex mechanisms and is a co-substrate for various enzyme families [225], one of which are sirtuins (SIRT). Sirtuins are a family of protein-deacetylating enzymes that act on proteins found within the mitochondrion, and those found within mitochondrial and nuclear genomes (e.g., histones). NAD^+ is an important mediator of cellular proliferation as it participates in the SIRT-mediated regulation of gene expression and histone acetylation. For example, human class III lysine deacetylases such as SIRT-1 are highly dependent on this ratio compared to other acetyltransferases (including histone acyltransferases and deacetylase proteins). SIRTs transcriptionally activate numerous gene families, including peroxisome proliferator-activated receptor-γ, tumour suppressor protein (p53), and the FOXO forkhead family of transcription factors [226]. The overexpression of the Sirt1 gene has the beneficial effect of ameliorating metabolic syndrome phenotypes in animal models. Sirt1 transgenic mice had reduced circulating free fatty acids, total cholesterol, adipokines, insulin and fasted glucose, and displayed metabolic and behavioural phenotypes resembling calorie-restricted and lean mice [227]. Sirt1 knockout mice display reduced PGC-1α-mediated transcription of mitochondrial, fatty acid utilization, and transcription regulatory genes compared to control, implicating Sirt1 as a vital regulator of the switch for substrate use. Additionally, CAC activity was reduced when Sirt1 is inhibited [122]. NAD^+ also interacts with Sirt3 which is a well-characterized regulator of lipid metabolism and energy production through its deacetylase activity on numerous enzymes including

pyruvate dehydrogenase [228,229]. Sirt3 knockout in muscle cells leads to inhibition of PDH, shifting substrate utilization from glucose oxidation to fatty acid oxidation in the fed state, commonly known as 'metabolic inflexibility' [230]. This is summarized in Fig. 3.

Since NAD^+ concentrations fluctuate in a circadian manner and decrease with age, this has immense downstream consequences for the regulation of protein acetylation and transcriptional regulation that correlates with altered metabolism during the sleeping state and in the mitochondrion throughout an organism's life cycle. Furthermore, NAD^+ can be salvaged by the NAMPT-NMNAT pathways which convert nicotinamide back into NAD^+. Thus, the levels of NAMPT and NMNAT within cells also largely determine their ability to maintain a steady NAD^+/NADH ratio when presented with metabolic stress [231,232]. The fasted versus fed condition of the cell also highly regulates the concentration of NAD^+: in the fasted state, NAD^+ levels increase, which would assist SIRTs in deacetylating OXPHOS proteins, and increasing the basal metabolic rate within the cell; however in the fed state, the opposite is suspected to be true, where the level of β-oxidation of stored fats for ATP generation would be suppressed [229,233]. These salvage mechanisms are summarized in Fig. 3.

Targeted metabolomics has been widely used to quantify intracellular NAD^+/NADH ratio in cells both via direct screening for NAD^+ and/or NADH [187], or by screening for metabolites whose synthesis is dependent on this ratio. The latter method has the advantage of visualizing the direct and compensatory mechanisms for alterations in this ratio. For example, as NAD^+ regeneration is needed for the generation of acetyl-CoA via numerous metabolic pathways (glycolysis, fatty acid oxidation, amino acid catabolism, etc.), a lower level of acetyl-CoA observed in OXPHOS dysfunction is expected to be reflective of a lower NAD^+/NADH ratio. In addition, metabolic compensation for a

