The Arylamine N-acetyltransferases as therapeutic targets in metabolic diseases associated with mitochondrial dysfunction.

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Abstract
In humans, there are two arylamine N-acetyltransferase genes that encode functional enzymes (\textit{NAT1} and \textit{NAT2}) as well as one pseudogene, all of which are located together on chromosome 8. While they were first identified by their role in the acetylation of drugs and other xenobiotics, recent studies have shown strong associations for both enzymes in a variety of diseases including cancer, cardiovascular disease, and diabetes. There is growing evidence that this association may be causal. Consistently, \textit{NAT1} and \textit{NAT2} are shown to be required for healthy mitochondria. This review discusses the current literature on the role of both \textit{NAT1} and \textit{NAT2} in mitochondrial bioenergetics. It will attempt to relate our understanding of the evolution of the two genes with biological function and then present evidence that several major metabolic diseases are influenced by \textit{NAT1} and \textit{NAT2}. Finally, it will discuss current and future approaches to inhibit or enhance \textit{NAT1} and \textit{NAT2} activity/expression using small molecule drugs.

Significance statement
The arylamine N-acetyltransferases, \textit{NAT1} and \textit{NAT2}, share common features in their associations with mitochondrial bioenergetics. Together, they are potential drug targets for diseases where mitochondrial dysfunction is a hallmark of onset and progression.
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Perturbations in mitochondrial function are common features in the pathogenesis, progression, and complications of various metabolic diseases. Recent studies have shown that the N-acetyltransferases (NATs) are important in mitochondrial biogenesis and cellular metabolism. Through this review, we will consider various aspects of mitochondrial function as it relates to health and disease, before discussing how NAT could contribute to mitochondrial function, and dysfunction. Our primary aim is to provide an up-to-date summary of the role that NATs may play in metabolic diseases, while offering perspectives that are often overlooked. Throughout the review, we compare NAT1 and NAT2 to highlight their functional similarities and differences.

I. History and background of arylamine N-acetyltransferases

A. Brief history.

The NATs are a family of highly conserved enzymes found in prokaryotes and eukaryotes (Butcher et al., 2002). In humans, there are two genes that encode NAT1 and NAT2. Both are located on the short arm of chromosome 8 at 8p21.3-23.1 (Blum et al., 1990) and share approximately 87% nucleotide sequence identity (Kawamura et al., 2005). They were first identified for their role in the metabolism of drugs such as isoniazid, dapsone, and sulfamethoxazole. In 1953, Bonicke and Reif reported significant inter-individual variation in the metabolism of isoniazid in humans and animals, which was later shown to be caused by genetic variation in the NAT2 gene (Bonicke and Reif, 1953). This discovery pioneered the era of pharmacogenomics and was the basis for many future studies into the relationship between genetic variation in drug metabolizing enzymes and clinical outcomes (Hein and Millner, 2021). Later, it was discovered that NATs could bioactivate carcinogenic aromatic hydrocarbons (Glowinski et al., 1978) and food-derived heterocyclic amines (Snyderwine et al., 1988). This led to numerous population studies associating NAT polymorphisms with cancer risk. The role of the NATs in carcinogenesis has been extensively reviewed elsewhere (Butcher and Minchin, 2012; Hein, 2002; King et al., 1997).

A major contribution to our understanding of the NATs was the solution of their crystal structures along with an in-depth analysis of the similarities and differences between the two proteins (Wu et al., 2007). Firstly, the human NATs have very similar three-dimensional structures to each other, especially in the N-terminus. Moreover, they show structural similarities to the prokaryotic NAT proteins (Sinclair et al., 2000). Both NAT1 and NAT2 have catalytic triads but their active sites show distinct differences. NAT1 has a smaller binding pocket (~162 Å) compared to NAT2 (~257 Å) and prefers smaller substrates (Wu et al., 2007). The C-terminus of the NATs is located across the cavity in which the active site resides, possibly regulating catalytic activity (Sim et al., 2008). The two proteins have similar overall structures but with important subtle differences that may have implications for their individual biological roles. One of these differences is the binding of acetyl-CoA, which may have a higher affinity for NAT1 than for NAT2 (see Section IF).

B. NAT regulation – update

Expression of the NATs is regulated at transcriptional, post-transcriptional, and post-translational levels and has been reviewed previously (Butcher and Minchin, 2012). However, recent studies have expanded our understanding of NAT regulation,
particularly on how NAT1 and NAT2 enzymatic activities are affected by post-translational mechanisms such as protein lysine acetylation, microRNA expression, thermoregulation, and modulation by endogenous molecules such as glucose and ATP. These post-translational effects may be as important as genetic variation, especially in metabolic diseases where mitochondrial dysfunction is evident.

The role of lysine acetylation in NAT1 activity was first reported in studies using the pan histone deacetylase inhibitor trichostatin A (TSA) (Paterson et al., 2011). Cells treated with TSA increase NAT1 transcription by recruiting Sp1 to the proximal promoter suggesting the NAT1 gene is partially repressed by chromatin condensation. Both TSA and the pan histone deacetylase inhibitor suberoylanilide hydroxamic acid induced NAT1 expression in both MDA-MB-231 and ZR-75-1 cells (Salazar-González et al., 2022). These results are supported by observations in blood cells from patients with acute lymphoblastic leukemia, who had significantly lower NAT1 activity compared to controls and lower acetylated histones associated with the NAT1 promoter (Hernandez-Gonzalez et al., 2023). Direct acetylation of the NAT proteins may also affect their activities. Treatment of blood cells with nicotinamide, a pan inhibitor of sirtuins, increased NAT2 activity without apparently affecting NAT1 activity (Turiján-Espinoza et al., 2018). By contrast, nicotinamide decreased NAT1 activity in cancer cells (Butcher et al., 2020). Moreover, transient knockdown of SIRT1/2 with siRNA decreased NAT1 acetylation and activity (Butcher et al., 2020; Salazar-Gonzalez et al., 2018). Acetylation of NAT1 protein is catalysed primarily by p300/CREB-binding protein, which targets lysine 100 and lysine 188 (Butcher et al., 2020; Minchin et al., 2018). Lysine 100 may be involved in acetyl coenzyme A (acetyl-CoA) binding suggesting protein acetylation modulates the first step in the acetylation reaction (Minchin and Butcher, 2015). Collectively, these studies illustrate regulation of NAT1 activity by protein acetylation. It is noteworthy that the rate of intracellular protein acetylation is dependent on the abundance of acetyl-CoA, which is primarily generated in the mitochondrial matrix (Pietrocola et al., 2015). Intracellular acetyl-CoA changes depending on the metabolic status of the cell. Thus, acetyl-CoA may provide a link between mitochondrial function and NAT1 expression through histone acetylation, and NAT1 activity through direct protein acetylation (Fig. 1). Hernandez-Gonzalez et al. suggested that an imbalance in acetyl-CoA and CoA due to abnormal NAT1 activity could explain its effects on growth and cell death (Hernández-González et al., 2022).

Sirtuins may also have a role in regulating NAT2 activity. When peripheral blood mononuclear cells were treated with nicotinamide for 3 hours, NAT2 activity increased (Turiján-Espinoza et al., 2018). Moreover, up-regulation of SIRT1 with SRT1720 decreased NAT2 activity whereas activity increased in cells treated with SIRT1 siRNA (Salazar-González et al., 2022). Like NAT1, NAT2 is acetylated, although the specific lysine residues modified remain unknown. Nevertheless, the current literature indicates a stark difference in the effects of protein acetylation between NAT1 and NAT2, suggesting reciprocal regulation.

Not surprisingly, the NATs are targets for microRNAs (miRNAs). Prediction algorithms have identified numerous potential miRNAs, but few studies have been validated as directly targeting NAT1 or NAT2 mRNA. The Hormonizome web site (Rouillard et al., 2016) lists 49 potential miRNAs for NAT1 and 27 for NAT2, providing a starting point for identifying miRNAs important in NAT gene silencing (Table 1). Other available sites confirm most of the listed miRNAs. For NAT1, mir-1290 (Endo et al., 2013) and mir-6744-5p (Malagobadan et al., 2020) have been directly implicated in its functional regulation. Of these, mir-1290 has been
investigated in greatest detail. Its putative binding site is located approximately 550 bases downstream of the stop codon. mir-1290 interaction with NAT1 was originally identified using MCF-7 and T-47D breast cancer cells where transfection of the miRNA decreased NAT1 mRNA (Endo et al., 2013). In addition, these investigators reported a negative association between mir-1290 and NAT1 expression in breast cancer tissue. In a follow-up study, a luciferase reporter containing the NAT1 3′-UTR was used to assess the effects of the miRNA (Endo et al., 2014). Mutation of the mir-1290 binding site hindered downregulation by the miRNA supporting a direct effect on NAT1 mRNA levels. However, the regulation of NAT1 by mir-1290 may not be that straightforward. Different cells use different NAT1 polyadenylation sites but only the most distal site includes the putative mir-1290 binding sequence (Choudhury et al., 2022). The effect of a mir-1290 mimic on NAT1 activity was similar regardless of the presence or absence of the miRNA binding site. Furthermore, treatment of SH-SY5Y cells with retinoic acid enhanced mir-1290 expression but did not decrease NAT1 mRNA. Further studies are needed to fully understand how mir-1290 affects NAT1 expression.

The role of miRNAs in NAT2 regulation has not been thoroughly investigated. mir-15a-3p is a pro-apoptotic miRNA (Druz et al., 2013) that is frequently deleted in chronic lymphocytic leukemia (Calin et al., 2002). It binds to a sequence in the 3′-UTR of NAT2 approximately 200 bases downstream of the stop codon and suppresses NAT2 expression, and isoniazid toxicity, in human liver cells (Li et al., 2021).

An interesting difference between NAT1 and NAT2 is their inherent stabilities. First reported by Grant et al in 1991, NAT1 purified from human liver had a half-life of 3.5 hours compared to more than 60 hours for NAT2 (Grant et al., 1991). Similar differences in stability are seen when the NAT proteins are exposed to elevated temperatures (Deng et al., 2014; Leff et al., 1999; Tsirka et al., 2018; Walraven et al., 2006). Since NAT1 and NAT2 evolved along separate pathways, this difference may be associated with their physiological functions. For example, labile proteins, such as NAT1, show structural flexibility that is often stabilized following ligand or substrate binding (Richard, 2019). This was recently demonstrated with NAT1 where folate binding attenuated temperature-induced inactivation (Choudhury et al., 2023). Folate is an allosteric modulator of NAT1 that shifts the enzyme from an acetylase to a hydrolase (Laurieri et al., 2014a).