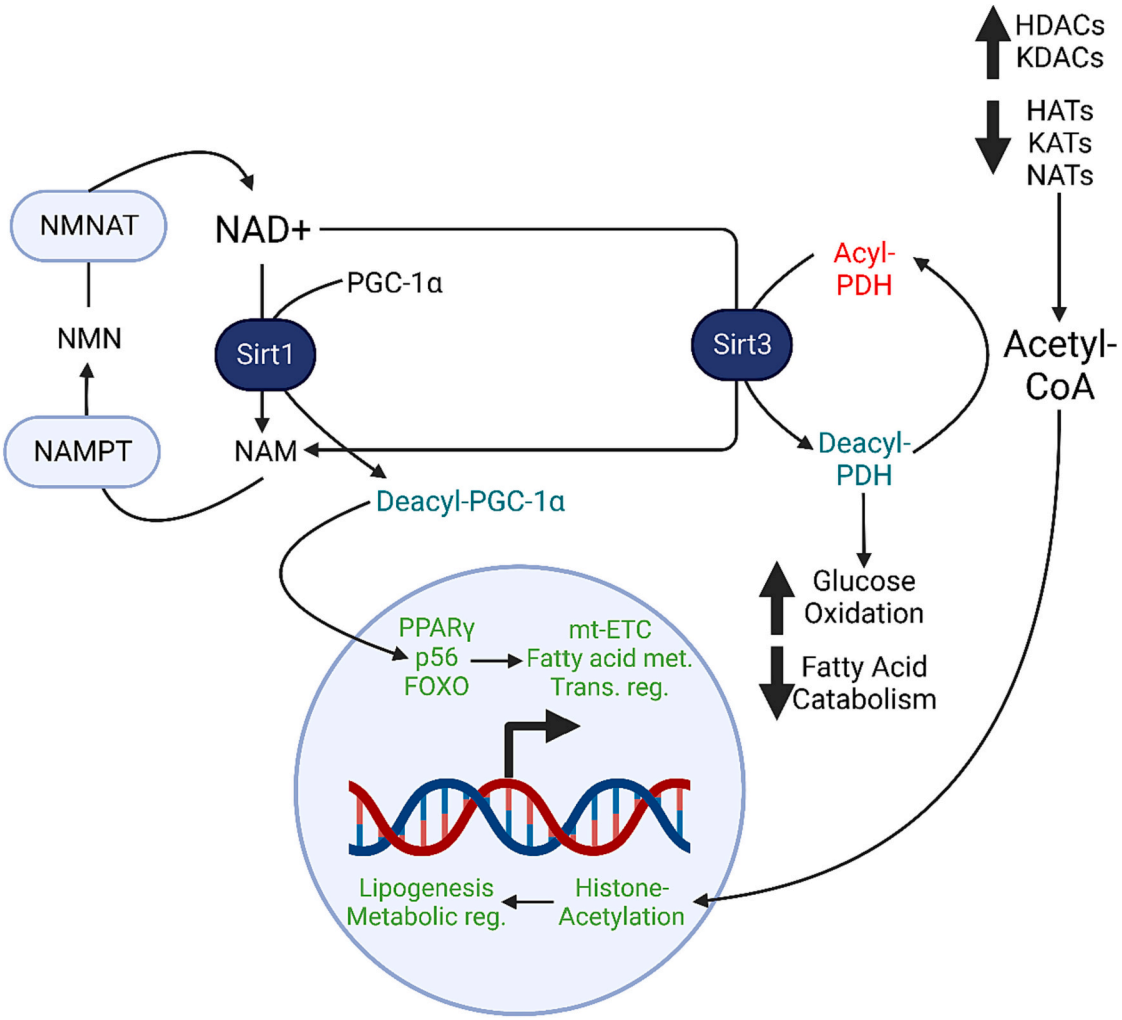

Fig. 3. Regulation of epigenetics and transcription through Sirt-mediated acetylation by NAD+ and Acetyl-CoA.
NAD+ is involved in the Sirt-1 mediated conversion of PGC-1α to Deacyl- PGC-1a, its activated form. Deacyl- PGC-1a increases the expression of numerous regulatory genes including PPAR-γ, p56, and FOXO, which are involved in altering the transcription of mitochondrial electron transport chain, fatty acid synthesis and metabolism, and transcriptional regulation genes. NAD+ is recycled through this process, and therefore strict maintenance of NAD+ levels is critical for transcriptional and metabolic coordination. High levels of acetyl-CoA, controlled by abnormal activity of various acyltransferases and deacetylase enzymes in the CMD state, promotes acylation of PDH, a key regulator of glycolysis. Sirt3-mediated deacylation of PDH alters energy utilization through histone modifications and transcriptional regulation, which leads to alterations in substrate utilization favouring glucose instead of fats in the fed state. These two mechanisms broadly alter substrate utilization leading to metabolic inflexibility (one of many outcomes), which is a hallmark of the CMD phenotype. Abbreviations: Acetyl-CoA - Acetyl-Coenzyme A; FOXO – forkhead box members of class O; HAT – histone acetyltransferases; HDAC - histone deacetylase; KAT - lysine acetyltransferase; KDAC - lysine deacetylase; mt-ETC – mitochondrial-electron transport chain; NAD+ – nicotinamide adenine dinucleotide (oxidized); NAM - nicotinamide; NAMPT - nicotinamide phosphoribosyltransferase; NAT - N-alpha-acetyltransferase; NMN - Nicotinamide mononucleotide; NMNAT - nicotinamide mononucleotide adenylyltransferase; p56 - interferon-inducible protein; PDH - pyruvate dehydrogenase; PGC-1α - peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PPAR-γ - Peroxisome Proliferator-Activated Receptor-γ; Sirt1 – sirtuin 1; Sirt3 - sirtuin 3.

lack of NAD^+ can be examined through the increased activity of lactate dehydrogenase, where a larger lactate/pyruvate ratio has been observed in metabolically stressed cells compared to controls, which salvages the levels of NAD^+ by oxidizing NADH to generate lactate from pyruvate [234]. A general problem with quantifying NAD^+/NADH ratios within cells using traditional metabolomic techniques is the consistent interconversion between NAD^+ and NADH that cannot be controlled at the time of extraction. However, effective protocols have been established which make real-time metabolomic analyses of these molecules accurate and reliable [235].

4.2. Acetyl-CoA

Acetyl-CoA is a central metabolite that is common to many metabolic pathways. It is produced from the catabolism of carbohydrates, fats, and amino acids, and is the primary conveyor of carbons into the mitochondria for metabolism through the CAC [236]. In glycolysis, glucose is ultimately broken down into two pyruvate molecules, which can be transported into the mitochondrial matrix via MPC1 and MPC2. The intramitochondrial PDH complex then decarboxylates pyruvate to Acetyl-CoA, through the recruitment of Coenzyme A (CoA). In addition, the β-oxidation of fatty acids produces an acetyl-CoA molecule at each iteration. Acetyl-CoA is also produced in the cytosol largely through two mechanisms: in a highly fed state, an increase in mitochondrial citrate is exported into the cytosol and is converted by citrate lyase into acetyl-CoA; in the fasted state, acetate, amino acid carbon backbones, ethanol, and ketone bodies can all be used to regenerate cytosolic acetyl-CoA to restore adequate CAC flow and energy production[236]. Therefore, the regulation of acetyl-CoA production, and the Acetyl-CoA to Unbound CoA ratio (acetyl-CoA/CoA) is directly determined by the abundance of glycolytic input and the bioenergetic efficiency of the mitochondrial ETC, similar to that of NADH.

During the initial period of the fasting state and during the fed state, adipose tissues release fatty acids into circulation which are taken up by cells. Intracellular fatty acid catabolism results in an abundance of acetyl-CoA in peripheral tissues, which increases the production of fatty acids and ketone bodies in organs where metabolic homeostasis is vital (the brain and heart) [236]. This buildup of acetyl-CoA within the mitochondria has been strongly associated with acetylation of lysine residues found within mitochondrial proteins, both in fasting and fed states [237–240]. It has also been suggested that the presence of acetyl-CoA itself promotes enzyme-independent protein acetylation [241].

Acetyl-CoA has largely been studied in relation to its role in modulating gene expression through the modification of histone proteins. As mentioned previously, a key regulator of acetyl-CoA availability in the cytoplasm is the citrate lyase enzyme. This enzyme is also found present in the nucleus. Knockdown of citrate lyase in human cells decreases the amount of histone acetylation in the nucleus, but does not change global protein acetylation in a significant way. In the fed state, the flux of acetyl-CoA is directed out of the mitochondrion to facilitate fatty acid and sterol synthesis and histone acetylation; in the fasted state, the generation of a stable and abundant acetyl-CoA within the mitochondrion and cytosol is vital, so the flux of acetyl-CoA is directed to the CAC and the synthesis of ketone bodies [242].