The effect of endogenous small molecules on the expression and activities of both NAT1 and NAT2 has gained increasing attention. A recent study using human liver HepG2 and Hep3B cells showed induction of NAT2 mRNA following glucose and acetate treatment (Hong et al., 2022b). Little or no change in NAT1 mRNA was observed. The exact mechanism for this regulation of NAT2 transcription is currently unknown but could be related to the production of acetyl-CoA. Glucose feeds into the synthesis of acetyl-CoA in the mitochondria while acetate is used to produce acetyl-CoA by cytosolic acyl-CoA synthetase short-chain family, member 2 (ACSS2) (Pietrocola et al., 2015). Both pathways are stimulated by insulin treatment (Karwi et al., 2020; Sone et al., 2002). Interestingly, insulin increases NAT2 mRNA in hepatocytes (Hong et al., 2022b).

NAT1 directly binds ATP, the primary product of mitochondrial respiration, and decreases activity by competing for acetyl-CoA (Minchin et al., 2018). The docking of ATP to NAT1 suggested it binds within the active site cleft of the enzyme with its triphosphate tail in the vicinity of arginine 127. Lysine 100, located at the rim of the active site cleft, is also important for ATP binding. When this amino acid was
mutated to glutamine or arginine, the effects of ATP were diminished. The binding of ATP to NAT1 may be one mechanism that links cell metabolism to NAT1 function.

C. NAT protein-protein interactions
Most proteins including enzymes have binding partners that influence their activities, stabilities, or cellular localization. The binding of the NATs to other cellular components has not been investigated in detail. Identifying possible interactions with other proteins may enhance our understanding of the endogenous roles of the NATs and explain why some cell phenotypes do not correlate well with NAT activity.

The detection of protein-protein interactions has progressed significantly with the advent of techniques such as high throughput two-hybrid systems, protein chip technology and phage display (Rao et al., 2014). The outcome has been the development of interactome databases such as the Human Interactome Project, BioGrid and IntAct that list known protein-protein interactions. NAT1 has been shown to interact with Intracellular Adhesion Molecule 1 (ICAM1) in HEK293T cells using co-immunoprecipitation assays (Huttlin et al., 2021; Huttlin et al., 2017; Rao et al., 2014). ICAM1 is found in lamellipodia and is required for cell motility and spreading (Pizza et al., 2017). Interestingly, NAT1 is also localized to lamellipodia and knockdown of NAT1 decreases filopodia protrusions (Tiang et al., 2015). NAT1 also may interact with Leucine-Rich Repeat Kinase 2 (LRRK2) (Martin et al., 2014). LRRK2 is a kinase best understood for its prevalence in Parkinson’s disease where mutations in the gene are found in both familial and sporadic Parkinson’s patients. Mutant protein is associated with increased reactive oxygen species (ROS) production, changes in mitochondrial fission and fusion, and loss of calcium homeostasis (Singh et al., 2019).

The NAT2 interactome is less well defined, in part because cell models used to identify interactions often have little or no NAT2 expression. In a two-hybrid system, NAT2 interacted with Ataxia Telangiectasia Mutated ATM (Arroyo et al., 2015), methylCpG Binding Domain Protein 4 (MBD4) (Luck et al., 2020) and RAD51 recombinase (Arroyo et al., 2015). ATM is important in mitochondrial biogenesis and respiration (Ambrose et al., 2007).

Identifying and validating protein partners for the NATs should be a high priority as it may reveal intracellular pathways that help explain how the NATs influence mitochondrial function.

D. NAT coordination, and reciprocity
In some cells, expression of NAT1 and NAT2 may be coordinated. For example, a positive correlation between NAT1 and NAT2 mRNA levels in primary breast tumour tissue, breast cancer cell lines, and normal breast tissue was reported by Carlisle and Hein (Carlisle and Hein, 2018). Coordinate expression implies common transcriptional mechanisms, although this is not supported by their different tissue distributions. Knockout of mNat2 (homolog of human NAT1) in mice did not affect the activity of mNat1 (homolog of human NAT2) in liver, gut, pancreas, or bladder (Loehle et al., 2006).

A recent publication reported an inverse association between NAT1 and NAT2. In breast cancer MDA-MB-231 cells where NAT1 was deleted using CRISPR/Cas9, NAT2 mRNA was elevated by almost 10-fold (Carlisle et al., 2022). It was proposed that the NAT2 transcription might compensate for the loss of NAT1 (Carlisle et al., 2022). This reciprocity has significant implications, especially in metabolic diseases where both NAT1 and NAT2 may have similar or opposing roles.
(see below). It will therefore be essential to determine whether the loss of NAT1 or the gain of NAT2 is responsible for any resulting changes in cellular phenotype.

Up-regulation of NAT2 was only observed following NAT1 deletion, not knockdown using siRNA. There are major differences between these two models. NAT1 and NAT2 are in close proximity on chromosome 8 so their expression may be coordinated, in part, depending on the surrounding chromatin. CRISPR/Cas9 gene deletions favour open chromatin regions (Liu et al., 2020a) and clones that are selected by this technique may be biased for cells with less condensed DNA in the vicinity of the target gene. Moreover, CRISPR activators (Cas9) themselves can locally open chromatin (Barkal et al., 2016) and increase expression of adjacent non-targeted genes (Weltner et al., 2018). This may explain the observed increase in NAT2 following NAT1 deletion with CRISPR/Cas9 but not following knockdown with siRNA, which does not require chromatin remodelling.

NAT1 and NAT2 both affect mitochondrial fuel usage and mitochondrial respiration, although the generality of these observations across different cells and tissues is currently not known (Chennamsetty et al., 2016; Denis et al., 2019; Wang et al., 2019). The paucity in work on co-expression of NAT1 and NAT2 emphasises the need to better understand how these proteins are regulated, whether their expressions are coordinated, and whether they show any functional redundancy.

E. Evolutionary evidence suggests different functions for the human NATs

The evolutionary differences between the two human NATs may explain many of their functional differences. Both genes have similar mutation rates (Ensembl) but the adverse effect of these mutations on function is more common for NAT2 than NAT1 (Patin et al., 2006). Each gene arose following separate duplication events from a common ancestorial gene (Sabbagh et al., 2013). Interestingly, NAT1 appears to have evolved through negative selection, which generally hinders the emergence of deleterious alleles, whereas NAT2 evolved through positive selection (Sabbagh et al., 2013). This fundamental difference in evolutionary history is consistent with different biological roles for the two enzymes.

The NAT1 gene shows two divergent lineages that separated approximately two million years ago, well before the emergence of modern humans (Patin et al., 2006). By contrast, a common ancestor for NAT2 emerged more recently (~ one million years ago). Negative or purifying selection of NAT1 suggests conservation of an important role in cell biology whereas positive selection of NAT2 argues for advantageous genetic changes in response to environmental changes (Sabbagh et al., 2013). Several researchers have suggested that dietary changes associated with a shift from hunter/gather subsistence to pastoralism favoured emergence of the slow acetylator phenotype, which is the result of low or negligible enzyme activity (Luca et al., 2008; Mortensen et al., 2011; Patillon et al., 2014; Patin et al., 2006; Podgorná et al., 2015; Sabbagh et al., 2008; Sabbagh et al., 2013). Early work by Patin et al found a significant increase in the NAT2*5B haplotype in western and central Eurasians. Expansion of the T$_{341}$C mutation, which causes the slow acetylator phenotype in NAT2*5B, coincided with the emergence of agriculture in these geographic regions (Patin et al., 2006). The effect of diet is consistent with the tissue-specific expression levels of the NATs. NAT2 is highest in the liver and upper GI tract, which are the major sites of dietary absorption while NAT1 is more broadly expressed. This also suggests different, independent transcriptional regulation of the two genes (Butcher and Minchin, 2012) and that slow acetylators had a significant survival advantage compared to rapid acetylators. However, as discussed later in
this review, greater risk of metabolic disorders has been associated mostly with slow NAT2 acetylator status.

F. The interaction of the NATs with acetyl-CoA

Acetyl-CoA is synthesised in the mitochondria and the cytosol, and these two pools are inter-connected via the citrate–malate shuttle. Acetyl-CoA is essential for numerous metabolic processes and its levels fluctuate depending on the metabolic state of the cell (Pietrocola et al., 2015) and the health of its mitochondria (Jankowska-Kulawy et al., 2022). Acetyl-CoA is also required for NAT enzymatic activity. Because the NATs catalyse the acetylation of substrate via a double displacement reaction, acetyl-CoA must bind to the enzyme and acetylate the active site cysteine before the acetyl moiety can be transferred to the substrate (Kilbane et al., 1991; Minchin and Butcher, 2015).

Recently, Hein et al. reported a difference in the affinity of acetyl-CoA for NAT1 and NAT2 (Hein et al., 2022). Using various substrates, they quantified the apparent acetyl-CoA Km for both enzymes and then inferred differences in binding affinity. Their results suggested at least a 10-fold higher affinity for acetyl-CoA by NAT1, and suggested further that NAT1 activity is more likely that NAT2 activity to be influenced by intracellular acetyl-CoA concentrations. This is an important observation that warrants further investigation in cell models where acetyl-CoA levels can be manipulated. The relationship between Km and binding affinity for a double displacement reaction is complex as the Km is dependent on the off-rate of the acetylated substrate (Minchin and Butcher, 2015; Wang et al., 2005). In the study by Hein et al., the off-rates, at least for two substrates (N-hydroxy-aminofluorene and N-hydroxy-animobiphenyl), were much slower for NAT1 than for NAT2 (Hein et al., 2022). However, this does not explain the differences in acetyl-CoA apparent Km, supporting the conclusion that it has a higher affinity for NAT1 than NAT2. Thus, intracellular acetyl-CoA levels may be a link between NAT1 expression and mitochondrial function.

II. Mitochondria and metabolic disorders – overview

Mitochondria are best known for their role in converting nutrients into energy in the form of ATP. However, they have other important functions including the regulation of programmed cell death, ROS production and intracellular calcium homeostasis (Murphy et al., 2016). Many metabolic disorders are associated with altered ATP generation through changes in mitochondrial respiration. To understand how the NATs are involved in mitochondrial function, it is important to first consider mitochondrial respiration and its dependence on fuels such as glucose and fatty acids.