This implies that there are compensatory mechanisms to generate cytosolic acetyl-CoA after the acetyl group is consumed, likely through increased funneling of citrate out of the mitochondria, or through cytosolic acetyl-CoA synthetase. In addition, the loss of citrate lyase also inhibited genes related to glucose metabolism, including Glucose transporter Glut4 and PFK-1 [243]. Therefore, a depletion of acetyl-CoA links the cellular metabolic environment to gene expression responses, specifically decreasing glycolysis and relying on fatty acid synthesis for long term energy storage. When fed a high-fat diet, the cell switches from fat storage to oxidation, and therefore the pool of acetyl-CoA increases. The rise in fatty acid oxidation-dependent elevation of acetyl-CoA is directly proportional to the acetylation (and therefore activation) of key β-oxidation proteins including acyl-CoA dehydrogenases and pyruvate dehydrogenase [244,245]. Therefore, it appears as though acetyl-CoA regulates fatty acid oxidation as a positive feedback loop through the persistent activation of oxidation-related enzymes through acetylation. This has deleterious effects however as a rise in β-oxidation is associated with uncontrolled ROS generation [246], which is known to cause OXPHOS dysfunction.

The general trend therefore observed from acetyl-CoA as a communicator between cellular energetics and gene transcription is as follows: in times of carbohydrate and fat abundance, acetylation of DNA-binding proteins and increased expression of positive metabolic and proliferative genes are encouraged, whereas in the starvation state, the lack of acetylation (due to a lack of acetyl-CoA) and a rise in NAD^+ (increasing Sirt-mediated deacetylation) reduces gene expression and cellular proliferation.

Cellular acetylation can be measured through metabolomic screening, particularly in terms of post-translational modification analyses of acetylated residues in protein fractions. Pougovkina and colleagues utilized a novel LC-MS/MS method that allows for the stoichiometric ratio of acetyl-lysine to lysine in individual proteins and in total lysates to be analyzed. Their targeted analysis in human fibroblasts allowed for the precise quantification of acetylated mitochondrial proteins which was validated through western blot [240]. Similar protocols have also been developed by other authors, to both understand the absolute levels of cellular acetyl-CoA and correlate these levels with downstream acetylation [247,248]. Acetylation may be facilitated through the use of acetyltransferase enzymes (to acetylate histones or other regulatory proteins) or through the activity of Sirtuins such as Sirt3; when acetylated, proteins are in the inactive state and this aberrant acetylation can be resolved through NAD^+-mediated deacetylation as mentioned previously [242]. This interplay between acetyl-CoA overabundance – which is pronounced in disease states of hypernutrition – and low NAD^+/NADH ratios emphasize the opposing roles that NAD^+ (and NAD metabolites) and acetyl-CoA play in regulating metabolic flux through protein activity regulation [249]. This relationship is summarized in Fig. 3. This overabundance is mediated by the dysregulation of acetylation-mediates enzymes including histone acetyltransferase (HATs) and lysine acetylases and deacetylases (KAT and KDAC, respectively); for instance, depletion of N-alpha-acetyltransferase 40 (NAA40), a high conserved HAT catalyzes the addition of acetyl groups to histone H4 and H2A causes increased cellular acetyl-CoA concentrations, de novo lipogenesis, impaired insulin signalling, and decreased glucose uptake into hepatocytes. Additionally, the increased acetylation of histones H2A and H4 was observed and a significant number of lipid biosynthesis genes were induced; however, authors noted that the induction of genes occurred subsequent to the generation of DAG and triacylglycerol (TAG) lipid species, meaning that there is an immediate metabolic rewiring that occurs as a consequence of increased acetyl-CoA stores, which is followed by transcriptional deregulation [250]. NAA40, along with other HATs belonging to the N-terminal acetyltransferase (NAT) class have been implicated in numerous cancers and diseases involving aberrant lipid metabolism [251–253]. Therefore, an investigation into enzymes involved in acetylation/deacetylation – including the movement of other acyl groups (i.e. succinyl, propionyl) – can help elucidate the regulatory and pathological roles of these groups.

4.3. SAM

S-Adenosylmethionine (SAM) is a molecule produced in the cytosol through the reaction of L-methionine and ATP. SAM is largely involved in histone methylation at lysine and arginine sites and acts as a key regulator of DNA packaging. SAM synthesis follows numerous conjoined steps summarized briefly here: abundant cytosolic methylene tetrahydrofolate becomes reduced into methyl-tetrahydrofolate, which in a series of methyl-transferase-mediated events, helps convert homocysteine into methionine, which can be used to synthesize SAM and

glutathione. SAM is a key metabolite in a collection of processes known as 'one-carbon metabolism', which is a converging of numerous cell processes that are dependent on molecules that fundamentally involve the transfer of one carbon to propagate a cellular process; in this case, one-carbon metabolism allows for the transfer of a methyl group to create SAM. SAM levels are also dependent on the NAD^+/NADH ratio; a lower ratio (as in the case of OXPHOS dysfunction) translates to the depression of methylene tetrahydrofolate levels which is an important step in SAM generation, directs THF to be used in purine synthesis pathways, and reduces the recycling of SAM into S-Adenosyl Homocysteine (SAH) which then can be hydrolyzed to form Homocysteine (Hcy), which is used in a NAD^+ -mediated pathway to regenerate SAM [254,255]. Because this epigenetic process is highly regulated by multiple metabolites, understanding SAM and intermediates in the SAM synthesis pathway are important in evaluating the metabolic and bioenergetic control of gene expression through epigenetics. Using metabolomic methods, Mentch and colleagues demonstrated that the status of methionine metabolism was significantly correlated with levels of SAM and SAH. In addition, the modulation of methionine levels in diet led to alterations in H3K4me3 methylation compared to other methylation sites, which was detected using LC-MS [256]. The use of metabolomics to detect SAM, SAH, and histone methylation levels of individual histone proteins in cells and tissues has been fairly recent, although well-validated protocols do exist [257,258]. Hcy, and by association SAM which are both sulfur-containing metabolites, can be used to generate GSH, an endogenous antioxidant central to the regulation of autophagy and cellular redox status, which links SAM concentrations to the level of GSH [259–261]. Increased cellular oxidative stress is central to the cardiovascular disease and cancer state. Ouyang and colleagues have explored SAM's multi-faceted role in regulating autophagy through epigenetic regulation of protein, histone, DNA, and RNA-mediated autophagy pathways, through GSH antioxidant synthesis, and through the management of the levels of various SAM metabolites including Hcy and decarboxylated SAM (dcSAM) [262]. Hcy inhibits mTORC through the AMPK/mTORC signalling pathway, which reduces autophagy [263]. dcSAM, whose concentrations are tightly controlled, is used to generate the polyamine spermidine (SPD) which is known to induce the transcription of autophagy genes by inhibiting histone acetyltransferase activity [264]. It has recently been found that activation of the pro-inflammatory phenotype within macrophages is mediated by SAM-induced trimethylation of histone H3K36 which induces Il-1B production through the integration of the folate and methionine cycles [265]. The circulating SAM/SAH ratio has been widely regarded as an indicator of cellular methylation status and has been shown to be a sensitive indicator of a variety of diseases [266–268]. In ApoE−/− mice, the SAM/SAH ratio was higher compared to controls; the methylation of numerous genes implicated in atherosclerotic plaque generation was decreased [269]. Therefore, de novo generation of SAM through overnutrition (over availability of methionine and amino acid backbones for SAM generation) may contribute to the function or dysfunction of various cell types, and SAM and other sulfur-containing SAM metabolites must be highly regulated to prevent the autophagic phenotype of various diseases.

Indeed, the three metabolites mentioned in Section 3 are also found in circulation. Here, we will focus on NAD^+ as an emerging redox biomarker indicated in a variety of disease types [270–272]. NAD^+ has been previously established as a key aging-related biomarker. Its depletion is highly correlated poly(ADP-ribose) polymerase (PARP), a DNA-damage repair family of proteins that consumes a large amount of NAD^+. Therefore, NAD^+ presents as an attractive marker of both oxidative stress and DNA damage. However, there are numerous mechanisms for the export of NAD^+ across the plasma membrane, which include connexins and exocytotic release. Cx43 hemichannels in the plasma membrane allow for passive bidirectional transport of NAD^+, and are regulated by cytosolic Ca2 binding [273]. NAD^+ release has also been noted in dying cells, due to pathogen-mediated leaks or hyperosmotic forces that disrupt the plasma membrane, releasing NAD^+ into the extracellular space [274]. Interestingly, enzymes involved in the biosynthesis of NAD^+, including NAMPT and NARPT, are also found in the extracellular space, and act as pro-inflammatory factors on target vascular and immune cells [275].

Extracellular NAD^+ has been implicated in the contractility of mammalian vascular tissues. NAD^+ infusion on rat thoracic aorta, porcine coronary artery, and porcine mesenteric arteries that were mounted for isometric tension recording invoked smooth-muscle dependent vasorelaxation through A2A adenosine receptors on the aorta and coronary artery tissue studied but contrastingly invoked vasoconstriction in mesenteric arteries mediated by P2X-like receptors. Therefore NAD^+ has actions that are highly tissue- and species-specific, which would serve as a point of caution for studies translating NAD^+ signalling endpoints between organism models and in the biological system studied. However, since NAD^+ has the property of mediating vasoregulation, elucidating the effect of circulating NAD^+ levels in manifestations of smooth muscle dysfunction (e.g. in the hypertensive state) would be interesting.

It is clear however that circulating levels of NAD^+ and associated molecules change dramatically with age and in age-related diseases. By performing a targeted metabolomic screening of human plasma, Clements and colleagues demonstrated that absolute levels of NAD-related metabolites (NAD^+ and $NADP^+$), as well as the NAD^+/NADH ratio were decreased during aging. It was also noted that NAD^+ was most sensitive to age-related decreases [276]. It is known that aging increases the risk of metabolic dysfunction within highly metabolically active cell types [277,278]. In addition, as previously mentioned, the NAD^+/NADH ratio decreases with increased OXPHOS dysregulation and ETC disruption. Thus, it is interesting that the decrease in the NAD^+/NADH ratio seen within those cells can be directly emulated in the plasma environment. Sex differences exist in the plasma NAD^+/NADH ratio; female adults have a higher ratio compared to male adults, although this discrepancy reduces significantly with increasing biological age. Although sex-related differences in vascular function (among other markers of CMD phenotypes) related to mitochondrial regulation are largely uninvestigated, there is evidence that vascular mitochondria derived from female rats display a larger resistance to oxidative damage and healthier bioenergetic profile compared to males [279]. Estrogen may have a protective effect here by modulating antioxidant activity through PGC-1a modulation and increased ETC subunit biogenesis [280]. It appears then that age and sex are both modulating factors in the cellular redox state that is reflected through the balance of both cellular and circulating pools of NAD^+ and NADH.

5. Mitochondrial DNA mutations and metabolic dysfunction.

The mitochondrial genome contains 37 genes which encode for 13 proteins, 22 tRNAs, and 2 rRNAs; all of these encoded proteins are involved in the production of ETC subunits in the OXPHOS system [281]. Mitochondrial DNA (mtDNA) has a high mutational load relative to its nuclear counterpart, and the accumulation of point mutations in the genome has been associated with alterations in the makeup of ETC subunits at the amino acid level (missense mutations) [282]. The relationship between mtDNA mutations and disease outcomes can be modeled using the mitochondrial disease, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) as an example. MELAS is a multi-system disorder derived from mutations to the mt-TL1 gene, which encodes for a leucine transfer RNA [283]. The m.3243A>G variant is the most prevalent and therefore most studies evaluating biomarkers for MELAS utilize cell-based model systems with m.3243A>G mutations. MELAS has been associated with deficient Complex I assembly and reduced OXPHOS function, as well as decreased protein levels of Complex I, III, IV, and V subunits [284,285]. Cells with increasing m.3243A>G heteroplasmy (increasing ratio of mutated to non-mutated cells) display a decline in the nuclear NAD^+/NADH ratio,

only in the 20–30 % range [286]. Additionally, increased heteroplasmy induces glycolytic gene expression and alters mitochondrial morphology and reduces the OXPHOS and ETC mRNA levels of genes transcribed from mtDNA [287]. In the higher ranges of mutation, NAD^+ compensatory mechanisms are activated raising nuclear NAD^+ levels but mitochondrial NAD^+ levels decline significantly. Acetyl-CoA levels slightly decrease and AKG levels fluctuate significantly with increased heteroplasmy, which leads to increased H3 and H4 histone acetylation [286]. This may be mediated directly through non-enzymatic acetylation caused by generally high Acetyl-CoA levels because of lower OXPHOS activity consuming acetyl-CoA through the CAC and depressed HDAC activity in highly heteroplasmic cells [287]. These in vitro findings are summarized in Fig. 4. Human studies have also corroborated MELAS-mediated OXPHOS dysfunction and redox balance; elevated levels of β-hydroxy acylcarnitines, β-hydroxy fatty acids, several N-lactylated BCAAs and aromatic amino acids, and lactate were found in the plasma of MELAS patients vs controls [288]. Li and colleagues (2022) have also found decreased arginine metabolism through integrated proteomic-metabolomic plasma profiling [289]. Therefore, there is a potential link between mtDNA mutation, ETC and OXPHOS activity, and the levels of various key metabolites involved in pathological progression.