A. Mitochondrial respiration

Mitochondria convert glucose, the primary fuel source for most cells, into ATP (Fig. 2A). Glucose is first converted to pyruvate in the cytosolic glycolysis pathway, which produces ATP independent of the mitochondria, albeit at a much lower capacity (for review, see (Chaudhry and Varacallo, 2022)). Pyruvate is shuttled into the mitochondria to drive the tricarboxylic acid (TCA) cycle, which feeds electrons into the electron transport chain (ETC) to establish the proton gradient across the mitochondrial inner membrane. This gradient then drives ATP synthesis by the enzyme ATP synthase in a reaction that consumes oxygen. In conditions of limited...
oxygen supply, pyruvate is diverted to lactic acid before entering the mitochondria, which decreases mitochondrial respiration.

Electrons from NADH and FADH$_2$ produced in the TCA cycle can directly react with oxygen to generate ROS. At low concentrations, ROS behave as signalling molecules to regulate cell stemness, proliferation and reprogramming, especially during development (Sinenko et al., 2021). At high concentrations, ROS causes oxidative stress, which damages proteins, lipids, and nucleic acids (Auten and Davis, 2009).

B. Mitochondrial respiration as a measure of metabolic dysfunction
Mitochondrial respiration is normally measured by the rate of oxygen consumption, which changes in many metabolic disorders (Avram et al., 2022). There are three common parameters used to measure mitochondrial health in cells: basal respiration, ATP production and reserve respiratory capacity (RRC). Basal respiration is the minimum oxygen consumption required to maintain cellular ATP levels. It varies depending on fuel source (see below) and cell type. For example, basal respiration is as low as 10 pmol/min/µg protein in hepatocytes and as high as 30 pmol/min/µg protein in cardiomyocytes (Hill et al., 2012). Inhibition of ATP synthase with oligomycin shuts down oxygen consumption by the ETC, and the resulting decrease in respiration is an indirect measure of ATP synthesis. Finally, the RRC quantifies the capacity of a cell to respond to increased metabolic demand. It is measured as the rate of oxygen consumption following the dissipation of the proton gradient across the inner mitochondrial membrane (Marchetti et al., 2020). Many cancer cells (e.g. HeLa) have very little RRC (Sacoman et al., 2017) whereas healthy cardiomyocytes show RRC that is at least twice the basal respiration rate (Hill et al., 2012). RRC diminishes with age and in diseases where mitochondrial function is compromised (Marchetti et al., 2020).

C. Mitochondrial fuel usage
Mitochondrial substrates, or fuels, provide precursors for the TCA cycle. The two most important fuels are glucose and fatty acids which are sources of acetyl-CoA. Other potential fuels include glucogenic and ketogenic amino acids (Fig. 2B). The fuel that a cell uses depends on its metabolic state and its environment. Mitochondrial capacity measures the extent that a particular fuel is used by a cell whereas flexibility measures its ability to switch between fuels when needed.

As described above, glucose enters the mitochondria following metabolism to pyruvate (Fig. 2A). Fatty acids contribute to the acetyl-CoA pool by undergoing β-oxidation in the mitochondrial matrix which also produces ROS that can cause endoplasmic reticulum stress and mitochondrial dysfunction characteristic of metabolic diseases with dysfunctional lipid homeostasis (Yang et al., 2021b).

As acetyl-CoA in the mitochondrial matrix increases, ketone bodies (acetoacetate and β-hydroxybutyrate) are formed through ketogenesis (Fernandes and Pikaar, 1972). Ketone bodies can act as a fuel source for the cell by catabolism, via ketolysis, to form acetyl-CoA primarily during nutrient deprivation (Yang et al., 2018). But β-hydroxybutyrate also moderates gene expression including genes involved in mitochondrial biogenesis (Fig. 2B). It inhibits class I and II histone deacetylases (Bough et al., 2006; Kolb et al., 2021; Shimazu et al., 2013b) and promotes FOXO3a expression, which increases sirtuin activity (Shimazu et al., 2013a). Since NAT activity is sirtuin-dependent (see below), β-hydroxybutyrate may be important in regulating NAT levels in cells.
Mitochondria can also derive TCA cycle intermediates from amino acids. Amino acids are classified as either ketogenic, glucogenic, or both. Ketogenic amino acids are catabolized to acetyl-CoA whereas glucogenic amino acids are catabolized to pyruvate or intermediates of the TCA cycle (Litwack, 2018). A large amount of research investigating amino acids in mitochondrial respiration has focussed on glutamine, as it is the most abundant and versatile amino acid in the body (Cruzat et al., 2018).

The ability for cells to transition between fuels is an important survival mechanism (Muio, 2014). It is controlled by signalling events that link glucose and lipid metabolism. Central to this regulation is acetyl-CoA. When carbohydrate supply is limited, cells switch to fatty acids to drive mitochondrial ATP production, resulting in an increase in acetyl-CoA, which inhibits pyruvate dehydrogenase to limit pyruvate entry into the mitochondria. By contrast, elevated glucose supply increases malonyl-CoA production, which inhibits fatty acid uptake into mitochondria by the transporter CPT1 (Muio, 2014). These mechanisms, and others, allow cells to switch between glucose, fatty acids and amino acids as required. In chronic overfeeding, or ‘metabolic congestion’ (Muio, 2014), mitochondria lose cooperativity and flexibility of fuel usage and burn glucose, fatty acids and amino acids continually regardless of the metabolic status of the cells.

D. Mitochondrial response to cell stress – role of SIRT1
Normally, cells exist in a state of metabolic homeostasis. However, when this balance is disturbed by an increase in energy demand and/or decreased energy supply, a metabolic stress response is initiated (Fang et al., 2021). One response is the up-regulation of the sirtuin SIRT1 (Hong et al., 2020b). SIRT1 acts to limit mitochondrial stress by changing the acetylation status, and consequently, the activity, of its substrates such as histones, transcription factors and coactivators (Fang et al., 2021). SIRT1 also deacetylates and activates acetyl-CoA synthetases, which increases the production of acetyl-CoA from acetate (Hallows et al., 2006). As noted above, SIRT1 deacetylates NAT1 and NAT2 but with opposing effects on their activities (Section 1C). While SIRT1 activates NAT1 (Butcher et al., 2020; Salazar-González et al., 2018), it inactivates NAT2 (Salazar-González et al., 2022).

SIRT1 also deacetylates the transcriptional coactivator peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α), which increases its transcriptional activity (Rodgers et al., 2005). PGC-1α coordinates the expression of many genes involved in mitochondrial biogenesis, as well as glucose and fatty acid metabolism (Rodgers et al., 2005).

E. Mitochondria and metabolic disorders
In 2011, Wallace published a meticulous summary on the role of bioenergetics and human disease. He argued that inherited and epigenetic mutations in mitochondrial DNA, along with an accumulation of mutations in nuclear DNA, underpins the age-related decline in mitochondrial health that is associated with an increased risk of major diseases including neurodegeneration, diabetes, cardiovascular disease and cancer. In addition, it was proposed that environmental factors such as dietary energy sources and exposure to mitochondrial toxins exacerbate this decline (Wallace, 2011).

Metabolic disorders in humans arise when the supply or utilization of metabolic substrates is compromised. Most are associated with mitochondrial dysfunction and can be inherited or acquired. Inherited metabolic disorders, or inborn
errors in metabolism, are individually quite rare but collectively include more than 1400 different syndromes that involve changes in carbohydrate, amino acid and lipid metabolism (Ferreira et al., 2021). These diseases are most prevalent in children because they are caused by inherited mutations in genes responsible for the delivery and/or use of metabolic substrates. Mutations in both mitochondrial and nuclear DNA affect mitochondrial activity.

Acquired metabolic disorders are mostly diagnosed in adults. They arise from a combination of accumulated gene mutations and environmental factors including diet and lifestyle. A common feature of acquired metabolic disorders is a decline in mitochondrial function, either before or secondary to the onset of disease (Prasun, 2020). Mitochondrial dysfunction increases with age and has been associated with metabolic disorders such as diabetes, heart disease, obesity, various forms of cancer and neurological syndromes including Alzheimer’s disease. This has led to the pursuit of new therapeutics and the repurposing of existing therapeutics that target mitochondria for treatment of common underlying mitochondrial dysfunction (Pillai et al., 2023).

III. Effects of arylamine N-acetyltransferases on mitochondrial function

Mitochondrial function is essential for cell division and differentiation, especially during development. In disease, mitochondria also help drive growth and invasiveness of cancer cells (DeBerardinis et al., 2008). The first studies that hinted at a role for the NATs in cell metabolism showed an association of NAT mRNA with proliferative (epithelium) but not quiescent (stromal or endothelial) cells in the rat prostate (Archer et al., 1999). Later studies using cancer cell lines showed that inhibition of NAT1 decreased cell proliferation (Tiang et al., 2010). Over the past decade, the role of the NATs in mitochondrial function has been more clearly defined both in vitro and in vivo. An interesting facet of these studies is the discovery that both NAT1 and NAT2 may be associated with cell metabolism. Given the separate evolutionary pathways for the 2 genes, this is somewhat unexpected. In the following sections, the current knowledge of the NATs and their association with mitochondrial function will be reviewed.

A. NAT1 studies with cell lines

In 2010, Tiang and colleagues showed that decreasing NAT1 activity with Rhod-o-hp, a competitive inhibitor of the enzyme, reduced the proliferation of MDA-MB-231 cells and limited their growth in soft agar. These changes were also observed following down-regulation of NAT1 with lentivirus-based shRNA (Tiang et al., 2010). A decrease in growth, and a shift from a mesenchymal to epithelial phenotype, was also seen in HT-29 and 22Rv1 cells following knock down of NAT1 (Tiang et al., 2011). In triple negative breast cancer cells (MDA-MB-231, MDA-MB-436, BT-549), NAT1 was physically associated with lamellipodia, and loss of the protein led to a decrease in filopodia formation and invasiveness both in vitro and in vivo (Tiang et al., 2015). Subsequent studies have firmly established the role for NAT1 in cell proliferation (Li et al., 2020; Stepp et al., 2019b; Wang et al., 2018). The proliferative attributes of a cell are controlled by mitochondrial dynamics (Zhao et al., 2013) and therefore suggests NAT1 affects mitochondrial function.