As several studies have shown redox imbalance and OXPHOS dysfunction to be hallmarks of the CMD state, an understanding of the mtDNA mutations that may drive these imbalances in OXPHOS protein assembly and activity is warranted [228–230]. Several preclinical studies have displayed the effects of pathological mtDNA mutations on systemic metabolism and OXPHOS function. Mitochondrial-Nuclear eXchange (MNX) mice are now a commonly utilized model for understanding the in vivo alterations to metabolic function caused by known mutations to the mitochondrial genome. The MNX mouse model utilizes C57BL/6J and C3H/HeN mice, which have known mtDNA mutations that make them susceptible and resistant to CMD pathogenesis, respectively [290–298]. Through pro-nuclear transfer, the nuclear and

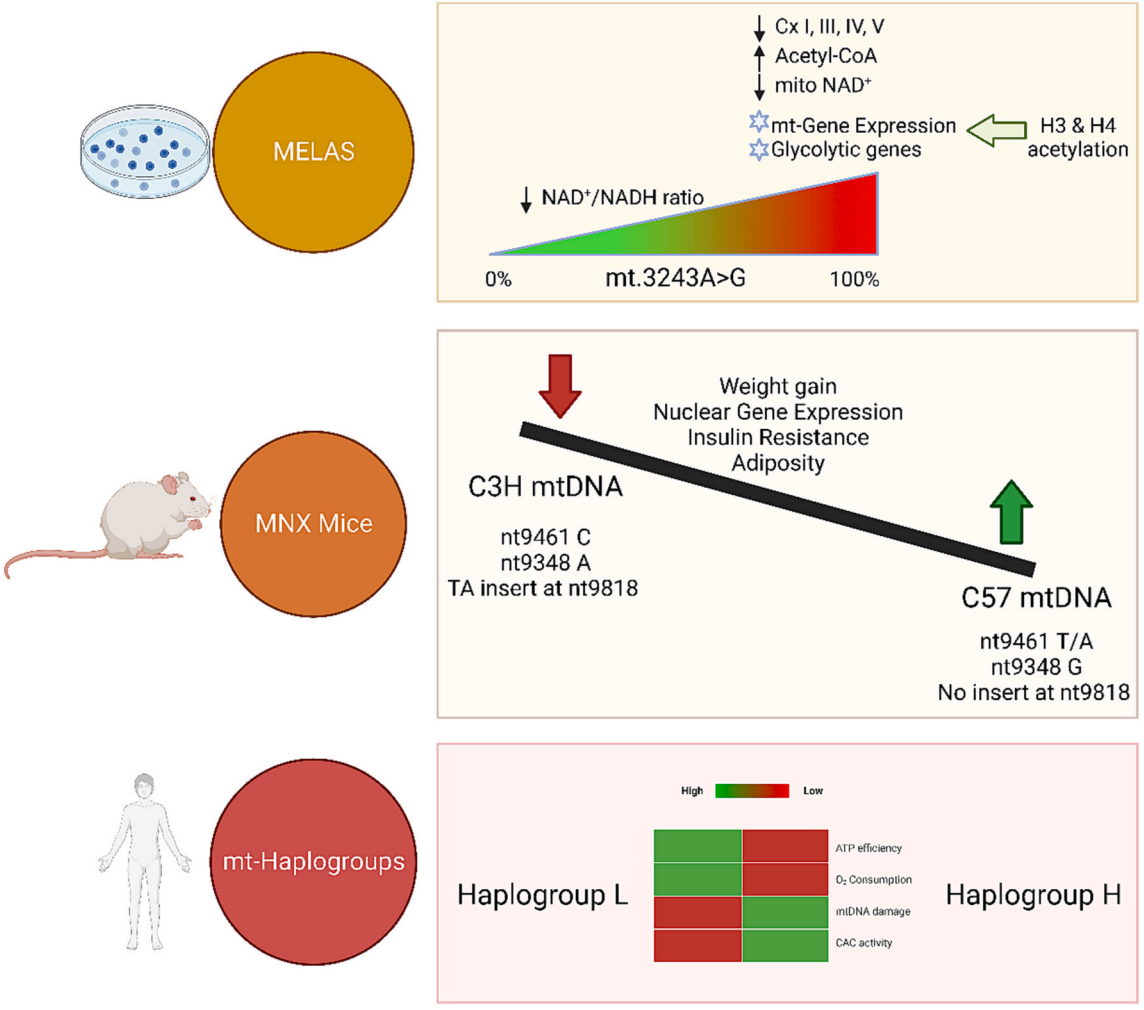

Fig. 4. Cell, Animal, and Human manifestations of mtDNA mutation-directed disease and effects on metabolic and redox control.
Studies of MELAS utilize increasing proportions of heteroplasmy (as viewed through the proportion of mtDNA with mt3234A > G mutation) to evaluate the metabolomics and epigenomic changes driven by this mutation. When approaching high levels of heteroplasmy, the expression of various electron transport chain proteins and mRNA, the levels of mitochondrial NAD+, and the acetylation of histones H3 and H4 are significantly altered, leading to irregular metabolic and mitochondrial gene expression. In studies of MNX mice, the presence of C57 mtDNA has been noted to negatively impact phenotypic and nuclear transcriptional changes leading to the increased prevalence of CMD, regardless of nuclear background; these pathological effects are relatively absent in diet studies of mice possessing C3HmtDNA. In humans, mitochondrial haplogroup studies have noted wide variations in the levels of oxygen consumption, energy utilization, and the efficiency of metabolic processes especially in the comparison of haplogroups L and H. Abbreviations: Acetyl-CoA – Acetyl-Coenzyme A; ATP – Adenosine triphosphate; CAC – Citric Acid Cycle; C3H – C3H/HeN strain; C57 – C57/BL6J strain; Cx – Complex; H3 – Histone H3; H4 – Histone H4; MELAS - Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes; MNX – Mitochondrial Nuclear eXchange; mt – mitochondrial; mtDNA – mitochondrial DNA; NAD+ – Nicotinamide adenine dinucleotide (oxidized); NADH - Nicotinamide adenine dinucleotide (reduced); nt – nucleotide.