Evidence for a direct role for NAT1 in cellular respiration emerged in 2018 when Wang et al reported that NAT1 was essential for maintaining non-toxic ROS levels during glucose starvation (Wang et al., 2018). ROS are a consequence of
aerobic metabolism and are formed in the ETC where electrons leak to form superoxide (Ren and Shen, 2019). During glucose starvation, mitochondria can switch to using fatty acids to supply reducing equivalents in the mitochondria, which increases ROS production (Ly et al., 2017). NAT1 is required for normal glucose use and deletion of the *NAT1* gene decreased glucose flux through the TCA cycle without affecting glycolysis (Wise et al., 2023). This may be explained, in part, by a significant loss of ATP synthase in NAT1 knockout cells, which should affect mitochondrial respiration (Hong et al., 2022a). It was also noted that glucose flux through acetyl-CoA was not affected by *NAT1* deletion, even though intracellular acetyl-CoA increases under these conditions (Stepp et al., 2019a) and decreases when NAT1 is over-expressed (Carlisle et al., 2016). This suggests that changes in acetyl-CoA with NAT1 levels are not associated with synthesis from pyruvate in the mitochondria but is the result of either changes in acetyl-CoA catabolism, or its synthesis from acetate and/or fatty acids. The effects of NAT1 on glucose flux is an important observation as it suggests NAT1 may be involved in fuel usage by mitochondria.

Cellular respiration changes in cells following *NAT1* deletion. However, the changes are not entirely clear from current work in the literature. In 2018, Carlisle et al reported that deletion of NAT1 in MDA-MB-231 cells caused no change in basal respiration or ATP production, but increased RRC, which was replicated in 2 independent clones. The study suggests inhibition of NAT1 enhances the ability for cells to adapt to increased energy demand (Carlisle et al., 2018), although how this might be related to a decrease in glucose flux and loss of ATP synthase requires further investigation. By contrast, a separate study in MDA-MB-231 and HT-29 NAT1 knockout cells reported reduced basal respiration, ATP production and RRC in the mitochondria (Wang et al., 2019). Glycolysis was also diminished in both cell lines. The loss of NAT1 decreased pyruvate dehydrogenase (PDH) activity, which would reduce glucose flux through the TCA cycle. Moreover, the reduced RRC was reversed when cells were treated with dichloroacetic acid, an inhibitor of pyruvate dehydrogenase kinase that activates PDH by limiting its phosphorylation (Wang et al., 2019). The opposing results from these two studies warrants further investigation to determine whether NAT1 is advantageous or detrimental to mitochondrial function, a particularly important issue if NAT1 is a drug target in the future.

There is recent evidence that NAT1 may be required for cytochrome C release from mitochondria. Following NAT1 deletion in T-47D and MDA-MB-231 cells, cytochrome C release in response to cytotoxic drugs was diminished. There was also a decrease in BAX as well as activated caspase 8 and 9 that resulted in chemoresistance (McAleese et al., 2022). Caspase 8 normally suppresses necroptosis by inhibiting essential components of the pathway including receptor-interacting kinase 3 (RIPK3). In the absence of NAT1, caspase 8 expression is normal, but its activation is inhibited. Consequently, NAT1 deficiency inhibits mitochondrial-dependent intrinsic apoptosis through loss of activated caspase 9 and increases necroptosis through loss of caspase 8 activation (Fig. 3).

### B. NAT2 studies with cell lines

The effects of NAT2 on mitochondrial function in cell models were first identified using murine 3T3-L1 adipocytes where the mouse homolog of NAT2 (mNat1) was knocked down using lentivirus shRNA constructs (Chennamsetty et al., 2016). Knockdown increase ROS production and decreased mitochondrial membrane potential, which led to a decrease in intracellular ATP. Basal respiration was slightly
less but RRC decreased by approximately 30%. Similar changes were seen in white adipose tissue isolated from mNat1-deficient mice. In addition, mitochondrial fragmentation increased while mitochondrial mass decreased in the 3T3-L1 cells following mNat1 silencing. A similar decrease in mitochondrial respiration was independently reported for brown adipose tissue and hepatocytes isolated from mNat1 deficient mice (Camporez João et al., 2017). These studies show that mNat1 may be necessary for optimal mitochondrial function, a finding that is similar to that for human NAT1. Further work is needed for human NAT2. While mNat1 is often viewed as a functional homolog of human NAT2, Laurieri et al. suggested this may not be the case. They showed very good similarities in substrate specificities for human NAT1 and mouse mNat2, but not for human NAT2 and mNat1 (Laurieri et al., 2014b). Moreover, the environmental pressures over the past 6500 years thought to have influenced NAT2 gene evolution (see Section 1B) are unlikely to have influenced mouse mNat1 evolution. Hong et al. noted that co-expression of genes with NAT2 in human HepG2 cells and mNat2 in mouse adipose tissue differed, and also suggested these two genes may not be functional homologs (Hong et al., 2023).

C. Studies with mNat null mouse models

There are three NAT genes in mice – mNat1, mNat2 and mNat3. Both major mNATs (mNat1, mNat2), and the less well characterised mNat3, have been deleted with no apparent effects on viability (Chennamsetty et al., 2016; Cornish et al., 2003; Sugamori et al., 2007). Moreover, both mNat1 and mNat2 have been deleted simultaneously (Sugamori et al., 2003). Early studies established mouse lines primarily for the purpose of examining drug metabolism and chemical carcinogenesis. They showed that the knockout mice developed normally with no overt phenotype, suggesting the Nat’s are not critical for early development and growth (Sugamori et al., 2003).

In 2015, the first mNat1 null mice were created to investigate its role in whole body metabolism (Knowles et al., 2015). In the absence of mNat1, circulating glucose, insulin and triglycerides increased following fasting. In addition, responses to a glucose tolerance test and an insulin tolerance test indicated the mice were insulin resistant. A more detailed study of the mNat1 null mice showed a decrease in in vivo metabolic rates and energy expenditure that accompanied a decrease in exercise capacity. The mNat1 null mice had an increased respiratory exchange ratio indicative of a decreased ability to switch from carbohydrate to fat metabolism (Chennamsetty et al., 2016). Similar results were also reported by Camporez et al. who also showed decreased oxygen consumption rates in adipose tissue and hepatocytes isolated from the mNat1 null animals (Camporez João et al., 2017). Notably, hepatocytes from adult animals had lower mitochondrial basal respiration and a significant loss in reserve respiratory capacity. Finally, mNat1-/- mice showed increased blood-brain barrier damage associated with endothelial cell necroptosis (Zou et al., 2020a). Collectively, these studies show that mitochondrial function is compromised in the mNat1 knockout animals.

In double knockout mice (mNat1-/-, mNat2-/-) fed a high fat/high sucrose diet, there were no changes in body weight, energy expenditure or thermogenesis, and only modest changes in whole body respiration. A pyruvate tolerance test suggests the null animals had compromised hepatic gluconeogenesis. There was also a modest increase in hepatic CoA without a change in acetyl-CoA (Denis et al., 2019). Interestingly, this study did not identify major metabolic changes that were reported for the mNat1 null mouse.
D. NATs and mitochondrial function – causation versus association

Growing evidence from cell studies and animal models demonstrate that NAT expression is associated with mitochondrial function. Moreover, agreement between independent studies argues strongly that this association is not reliant on cell type and may be common to a range of metabolic diseases (see Section IV). There is also evidence that the association between NAT and mitochondria is causal; that is, the changes in NAT expression are responsible for the changes observed in mitochondrial function. Firstly, many of the mitochondrial changes are seen in multiple cell lines and in multiple clones from the same cell line (Carlisle et al., 2020; McAleese et al., 2022; Stepp et al., 2019b; Tiang et al., 2015). While this is not direct evidence, it strongly supports causation. Secondly, in animal models where mNat2 has been deleted, mitochondrial changes are consistently seen in multiple organs (Chennamsetty et al., 2016). Thirdly, many phenotypes that arise following NAT deletion are rescued by the re-introduction of the gene. This has been shown for changes in cell size and morphology (Li et al., 2020), chemoresistance (McAleese et al., 2022), MMP9 over-expression (Li et al., 2019) and up-regulation of the integrin ITGαV (Li et al., 2020). Rescue of the phenotype is strong evidence that NAT deficiency causes the different phenotypes. The consensus from studies to date support the hypothesis that, for both NAT1 and NAT2, high activity promotes healthy mitochondria while low activity promotes mitochondrial dysfunction. A challenge for the future is to identify the cellular mechanisms that underpin this hypothesis.

IV. Arylamine N-acetyltransferases and specific metabolic disorders

A. Cancer is a metabolic disease.

Recent research suggests that cancer is a metabolic disease where metabolic reprogramming and mitochondrial dysfunction are associated with the onset and maintenance of cancer cell proliferation and survival (Coller, 2014; Seyfried et al., 2014). Consequently, targeting pathways that regulate mitochondrial function provides novel treatment opportunities (Fulda et al., 2010).

Otto Warburg first proposed that changes in cell respiration are critical for tumour initiation (Warburg, 1956). Warburg hypothesized that cancer cells switch to lactic acid fermentation (aerobic glycolysis) for ATP synthesis, as oxidative phosphorylation is hindered by defective mitochondria (Chen et al., 2015), although later studies showed that many cancer cells have higher rates of lactate production because of altered mitochondrial function, not impaired mitochondrial function (Fantin et al., 2006; Moreno-Sánchez et al., 2007; Weinhouse, 1976). Although glycolysis is significantly upregulated in many cancer cells, oxidative phosphorylation still occurs at rates proportional to the oxygen levels (Chen et al., 2015). In addition to a switch towards aerobic glycolysis, specific mitochondrial dysfunction and defective mitochondria have been reported. Arismendi-Morillo and Castellano-Ramirez demonstrated aberrant mitochondrial structure in glioblastoma cells where loss of cristae was evident (Arismendi-Morillo, 2009; 2011; Arismendi-Morillo and Castellano-Ramirez, 2008). Similar changes in the ultrastructure of the mitochondrial membrane have been reported in breast cancer cell lines and patient tumour samples (Elliott and Barnett, 2011; Elliott et al., 2012).

Overall, changes to mitochondrial bioenergetics, fusion and fission, mitochondrial biogenesis and regulation of oxidative stress are commonly observed in cancers (Vyas et al., 2016). Mitochondrial dysfunction does not appear to increase the risk of
cancer; that is, its initiation (Lund et al., 2015). Rather, it may be important in cancer progression once a tumour is established (Hsu et al., 2016).

B. NATs and cancer

Early research identified robust correlations between NATs and cancer incidence, especially for NAT2 (Butcher and Minchin, 2012; Gong et al., 2011; Hein, 2002; Kadlubar and Badawi, 1995; Minchin et al., 1993; Ochs-Balcom et al., 2007). Both NAT genes carry mutations that affect activity, and the prevalence of these mutations is associated with cancer risk. Which phenotype is important (low or high activity) depends on the type of cancer. For example, NAT2 slow acetylators have a higher risk of bladder cancer compared to intermediate or rapid acetylators (García-Closas et al., 2005). By contrast, NAT2 rapid acetylators have a higher risk of colon cancer if associated with a diet high in well-done red meat (Hein et al., 2000). The underlying mechanism for these associations is the role of the NATs in activating and detoxifying arylamine and heterocyclic amine carcinogens found in the environment and in the diet (Hein, 2002; Ishibe et al., 2002). Thus, the NATs have a role in the initiation of human cancers by increasing DNA adducts that result in cell transformation.

The NATs are also important for cancer progression. One of the first studies to show that NATs are associated with disease outcome (recurrence, survival) reported increased median survival in breast cancer patients with an intermediate NAT2 genotype (Khedhaier et al., 2008). Since then, a number of studies have found that both NAT1 and NAT2 are associated with cancer progression (Andres et al., 2013; Cai et al., 2021; Endo et al., 2014; Liu et al., 2020b; Minchin and Butcher, 2018; Shi et al., 2019; Troy et al., 2013). Table 2 summarizes the influence of NAT1 and NAT2 expression on survival in eight different human cancers where full gene expression profiles using gene chip arrays or RNA-seq have been published. Three different groups can be identified: (1) cancers where low NAT1 expression predicted decreased overall survival, but NAT2 had no effect (breast, ovarian, lung), (2) cancers where both low NAT1 and NAT2 predicted decreased overall survival (gastric, colon, liver), and (3) cancers where high expression of either or both genes predicted decreased survival (acute myeloid leukemia, myeloma). Apart from acute myeloid leukemia, low NAT1 was always associated with decreased survival. This was greatest in gastric cancer (64%) and breast cancer (50%). Group 2 represents cancers of the digestive system. This includes organs such as the liver and gastrointestinal tract where NAT2 expression is highest in the body. It is not possible to identify whether NAT1 or NAT2 most affected survival since their expressions were highly correlated (Table 2). Finally, acute myeloid leukemia differed from the other cancers as decreased survival was associated with high NAT1 and high NAT2. Both enzymes appeared to affect survival independently since there was no correlation in their expression (Pearson’s r = -0.009). Acute myeloid leukemia also differs from most cancers as it relies heavily on oxidative phosphorylation, as opposed to aerobic glycolysis or lactate fermentation, for ATP production (Panina et al., 2021). This is consistent with a metabolic phenotype associated with elevated NAT1/2. Many of the effects of NAT1 on cancer cell metabolism have been described above, and a summary is provided in Table 3.

Typically, there is an inverse relationship between glycolysis and oxidative phosphorylation in cancer. This is commonly referred to as the Warburg effect (Vander Heiden et al., 2009), and has been reported across 22 different cancer cell lines (Teh et al., 2019). However, most cancer cells have an intact oxidative
phosphorylation pathway and demonstrate cooperativity with aerobic glycolysis, allowing for more diverse energy production pathways (Zheng, 2012). Low NAT1 (and perhaps NAT2 as well based on mouse studies) leads to mitochondrial dysfunction, although exactly how this is achieved is still unresolved. It might be predictable that the decrease in oxidative phosphorylation seen with low NAT1 requires increased aerobic glycolysis to maintain energy production. However, this does not appear to be the case (Carlisle et al., 2018; Chennamsetty et al., 2016; Wang et al., 2019). Identifying the molecular pathway(s) linking NAT expression with mitochondrial function is needed to better understand their role in cancer cell metabolism.

C. Diabetes and mitochondrial function

Type 1 diabetes occurs when pancreatic β-cells do not produce sufficient insulin, usually due to pancreatic cell dysfunction (Lee et al., 2021; Ozougwu, 2013). By contrast, Type 2 diabetes occurs when tissues develop resistance to insulin (Ozougwu, 2013; Park and Seo, 2020). Both diseases are metabolic disorders characterized by a reduction in mitochondrial number and morphology in skeletal muscle and other organs in the body. Moreover, mitochondrial biogenesis is compromised (Sivitz and Yorek, 2010). Hyperglycaemia contributes towards mitochondrial dysfunction by increasing oxidative stress in endothelial cells, in turn causing mitochondrial stress and damage (Petersen et al., 2004). Children of insulin-resistant patients have dysregulated fatty acid metabolism in the skeletal muscle, indicative of inherited defects in mitochondrial oxidative phosphorylation (Petersen et al., 2004).

Mice deficient in PGC-1α are prone to hypoglycaemia (Lin et al., 2004) and muscle biopsies from patients with Type 2 diabetes have lower levels of PGC-1α (Mensink et al., 2007; Mootha et al., 2003; Patti et al., 2003). PGC-1α coactivates transcription factors that regulate genes involved in mitochondrial biogenesis, oxidative phosphorylation, and glucose and fatty acid metabolism.

D. NATs and diabetes

Epidemiological evidence first linked NAT2 with diabetes in the early 1990’s (for review, see (Fathzadeh et al., 2017)). But results were variable and often limited by an incomplete understanding of NAT pharmacogenomics and by study power (Hernández-González et al., 2022). Nevertheless, those studies that showed an association between NAT2 polymorphisms and diabetes suggested that individuals with a slow acetylator phenotype were at increased risk (Al-Shaqha et al., 2015; Yalin et al., 2007). In 2015, Knowles et al reported an association between a single nucleotide polymorphism (SNP) at base 803 (rs1208) that is associated with insulin resistance, a condition that can lead to non-insulin dependent diabetes (Knowles et al., 2015). However, that SNP is not thought to alter NAT2 enzyme activity (Fathzadeh et al., 2017). It is in linkage disequilibrium with another SNP (rs1801280) that introduces an amino acid change (I114T) resulting in a less stable protein (Fretland et al., 2001). Knowles et al noted that insulin resistance in their population was not associated with NAT2 acetylator phenotype and suggested its effects may be independent of enzyme activity towards xenobiotics (Knowles et al., 2015). A summary of recent findings that relate NAT expression to mitochondrial function in insulin resistance and diabetes is provided in Table 4.

It is noteworthy that the ancestral gene ('A' at 803) was associated with increased risk, which is in line with the evolutionary evidence (Sabbagh et al., 2008).
Both mutations are present in the \textit{NAT2*5B} haplotype, which is much more common in European populations compared to East Asian populations. The frequency of the \textit{NAT2*5B} haplotype increased rapidly in Western and Central Eurasia in the last 6000 years suggesting a survival advantage for the slow acetylator phenotype (Patin et al., 2006). One of the selection pressures may have been diet-induced insulin resistance at a time when humans began a shift from hunter/gather subsistence to pastoralism.

The relationship between \textit{NAT2}, diabetes and insulin resistance has been strengthened by studies using mouse 3T3-L1 adipocytes following siRNA knockdown of \textit{mNat1}. Knowles \textit{et al} showed a decrease in insulin-dependent glucose uptake and an increase in free fatty acid release following knockdown. This was accompanied by a decrease in expression of adipogenic genes such as \textit{Pparg} and \textit{Cebpa}. Finally, they showed an increase in fasting blood glucose, circulating insulin and circulating triglycerides in mice deficient in \textit{mNat1} (Knowles \textit{et al}., 2015). \textit{mNat1} deficiency in adipocytes decreased PGC-1α levels, an important regulator of glucose and fatty acid metabolism (Chennamsetty \textit{et al}., 2016). PGC-1α is critical for the uptake and storage of glucose in skeletal muscle (Wende \textit{et al}., 2007). The protein is regulated by several post-translational mechanisms including acetylation, which inhibits its activity (Cantó \textit{et al}., 2009). Elevated PGC-1α favours fatty acid usage and increased exercise performance while low levels lead to cardiac mitochondrial defects and heart failure (Shoag and Arany, 2010). Collectively, these results suggest that cells shift from fatty acid β-oxidation to a greater reliance on glucose for energy supply when \textit{mNat1} is low or absent. They show convincingly that \textit{mNat1} is important in insulin resistance and, therefore, possibly in diabetes.

Less work has focussed on the possible role of \textit{NAT1} in insulin resistance and/or diabetes. Ung \textit{et al} found that \textit{NAT1} expression was diminished in retinal endothelial cells treated for 72 hours with high glucose (Ung \textit{et al}., 2017). This is opposite to the effect of glucose on \textit{NAT2} expression in liver HepG2 cells (Hong \textit{et al}., 2022b). \textit{NAT1} was not affected by 24 hour treatment with high glucose in HepG2 cells, but it did increase in response to glutamine (Hong \textit{et al}., 2022b). The \textit{NAT1} SNP C$^{559}\text{TT}$, which introduces a stop codon in the coding sequence, is more common in retinal epithelial cells from diabetic patients than in controls. The frequency of this SNP is less than 0.5% of the population. While a direct role for \textit{NAT1} was not established, the authors suggested that persistent hyperglycaemic conditions suppress \textit{NAT1} expression (Ung \textit{et al}., 2017). That may not be the case for insulin resistance, which leads to a decrease in intracellular glucose levels (Shulman, 2004). Indeed, a study on gene expression in skeletal muscle from insulin resistant non-diabetic individuals showed an 80% decrease in \textit{NAT1} mRNA compared to insulin sensitive individuals (GEO ID 758540) (Yang \textit{et al}., 2002). This loss in \textit{NAT1} could be associated with mitochondrial dysfunction. By contrast, no difference was seen in \textit{NAT2} expression (GEO ID 753721). Moreover, \textit{NAT2} expression in the liver of diabetic patients was not different to health controls (GEO IDs 71239744, 71077223) (Misu \textit{et al}., 2010; Pihlajamäki \textit{et al}., 2009). These results are extracted from annotated microarray studies and suggest that \textit{NAT2} expression is not altered significantly in insulin resistance whereas \textit{NAT1} levels may change, at least in skeletal muscle. Nevertheless, observations need confirmation by studies that directly investigate expression of the NATs in different organs. In addition, because both \textit{NAT1} and \textit{NAT2} undergo posttranslational modifications, activities might also be informative.
E. Cardiovascular disease and mitochondrial health

Cardiovascular disease has long been associated with changes in mitochondrial function (Murphy et al., 2016). Cardiac ATP requirements are high compared to most organs. Consequently, mitochondria in cardiomyocytes occupy more than one third of the cell volume (Kolwicz et al., 2013). These cells rely predominantly on fatty acids for energy production (Lee et al., 2017). When metabolic demand increases, however, cardiomyocytes can switch to other fuels such as glucose, lactate, and ketone bodies (Stanley et al., 2005). Recent studies have shown impaired mitochondrial biogenesis and reduced mitochondrial content in cardiomyocytes with onset of cardiovascular disease (Poznyak et al., 2020; Siasos et al., 2018).