mitochondrial genetic backgrounds of these wild-type mice are altered, resulting in the generation of MNX mice with nuclear and mitochondrial genomes with opposite susceptibilities. Dunham-Snary and colleagues (2018) have demonstrated that mice harboring the C57MNX mtDNA possess greater body fat, weight gain, pathological nuclear gene expression, and metabolic efficiency in a high-fat diet model of CMD [299]. C57 mtDNA possesses point mutations in the genes encoding for ETC Complexes I and IV subunits as well as a 'TA' insert in an arginine transfer RNA that differentiates it from C3HmtDNA. C57mtDNA-possessing mice were also observed to have lower baseline leptin levels, greater insulin resistance, and lower muscle phosphorylated Akt compared to controls [300]. These results are summarized in Fig. 4.

The efficient utilization of caloric input (carbohydrates, fats) into energy-dense molecules used as the energy currency of the cell (e.g., ATP, reducing equivalents) relies upon the efficient reduction of ubiquinone (CoQ) at Complexes I and II and the transport of electrons through the ETC. However, ETC complexes are prone to electron leakage, which manifest as superoxide production (ROS), which increase DNA damage and cell apoptosis [301]. In addition, this leakage and uncoupling reduces the efficiency at which ATP is produced given the same stimulus; this discrepancy is visualized in human mitochondrial haplogroups (ancestral genetic groups that are separated both by geographical space and evolutionary time providing separate accumulations of mutations within their respective mtDNA), where endothelial cells belonging to haplogroup L (found ubiquitously in the African continent) consume less oxygen to produce a larger amount of ATP compared to those in group H (found in northern Europe). Interestingly, individuals in group L also have less CAC activity and increased mtDNA damage, despite more 'efficient' ATP production [302]. This drop in CAC activity and rise in ROS in mtDNA variants implicated in features of CMDs is characteristic of cellular dysfunction not only in endothelial cells, but in cardiac cells as well [303]. Therefore, possession of particular mtDNA backgrounds may modulate CMD manifestation, and this relationship is summarized in Fig. 4 [304].

6. Harnessing metabolomics to drive translational CMD research

The plasma metabolome has been extensively studied to grow our understanding of markers for disease diagnosis and progression. Numerous preclinical and clinical studies have looked to circulating metabolomics to identify markers of disease that correlate with hypertension, a primary CMD [305]. Deitrich and colleagues (2016) identified 127 metabolites that were highly prevalent in serum profiling of participants that were followed up 10 years after initial observations; six of these metabolites were the most predictive of the development of hypertension, which included elevated serine and glycine, and the association was as nearly as strong as that of BMI and age on hypertension development [306]. Similarly, other metabolomic studies have pointed to elevated total glycerolipids, including well-characterized species such as triglycerides and diglycerides, ceramides, and oleic acids, as being largely associated with incident hypertension diagnosis. These biomarkers have been significantly associated with systolic blood pressure, diastolic blood pressure, and mean arterial pressure [307,308]. Interestingly, numerous biomarkers that are typically associated with BMI were also found associated with abdominal adiposity, insulin resistance, and dyslipidemia, which include numerous amino acids, intermediates in the CAC, and the urea cycle. However, many of these markers did not associate with changes in blood pressure or resting glucose [309]. This indicates two things: 1) researchers can cluster disease-associated biomarkers based on shared and unshared relationships to disease pathogenesis, and 2) novel biomarkers that align singularly with one disease type can be used in conjunction with shared markers to heavily inform and predict disease diagnosis, complementing traditional physical examination markers associated with diagnosis.

Several reviews have been published regarding the opportunities and challenges of integrating metabolomics modalities into the clinic laboratory and the process of clinical biomarker discovery in CMD [310]. Grebe and Singh (2011) outlined several benefits to the replacement of traditional radiolabelled immunoassays with LC-MS/MS, including limitations in the sensitivity of immunoassays themselves in capturing low molecular weight compounds, simpler workflows for target extraction, savings in reagent and maintenance costs compared to alternative techniques (immunoassays as well as GC–MS), as well as avoiding prolonged exposure to radiation. Limitations to the adoption of LC-MS/MS in clinical laboratories include the technical complexity of operating the instrumentation and the time necessary to train relevant staff on operation, among others [311]. These considerations and a biomarker discovery workflow can be found in Fig. 5.

However, there are certainly numerous challenges in implementing plasma metabolomics as an indicator of systemic pathology. Humans experience biological variation due to numerous factors such as diet, age, and sex that significantly change the abundance of circulating metabolites even in individuals presumed to be biologically similar, in addition to economic and environmental interventions such as pollution which widely alter the metabolome through various mechanisms [312–315]. Two factors that necessarily need to be considered include feeding and circadian rhythm when developing a metabolomics experimental design. Blood metabolomic studies in preclinical models and human subjects are often inconsistent in their reporting of whether their subjects are fasted or fed or what time of day blood is collected [316,317]. Differentially regulated metabolites that can be sub-grouped based on their presence or absence during various feeding and circadian times increase validity of these metabolites as biomarkers for disease in a translational context, as patients can be tested for blood-derived metabolites at various times of day under varying feeding conditions [82].