In a detailed review of cell metabolism and cardiovascular disease, Murphy et al describes the main features of mitochondrial dysfunction and changes in gene expression, oxidative phosphorylation, ROS production, NAD⁺/NADH ratio, calcium influx and post-translational modifications. In addition, post-translational modification of mitochondrial proteins is important as it affects β-oxidation, amino acid and ketone metabolism, ATP production and ROS catabolism (Murphy et al., 2016). One modification, acetylation, occurs on many mitochondrial proteins. There are two proposed mechanisms for protein acetylation in mitochondria: (1) enzymatic, and (2) non-enzymatic (Baeza et al., 2016). Enzymatic acetylation is a balance in activities between acetyltransferases, such as p300, and deacetylation catalyzed primarily by Sirt3. Non-enzymatic modification of protein lysine residues is thought to be predominant and is dependent on several factors including the concentration of acetyl-CoA (Wagner and Hirschey, 2014). Intracellular concentration of acetyl-CoA may be NAT1-dependent, which provides a possible link between NAT1 expression and mitochondrial function in metabolic diseases (Carlisle et al., 2016; Stepp et al., 2019a).

F. NATs and cardiovascular disease

Early epidemiological studies did not identify any association between NAT activity and cardiovascular disease (Ilett et al., 1993). Later work showed a higher incidence of the NAT2 slow acetylator phenotype in patients with coronary heart disease (Sun et al., 2016). Barth et al performed a genome wide expression study on myocardial septum samples from hearts with dilated cardiomyopathy (Barth et al., 2006). They found that NAT1 expression was significantly increased (GEO ID 27922224) whereas NAT2 was significantly decreased (GEO ID 27912760) in diseased hearts compared to controls.

Genetic variation in the NAT2 gene has been strongly linked to dyslipidaemia (changes in circulating cholesterol/triglyceride levels), a major risk factor in cardiovascular disease. Almost all mutations associated with risk are non-coding variants that are not known to directly affect enzyme activity. Nevertheless, many are associated with high NAT2 activity in vivo (Hong et al., 2023). Activity depends on the presence or absence of genetic mutations as well as transcriptional and post-transcriptional regulation. Hong and Hein recently observed that many of the non-coding mutations are located downstream of the NAT2 coding region and speculated that they could be part of a distal regulatory region for the gene (Hong and Hein, 2023). Interestingly, NAT2 is co-expressed with several genes important in lipid and cholesterol synthesis such as APOA5, APOB, APOC2, APOC3, ABCG8, ANGPTL3, FABP1, MOGAT2 and PLA2G12B (Hong et al., 2022b). This observation suggests these genes have a common transcriptional pathway. If this is the case, then the
level of NAT2 expression may be indicative of high circulating cholesterol and triglycerides but it may not be causative.

Glucose and fatty acid use by the heart is highly regulated, and many of the genes associated with this are regulated by peroxisome proliferator-activated receptor alpha (PPAR α) and PGC-1α (Rowe et al., 2010; Wagner and Wagner, 2020). Several studies have reported that suppression of PPARs and PGC-1 leads to heart failure (Sack et al., 1996; Sihag et al., 2009). Ablation of mNat1 decreases PGC-1α in adipose tissue but its effects in the heart are unknown (Chennamsetty et al., 2016). Inflammation and mitochondrial DNA damage induced by oxidative stress and production of ROS are also involved in the pathogenesis of cardiovascular disease (Poznyak et al., 2020; Siasos et al., 2018). Increased ROS production is a key mechanism to mitochondrial dysfunction (Poznyak et al., 2020; Siasos et al., 2018). Both NAT1 and mNat1 deficiency cause increases in ROS production (Chennamsetty et al., 2016; Wang et al., 2018). Further studies are needed to unravel the complex associations between the NATs and cardiovascular disease.

G. Neurodegenerative diseases and mitochondrial function

Neurodegenerative disorders comprise a group of heterogenous conditions of progressive degeneration of cells in the nervous system that affect coordination, cognition, and mobility. They include Alzheimer’s disease, ataxia, amyotrophic lateral sclerosis (ALS), Huntington’s disease, dementia with Lewy body disease, Parkinson’s disease, and spinal muscular atrophy. Most have been shown to involve negative changes in mitochondrial function and quality (Lezi and Swerdlow, 2012). Recent reviews on the subject highlight the complexity of mitochondrial dysfunction and the key role of mitochondrial dynamics in neurodegeneration (Gao et al., 2017; Panchal and Tiwari, 2019; Yang et al., 2021a). The net effect of these complications is a loss of mitochondrial function that eventually leads to cell death. Here, we discuss only changes in mitochondrial function seen in neurodegeneration that have also been reported in NAT deficiencies.

A reduction in mitochondrial respiration has been reported in many neurodegenerative diseases (Area-Gomez et al., 2019). Typically, a decrease in ATP synthesis and RRC is seen with age and neurodegeneration (Desler et al., 2012). Each of these measures of mitochondrial health are affected by NAT1 and NAT2 expression. Diminished neuronal uptake and metabolism of glucose are common in Alzheimer’s disease, Parkinson’s disease, ALS, and Huntington’s disease (Han et al., 2021). Glycolysis is also inhibited in the striatum of patients with early-stage Huntington’s disease (Powers et al., 2007). In ALS, mounting evidence highlights the importance of mitochondrial dysfunction and metabolic shifts as key pathophysiological factors.

Early reports identified changes in mitochondrial bioenergetics and oxidative stress in the frontal cortex of sporadic and familial cases of ALS (Bowling et al., 1993). Subsequent reports further delineated these mitochondrial disturbances, revealing reduced expression of genes crucial for oxidative phosphorylation, both nuclear and mitochondrial in nature, in the spinal cord of patients with ALS (Ladd et al., 2014). This shift in energy metabolism is not limited to oxidative phosphorylation alone. Palamiuc et al. noted a decrease in glucose utilization in ALS mouse muscle, favouring a metabolic shift towards fatty acid catabolism (Fig. 4A) (Palamiuc et al., 2015). This increased reliance on fatty acid β-oxidation can exacerbate oxidative stress (Tracey et al., 2018). Central to these metabolic alterations is the role of PGC-1α. Notably, its expression is downregulated in muscle and spinal cord tissues of
ALS patients (Russell et al., 2013; Thau et al., 2012). Further linking the importance of metabolic regulation to neurodegenerative conditions, PGC-1α is also down-regulated in mice deficient in mNat1 (Chennamsetty et al., 2016).

H. NATs and neurodegenerative disease

The relationship between NAT2 acetylator phenotype and neurodegenerative diseases has been of significant interest in recent decades. In a pioneering study, Bandmann et al. in 1997 highlighted a higher incidence of the NAT2 acetylator phenotype in patients with familial Parkinson’s disease when compared to controls (Bandmann et al., 1997). Their finding spurred a series of subsequent investigations, and numerous studies have supported the association by reporting a heightened incidence of slow acetylators in cases of sporadic and early-onset Parkinson’s disease across various ethnicities (Agúndez et al., 1998; Bandmann et al., 1997; Bandmann et al., 2000; Bialecka et al., 2002; Borlak and Reamon-Buettner, 2006; Chan et al., 2003; Chaudhary et al., 2005; Jiménez-Jiménez et al., 2016; Pandi et al., 2020; Singh et al., 2010; Tan et al., 2000). Several other studies, however, failed to find an association (Dupret et al., 1999; Harhangi et al., 1999; Maraganore et al., 2000; Nicholl et al., 1999; van der Walt et al., 2003). Offering a more comprehensive overview, a meta-analysis by Jiménez-Jiménez et al. (2016) revealed no significant association between the NAT2 slow acetylator phenotype and Parkinson’s disease within Caucasian populations. Their analysis, however, revealed a strong relationship for Asian populations. (Jiménez-Jiménez et al., 2016). Beyond the scope of Parkinson’s disease, the NAT2 genotype has emerged as a topic of study in other neurological and psychological conditions. Recent investigations have drawn connections between the NAT2 genotype and anxiety (Cacabelos et al., 2023), dementia (Gołab-Janowska et al., 2007) and Alzheimer’s disease (Zou et al., 2020b).

Few studies have considered the potential molecular role of the NATs in neurodegenerative diseases. In both mouse models of Alzheimer’s disease and human patient samples, Zou et al. found reduced levels of mNat1 and human NAT2, respectively. Interestingly, mouse models with reduced mNat1 levels, alongside Nat2-deficient mice, manifested blood-brain barrier damage and endothelial necroptosis, which were concurrent with mitochondrial fragmentation and heightened β-amyloid plaque deposits (Zou et al., 2020a). An upregulation of necroptosis has also been observed in human cancer cells lacking NAT1 (McAleese et al., 2022), suggesting a potentially broader, unifying role of NATs in multiple disease contexts.

A large study that examined gene expression in blood cells from 397 patients with ALS (cases) and 645 control individuals identified Copper Chaperone for Superoxide Dismutase (CCS) as the strongest predictor of survival in patients. However, other genes, including the NATs, showed differences between cases and controls. In this study, there was no correlation between NAT1 and NAT2 expression in blood cells suggesting they were independently regulated (Fig. 4B). When patients were divided into ‘low’ and ‘high’ expression using the median as a cutoff, patients with low NAT1 showed significantly higher median survival (Fig. 4C, p = 0.03) whereas those with low NAT2 had marginally better survival (Fig. 4D, p = 0.054). When contrasting patients with combined low NAT1 and low NAT2 against combined high NAT1 and high NAT2, a significant increase in median survival was observed for those patients with low expression of both genes (Fig. 4E, p = 0.018). These results suggest that the NATs are associated with overall survival in ALS patients, and that they may both contribute to effect on cellular processes that impact disease progression or outcome.
As briefly noted above, Palamiuc et al (2015) observed a shift from glucose to fatty acid-oxidation in the muscle of a mouse model of ALS. This is accompanied by increased the pyruvate dehydrogenase kinase PDK4, which phosphorylates pyruvate dehydrogenase, to inhibit pyruvate, a product of glucose metabolism, entry into mitochondria (Fig. 4A). Ultimately this would facilitate greater reliance on fatty-acid oxidation. Reduced NAT1 levels have been implicated in modulating the pyruvate dehydrogenase complex through an upregulation of pyruvate dehydrogenase kinase activity. Specifically, in cancer cells an increase in pyruvate dehydrogenase kinase activity leads to greater phosphorylation and consequent inhibition of pyruvate dehydrogenase (Wang et al., 2019), curbing the cell's ability to use glucose by limiting pyruvate entry into the mitochondria. Concurrently, diminished NAT2 levels adversely affect glucose uptake and promote lipolysis in adipocytes (Knowles et al., 2015), reinforcing a potential shift in energy metabolism. Considering the combined effect of decreased NAT1 and NAT2, one could hypothesize a metabolic transition in cells from glycolysis-dependent glucose utilization to an increased reliance on β-oxidation for energy production (Fig. 4A). However, experimental observations from the SOD1<sup>G86R</sup> mouse model of ALS, challenge this simplistic metabolic narrative. In this mouse, treatments that counteract the shift to β-oxidation ameliorate symptoms associated with mitochondrial dysfunction (Palamiuc et al., 2015). Consequently, while expression of the NATs impacts mitochondrial fuel preferences, their exact role in ALS progression and pathology is likely more intricate. The interplay between NATs and ALS may extend beyond mitochondrial fuel selection, and could involve a broader and more complex array of cellular mechanisms and responses.

V. Pharmacological regulation of NAT expression and activity

Because of the relationship between NAT expression and disease outcomes, these proteins may be novel drug targets (Butcher and Minchin, 2012; Rodrigues-Lima et al., 2010; Sim et al., 2014). Consequently, the development of small molecules that specifically target NAT1 and NAT2 has been the subject of several studies. In addition, various existing therapeutics and environmental chemicals can inhibit the NATs, albeit with limited specificity. The following discussion reviews approaches to both inhibiting activity and enhancing expression as potential methods to manipulate NAT.

A. Pharmacological inhibitors of arylamine N-acetyltransferase activity

The first attempt to systematically develop specific inhibitors of the NATs was performed by Sim’s group in 2009. They screened a small library of compounds against prokaryotic and eukaryotic NATs and isolated several rhodanine and thiazolidin-2,4-dione derivatives that selectively inhibited NAT1 or NAT2, some at sub-micromolar concentrations (Russell et al., 2009). A virtual screen of approximately 700 million compounds identified more than 20 candidate inhibitors, of which 40% showed activity against mNat2. Most of these compounds had IC<sub>50</sub> values in the low micromolar range suggesting they may be useful in biological experiments (Ballester et al., 2010).

Another recent virtual screen identified 60 potential NAT inhibitors (Leggett et al., 2022). When tested against recombinant NAT1 and NAT2, several potent inhibitors of NAT1 were identified. One of the compounds, alizarin (1,2-dihydroxyanthracene-9,10-dione), showed a 100-fold selectivity for NAT1 over NAT2 and was effective <i>in situ</i>. Alizarin is a synthetic anthraquinone found in the roots of
the madder plant. Its IC\textsubscript{50} for growth inhibition in a range of pancreatic cancer cells is approximately 12 µM (Xu et al., 2022), which is an order of magnitude greater than its NAT1 inhibitory concentration. This compound warrants further investigation both \textit{in vitro} and \textit{in vivo} as a possible selective NAT1 inhibitor.

Unfortunately, few structures that show selective inhibition of either NAT1 or NAT2 have progressed to studies \textit{in vivo}. Additional studies into the efficacy of selected inhibitors \textit{in vitro} and \textit{in vivo} are needed to validate their future use. Moreover, because of the similar effects that NAT1 and NAT2 may have on mitochondrial function, small molecule compounds that inhibit both enzymes simultaneously may be advantageous in some metabolic diseases.

B. Pharmacological activators of arylamine N-acetyltransferase expression

Direct activators of the NATs have not been identified to date, but two classes of drugs have been shown to increase their activities indirectly: histone deacetylase inhibitors and sirtuin inhibitors (Fig. 1). The mechanisms that underpin the increase in activities is explained in detail in Section IC. Here, the potential use of these drugs is discussed.

Based on available evidence, NAT1 activity is increased by sirtuin activators and decreased by sirtuin inhibitors whereas the opposite is the case for NAT2 (Butcher et al., 2020; Salazar-González et al., 2018; Turiján-Espinoza et al., 2018). Modulators of the sirtuins are under development for a range of metabolic disorders including Type 2 diabetes and neurodegeneration. Both activators and inhibitors have been developed for these different diseases (Dai et al., 2018). However, few have progressed to clinical assessment and of those tested in clinical trials, most show limited therapeutic effect due, in part, to a lack of specificity and sub-optimum \textit{in vivo} pharmacological profiles (Curry et al., 2021).

NAT1 expression is also induced in cancer cells by pan histone deactylase inhibitors, but their effects on NAT2 expression have not been reported. Histone deacetylase inhibitors have been developed for clinical use and include vorinostat, panobinostat and belinostat, which have received FDA approval mostly for cancer treatment. However, their repurposing for metabolic disorders has been widely suggested (King et al., 2021; Tang et al., 2013). Vorinostat is under investigation in Alzheimer’s disease as a monotherapy (NCT NCT03056495). However, the overt toxicity of currently available therapies, which includes high grade thrombocytopenia, cardiac abnormalities, and hepatic abnormalities, may limit their use in metabolic diseases (Bondarev et al., 2021).

There are good reasons to suggest that future development of both sirtuin and histone deacetylase inhibitors may generate novel therapeutics that regulate NAT activity and expression \textit{in vivo}. Studies on the combined used of these two classes of drugs might also reveal novel approaches in this field.

VI. Outlook and conclusion

Growing evidence indicates that the NATs are important in mitochondrial biogenesis and cellular metabolism. From the studies to date, both NAT1 and NAT2 appear to promote healthy mitochondria and NAT deficiency is associated with mitochondrial dysfunction. This association may be causal. Changes in cell metabolism is common in the pathogenesis of many diseases. It is therefore possible that the NATs contribute to the onset, progression and/or outcomes of these diseases, making them novel drug targets. However, further studies are required to better understand how and why NAT expression influences mitochondrial bioenergetics. These studies
need to interrogate the molecular as well as the cellular pathways that are influenced by the NATs using genomic and proteomic approaches. Identifying and validating legitimate binding partners may also shed light on NAT function. The possible role of intracellular AcCoA is intriguing but further evidence is needed to confirm that this substrate is regulated by either or both NAT1 and NAT2.

There is currently little in vivo evidence that manipulation of NAT will impact disease outcome. To what extent mouse models of NAT deficiency inform on the biological role(s) of the NATs needs to be determined. Finally, the development of tools such as small molecule inhibitors and activators that alter NAT activity/expression in vivo would greatly assist advancement in this area of research.
Data Availability Statement
The authors declare that all the data supporting the findings of this study are contained within the paper.

Authorship contributions
Literature search and collection of information: CC, MKG, RFM
Manuscript preparation: CC, MKG, RFM
Edited and proof-read draft manuscripts: CEM, NJB, STN, FJS

Competing interests
The authors declare no competing interests.

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VII. References


Cai J, Sun H, Chen L, Xie M, Zhuang J, Gao L and Wei XX (2021) NAT1 is a critical prognostic biomarker and inhibits proliferation of colorectal cancer through modulation of PI3K/Akt/mTOR. Future Oncol 17:2489-2498.


Hong KU, Walls KM and Hein DW (2023) Non-coding and intergenic genetic variants of human arylamine N-acetyltransferase 2 (NAT2) gene are associated with differential plasma lipid and cholesterol levels and cardiometabolic disorders. *Front Pharmacol* 14:1091976.


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### Table 1 Potential miRNAs that target the arylamine N-acetyltransferases.

<table>
<thead>
<tr>
<th>NAT1</th>
<th>NAT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-1264</td>
<td>hsa-miR-4529-5p</td>
</tr>
<tr>
<td>hsa-miR-1272</td>
<td>hsa-miR-4633-5p</td>
</tr>
<tr>
<td>hsa-miR-1290</td>
<td>hsa-miR-4647</td>
</tr>
<tr>
<td>hsa-miR-1972</td>
<td>hsa-miR-4662a-5p</td>
</tr>
<tr>
<td>hsa-miR-203</td>
<td>hsa-miR-4662b</td>
</tr>
<tr>
<td>hsa-miR-29a</td>
<td>hsa-miR-4699-3p</td>
</tr>
<tr>
<td>hsa-miR-29b</td>
<td>hsa-miR-4731-3p</td>
</tr>
<tr>
<td>hsa-miR-29c</td>
<td>hsa-miR-4748</td>
</tr>
<tr>
<td>hsa-miR-3122</td>
<td>hsa-miR-4792</td>
</tr>
<tr>
<td>hsa-miR-3149</td>
<td>hsa-miR-4795-3p</td>
</tr>
<tr>
<td>hsa-miR-3160-5p</td>
<td>hsa-miR-4801</td>
</tr>
<tr>
<td>hsa-miR-320a</td>
<td>hsa-miR-544b</td>
</tr>
<tr>
<td>hsa-miR-320b</td>
<td>hsa-miR-548ag</td>
</tr>
<tr>
<td>hsa-miR-320c</td>
<td>hsa-miR-548ai</td>
</tr>
<tr>
<td>hsa-miR-320d</td>
<td>hsa-miR-548d-3p</td>
</tr>
<tr>
<td>hsa-miR-323-3p</td>
<td>hsa-miR-548g</td>
</tr>
<tr>
<td>hsa-miR-338-5p</td>
<td>hsa-miR-582-3p</td>
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<tr>
<td>hsa-miR-3913-5p</td>
<td>hsa-miR-607</td>
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<td>hsa-miR-410</td>
<td>hsa-miR-640</td>
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<tr>
<td>hsa-miR-4426</td>
<td>hsa-miR-767-5p</td>
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<tr>
<td>hsa-miR-4429</td>
<td>hsa-miR-629</td>
</tr>
<tr>
<td>hsa-miR-4464</td>
<td>hsa-miR-651</td>
</tr>
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<td>hsa-miR-4487</td>
<td>hsa-miR-664</td>
</tr>
<tr>
<td></td>
<td>hsa-miR-921</td>
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</tbody>
</table>