Moreover, although the abundance of metabolomic data is ever-growing, current databases suffer from insensitive methods used in metabolome detection (including NMR), the existence of unknown metabolites that have not been characterized, and a lack of understanding of the utility of metabolomic screening given the complex data that are produced in the form of mass spectra or liquid chromatograms [318]. However, metabolomics offers a substantially novel perspective on medical diagnosis and pharmacological intervention planning that focuses on alterations in the individual metabolomic landscape compared to the 'usual' metabolite profile. Because of this individualization, numerous in silico models can be utilized to prepare pharmacological treatments to address elevations or depressions in circulating metabolites that are either reflective of downstream cellular pathology or are markers of the individual expression of systemic disease. An example of this has been demonstrated by Xu and colleagues (2021). After an untargeted metabolomics screening of the human hepatocellular carcinoma cell line HepG2, an integrated network combining relative metabolite concentrations and enzyme connectivity was built using pathway enrichment analysis. By outlining which enzymes were responsible for significant perturbations in key metabolites, an in silico docking methodology that proposes potential inhibitory molecules for these enzymes based on drug-enzyme kinetics and affinity binding evaluations was developed, and was validated by measuring endogenous substrate concentrations as well as activity assays for the enzyme [319]. This has the power of both expanding our understanding of drug targets for molecules that have known or unknown therapeutic activity and the enzymes that they likely affect, and using a bottom-up approach by outlining which pathways should be beneficiaries of drug targeting specifically. A biomarker discovery workflow integrating this network analysis is proposed in Fig. 5.

Systems biology refers to the integrated understanding of a biological system through inter-disciplinary analysis of component parts to identify emergent properties of the system. This inter-disciplinary analysis relies on the emergence of computational technologies that allow for associations and correlations between large but distinct datasets within biological systems. However, metabolite profiling using a systems biology approach faces many challenges in reliably quantifying changes

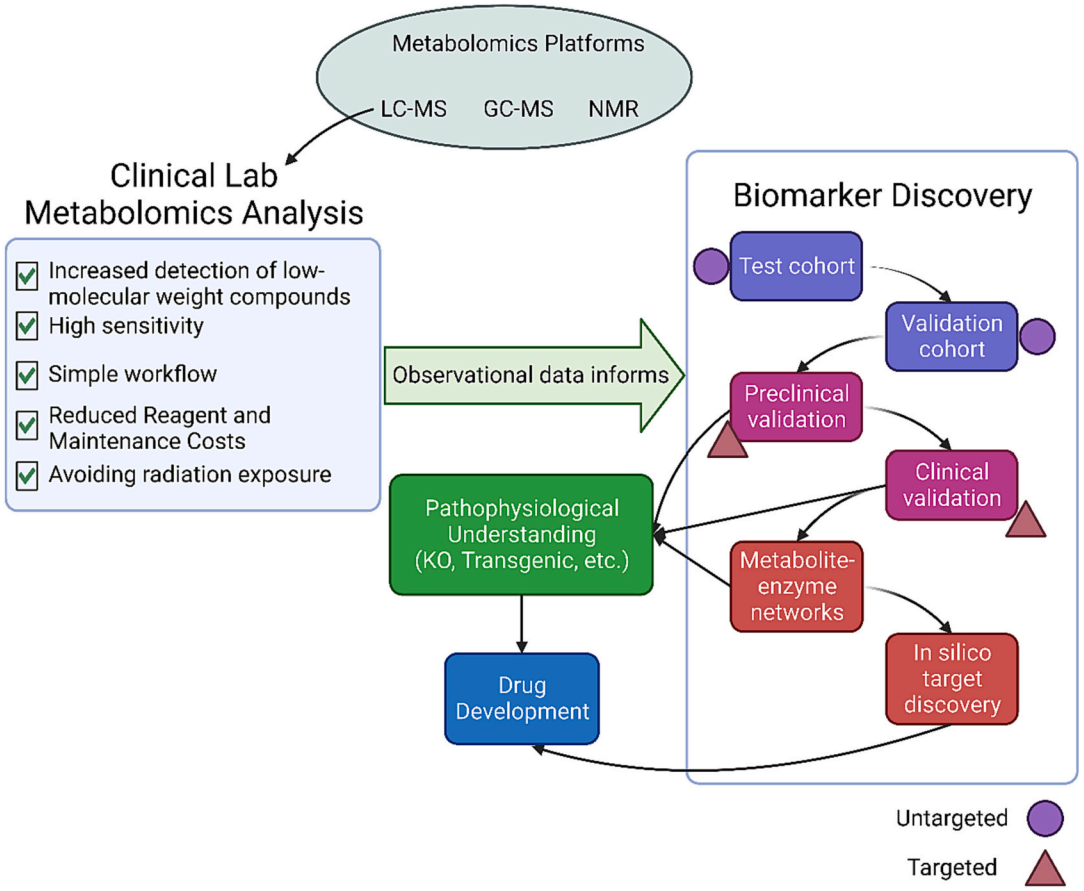

Fig. 5. Benefits of metabolomics implementation in the clinical laboratory and Biomarker Discovery workflow to elucidate biomarkers of interest and disease mechanisms through untargeted and targeted analyses.
In clinical use, metabolomics has numerous benefits over traditional marker or metabolite detection tools (i.e. immunoassays, radioactive isotope labelling). The metabolite profiles obtained from clinical samples can be used in a biomarker discovery pipeline, whereby a test cohort (from single- or multi-centre studies) can be utilized as pilot data. Untargeted metabolomic analysis from these pilot studies is validated through the use of specific patient populations. When metabolites of interest are elucidated, these metabolites are further manipulated in preclinical experiments (i.e. knockout, overexpression, metabolite labelling, fluxomics, etc.) and then validated in clinical samples using similar techniques. From this analysis, a greater understanding of disease mechanisms as they relate to the metabolite of interest can be gained, as well as a snapshot of the metabolite networks altered in disease states. These relationships and structural identification using targeted metabolomics and NMR studies can help identify drug targets in silico that may recapitulate the levels of these metabolites back to those found in patients without disease. Abbreviations: GC–MS – Gas Chromatography-Mass Spectrometry; KO – knockout; LC-MS – Liquid Chromatography-Mass Spectrometry; NMR – Nuclear Magnetic Resonance Spectroscopy.

in metabolite levels, discovering or annotating novel metabolites, and detecting low abundance metabolites [320].

The type of analysis technique is suitable for differing applications which poses a challenge for finding global metabolic profiles of biological samples, where a balance between adequate sensitivity and selectivity as well as reproducibility and dynamic range need to be met. A review of the considerations in various Chromatography-MS and NMR modalities is outlined in Table 1.When possible it is optimal to pair NMR analysis with MS analyses to increase metabolite coverage (in polarity and mass) [321]. Numerous studies have explored this pairing in novel ways. Bingol et al. (2015) developed a method called SUMMIT MS/NMR whereby mixtures are first analyzed by MS to identify chemical formulas for mixture components; these chemical formulas are translated into predicted NMR spectra and are compared to the experimental NMR spectra in order to match information from both MS and NMR sources [322]. Other groups have instead integrated metabolite identification results from both MS and NMR and counted overlaps between the two results or simply combined results together to generate a metabolite panel [323,324]. To some extent, this has the advantage of cross-validating metabolites with significant abundance alterations through two analytical techniques while leveraging the strength of MS to generate a broad dynamic range of possible metabolites. Gathungu et al. (2018) provide an excellent review of various physically coupled MS-NMR apparatuses [325]. A common theme arising from the multitude of approaches is the increased sensitivity and detection of low-abundance metabolites due to leveraging the broad dynamic range and sensitivity of MS and the resolution and reproducibility of NMR, which poses a significant benefit in metabolite profiling in CMD. Simultaneous quantitative metabolomic analysis of the plasma high-fat diet fed rats experiencing metabolic syndrome found that the sensitivity of plasma metabolome analysis and group separation of HFD exposure and time on diet were both improved when NMR and MS results were integrated [326].

To reliably create metabolite profiles for CMD states using a systems biology approach, certain conditions must be taken into consideration.

First, standardized practices must be maintained for metabolomic workflows of biofluids. The need to share pre-processing and processing steps in study methods with other investigators and the sharing of raw data for re-analysis is also instrumental in ensuring results are more reproducible. The use of integrated software with predetermined data treatment and filtering steps can be useful here. The use of standardized protocols and greater metabolome coverage through the use of contemporary MS techniques (e.g. UPLC-MS, GC–MS, 2D-NMR, MS-NMR) will increase the rate at which metabolite biomarkers can be

validated and implemented in patient care [327].

To validate and evaluate a metabolite as a biomarker for CMD states, implementation of other omics disciplines (i.e., genomics, transcriptomics, proteomics, etc.) can be useful. Genomic studies have outlined numerous metabolic loci associated with over 400 metabolites in human blood. This develops a hereditary framework from which to view metabolite differences in humans [328]. Further, implementation of a genetic risk score with metabolite biomarkers has improved the predictive accuracy of T2DM compared to using traditional clinical risk factors alone [329]. Price et al. (2017) demonstrated that combining genomic, metabolomic, proteomic, and microbiome analysis generated effective and personalized dietary interventions for patients with T2DM [330]. Therefore, multi-omics is a desirable systems biology approach integrating multiple fields to both provide detection tools and design interventions for CMD.

Lastly, practices involving the consistent updating, quality assurance, and scientific accuracy of online resources for metabolite discovery, identification, annotation, and pathway analysis must be made clear and conducted frequently. Mass spectral quality assurance and quality control practices in large databases as mentioned in Table 2 are largely variable due to oversights regarding curation, the deposition of low-quality mass spectra, inconsistent reporting of instrumentation and technical inputs, and the lack of data sharing may present hindrances to metabolite identification [74]. Fortunately, the use of tools such as MetaboAnalyst [77] presents open and transparent workflows that can be shared for spectral pre-processing to statistical analysis, and metabolite annotation from known libraries [327]. Furthermore, investigators performing pathway analysis through metabolite pathway enrichment analysis (MPEA), over-representation analysis (ORA), or other means rely on software programs to utilize updated databases and metabolite lists/terms as well as pathway databases to be informed about current literature on the metabolites of interest.

Although hopeful, metabolomic profiling in the context of our understanding of personalized medicine is largely ridden with knowledge gaps. Traditional medical signs of disease risk such as BMI circumference fail to capitulate inter-individual variation in susceptibility [331]. As it is noted that major bioenergetic changes occur during the cellular pathogenesis of numerous CMD states, an investigation into the controllers of mitochondrial variation is warranted to understand underlying disease mechanisms, and how these are presented and profiled within the circulation. The concept of the 'mitochondrial paradigm of disease susceptibility' has been proposed for decades [255,304,332–334], owing to our understanding of human migratory patterns and bioenergetic adaptations that occurred for early human populations to adapt to radically new environments (e.g. incredible thermal changes and food scarcity) [335]. We know that mtDNA variance underlies cellular dysfunction in cell types implicated in CMD pathogenesis [304]. Indeed, it has also been proposed that there is a form of 'cross-talk' that arises from the modulation of metabolite production within the mitochondria, that, through epigenetic mechanisms, regulates nuclear gene expression [286,299,336]. However, human studies in this area are unable to segregate the effects of mtDNA from nuclear DNA in terms of their control on bioenergetic dysfunction; additionally, numerous preclinical models often use cell lines that do not ensure complete segregation of the two genomes (e.g., cybrid cell lines). Fortunately, the use of the Mitochondrial-Nuclear eXchange (MNX) mouse model, an emerging and already highly-utilized model to completely segregate nuclear DNA- and mtDNA-specific effects on CMD progression, will be key in future in-vivo and in-vitro studies analyzing these concepts [337].

In addition, studies using the MNX mouse model largely rely on the bioenergetics, proliferation, or functionality of particular cell types implicated in CMD pathogenesis, but not in the plasma; studies that investigate the circulation have not been used for the discovery of new and confirmation of known metabolites of interest in predicting CMD progression, but rather have used classical markers of CMD (e.g. glucose and insulin) [300]. To fill this gap, a metabolomic screening and fingerprinting of the plasma of mtDNA-mutational models such as the MNX model is warranted, which will help elucidate systemic changes in key metabolites. The plasma metabolome, with or without modulation by the factors mentioned before, will help us understand the downstream consequences of large-scale metabolic dysfunction on the cellular and tissue levels, and will help us explore the use of plasma as a relatively non-invasive and reliable surrogate for understanding CMD onset and progression.

Declaration of competing interest

The authors declare no conflicts of interest.

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