Data obtained from the Hormonizome web site using NCBI Gene ID 9 (Rouillard et al., 2016)
<table>
<thead>
<tr>
<th>Group</th>
<th>Cancer Type</th>
<th>Number of Patients</th>
<th>Protein</th>
<th>Risk Phenotype</th>
<th>LogRank (p)</th>
<th>Median Survival</th>
<th>Pearson's r</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Breast</td>
<td>1879</td>
<td>NAT1</td>
<td>Low</td>
<td>1.7x10^{-5}</td>
<td>67</td>
<td>136</td>
<td>0.136* (Győrffy, 2021)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NAT2</td>
<td>ns</td>
<td>&gt;0.05</td>
<td>85</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Ovarian</td>
<td>1656</td>
<td>NAT1</td>
<td>Low</td>
<td>0.018</td>
<td>41</td>
<td>48</td>
<td>0.144* (Győrffy, 2023)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NAT2</td>
<td>ns</td>
<td>&gt;0.05</td>
<td>45</td>
<td>45</td>
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</tr>
<tr>
<td></td>
<td>Lung</td>
<td>2167</td>
<td>NAT1</td>
<td>Low</td>
<td>7.5x10^{-8}</td>
<td>57</td>
<td>84</td>
<td>0.041 (Győrffy et al., 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NAT2</td>
<td>ns</td>
<td>&gt;0.05</td>
<td>70</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Gastric</td>
<td>875</td>
<td>NAT1</td>
<td>Low</td>
<td>2.0x10^{-8}</td>
<td>25</td>
<td>30</td>
<td>0.613* (Szász et al., 2016)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>NAT2</td>
<td>Low</td>
<td>0.006</td>
<td>26</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Colon</td>
<td>550</td>
<td>NAT1</td>
<td>Low</td>
<td>1.5x10^{-4}</td>
<td>86</td>
<td>136</td>
<td>0.635* (Lánczky and Győrffy, 2021)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NAT2</td>
<td>Low</td>
<td>3.8x10^{-5}</td>
<td>72</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Liver</td>
<td>364</td>
<td>NAT1</td>
<td>Low</td>
<td>7.2x10^{-4}</td>
<td>41</td>
<td>71</td>
<td>0.555* (Menyhárt et al., 2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NAT2</td>
<td>Low</td>
<td>0.018</td>
<td>42</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Acute myeloid leukemia</td>
<td>1608</td>
<td>NAT1</td>
<td>High</td>
<td>0.008</td>
<td>17</td>
<td>73</td>
<td>-0.009 (Lánczky and Győrffy, 2021)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NAT2</td>
<td>High</td>
<td>0.002</td>
<td>17</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Myeloma</td>
<td>1416</td>
<td>NAT1</td>
<td>Low</td>
<td>3.9x10^{-4}</td>
<td>23</td>
<td>41</td>
<td>-0.121 (Lánczky and Győrffy, 2021)</td>
</tr>
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<td></td>
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<td></td>
<td>NAT2</td>
<td>High</td>
<td>&lt;10^{-16}</td>
<td>41</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

All data were divided into low and high NAT expression based on median mRNA values and were analyzed using KM Plotter (kmplot.com). Median survival is given in months. Asterisk indicates significant correlation between NAT1 and NAT2 expression (p<0.05).
### Table 3. Summary of studies investigating NAT1 and factors impacting cell metabolism.

<table>
<thead>
<tr>
<th>Research Area</th>
<th>Year</th>
<th>Finding</th>
<th>Conclusion</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA</td>
<td>2016</td>
<td>Increased NAT1 activity decreased flux of acetyl-CoA through palmitoleic acid synthesis pathway.</td>
<td>Loss of NAT1 increases acetyl-CoA levels</td>
<td>(Carlisle et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>2018</td>
<td>Reduction of NAT1 activity increased acetyl-CoA levels.</td>
<td></td>
<td>(Stepp et al., 2018)</td>
</tr>
<tr>
<td></td>
<td>2019</td>
<td>NAT1 KO increased acetyl-CoA levels in MDA-MB-231, MCF7, but not ZR-75-1 cells. NAT1 may regulate acetyl-CoA levels.</td>
<td></td>
<td>(Stepp et al., 2019b)</td>
</tr>
<tr>
<td>Folate metabolism</td>
<td>1995</td>
<td>Catabolite of folate, PABG, is an endogenous substrate of NAT1.</td>
<td>Folate binds to the NAT1 active site</td>
<td>(Minchin, 1995)</td>
</tr>
<tr>
<td>Lipid homeostasis</td>
<td>2020</td>
<td>NAT1 KO reduced pyrimidine biosynthesis and caused defective FA β-oxidation.</td>
<td>Loss of NAT1 results in a reduced capacity to utilise FA.</td>
<td>(Carlisle et al., 2020)</td>
</tr>
<tr>
<td>Mitochondrial function: respiration, glycolysis, stress response</td>
<td>2018</td>
<td>NAT1 KO increased mitochondrial reserve respiratory capacity and glycolytic reserve capacity.</td>
<td>NAT1 KO alters oxidative phosphorylation and glycolytic rates</td>
<td>(Carlisle et al., 2018)</td>
</tr>
<tr>
<td></td>
<td>2018</td>
<td>NAT1 KO increased ROS production and apoptosis and resulted in the loss of p53 during glucose starvation.</td>
<td></td>
<td>(Wang et al., 2018)</td>
</tr>
<tr>
<td></td>
<td>2019</td>
<td>NAT1 KO decreased oxidative phosphorylation and glycolysis by decreasing PDH activity.</td>
<td></td>
<td>(Wang et al., 2019)</td>
</tr>
<tr>
<td>Post-translational modification</td>
<td>2020</td>
<td>NAT1 was acetylated by p300/CBP and deacetylated by SIRT1 and 2 and this changed its enzymatic activity.</td>
<td>NAT1 enzymatic activity is altered by post-translational modifications</td>
<td>(Butcher et al., 2020)</td>
</tr>
<tr>
<td></td>
<td>2022</td>
<td>Inhibiting HDACs and sirtuins increased NAT1 enzymatic activity.</td>
<td></td>
<td>(Salazar-González et al., 2022)</td>
</tr>
</tbody>
</table>

KO, knockout; HDACs, histone deacetylases; PDH, pyruvate dehydrogenase; FA, fatty acid; PABG, p-aminobenzoyl glutamate.
<table>
<thead>
<tr>
<th>Year</th>
<th>Finding</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>2015</td>
<td>NAT2 rs1208 “A” allele (slow phenotype) is associated with insulin sensitivity and unfavourable changes in lipid levels. Individuals with the ‘slow’ allele less likely to develop insulin resistance.</td>
<td>(Knowles et al., 2015)</td>
</tr>
<tr>
<td>2016</td>
<td>Silencing of mouse Nat1 increases ROS production and mitochondrial fragmentation, decreases MMP, biogenesis, mass, cellular respiration, and ATP production. It also impairs exercise capacity and fatty acid utilization.</td>
<td>(Chennamsetty et al., 2016)</td>
</tr>
<tr>
<td>2017</td>
<td>Children with the NAT2 rs1208 “A” allele (slow phenotype) are at higher risk of impaired glucose tolerance, an indicator of pre-diabetes. No association was found between the NAT2 rs1208 “A” allele and insulin resistance or insulin sensitivity.</td>
<td>(Marzuillo et al., 2017)</td>
</tr>
<tr>
<td>2017</td>
<td>Human NAT1 RNA was significantly decreased after exposure to high glucose conditions, representative of hyperglycaemia and this may identify patients with diabetic retinopathy.</td>
<td>(Ung et al., 2017)</td>
</tr>
<tr>
<td>2017</td>
<td>Mouse Nat1 knockout is associated with whole-body insulin resistance, reduced whole-body energy expenditure, and decreased mitochondrial oxygen consumption.</td>
<td>(Camporez João et al., 2017)</td>
</tr>
<tr>
<td>2019</td>
<td>Double Nat1/Nat2 knockout mice have increased respiratory exchange rate compared to the control mice, indicating increased glucose oxidation, and decreased fatty acid oxidation.</td>
<td>(Denis et al., 2019)</td>
</tr>
<tr>
<td>2020</td>
<td>Rapid acetylator NAT2 rats (human NAT1) were more prone to developing dyslipidemia.</td>
<td>(Hong et al., 2020a)</td>
</tr>
</tbody>
</table>

Human NAT2 is homologous to mouse Nat1. ROS, reactive oxygen species; MMP, mitochondrial membrane potential; ATP, adenosine triphosphate.
Figure legends

**Fig. 1. Regulation of NAT1 activity by acetylation.**
(A) One of several modifications that regulate gene expression is acetylation of histones (orange circles). It is dependent on intracellular acetyl-CoA levels, which is the co-substrate for histone acetyltransferases and changes depending on the metabolic state of the cell. Deacetylation by HDACs condenses chromatin and represses gene expression, which can be overcome by HDAC inhibitors. NAT1 transcription increases when cells are treated with HDAC inhibitors such as TSA and SAHA. (B) NAT1 is directly acetylated at lysine 100 and 188 by p300/CBP, an acetyl-CoA dependent enzyme, and deacetylated by the sirtuins SIRT1 and SIRT2. The acetylated protein has diminished activity compared to the unacetylated form. Up-regulation of SIRT1 and/or SIRT2 shifts NAT1 to the deacetylated state thereby increasing its activity.

**Fig. 2. Mitochondrial respiration and ATP production.**
(A) Mitochondrial respiration is driven normally by glucose, which is converted to pyruvate in the glycolysis pathway, a minor source of ATP in most cells. Pyruvate enters the mitochondria to feed the TCA cycle that produces NADH and FADH$_2$ for the electron transport chain. Protons are pumped into the intermembrane space and establish an energy potential across the inner membrane. This provides the energy needed to produce ATP. (B) There are 4 main fuel sources for the TCA cycle: glucose, fatty acids, amino acids, and ketone bodies. Fatty acids are catabolized to acetyl-CoA via β-oxidation. Ketogenic amino acids are catabolized to acetyl-CoA while glucogenic amino acids are catabolized to various intermediates of the TCA cycle. Ketone bodies can be used to produce acetyl-CoA via ketolysis but can also be synthesized from acetyl-CoA via ketogenesis. The ketone body β-hydroxybutyrate can also inhibit HDACs, which up-regulates sirtuin expression.

**Fig. 3. Role of NAT1 in mitochondrial-dependent apoptosis and necroptosis.**
The intrinsic apoptotic pathway involves the release of cytochrome C from mitochondria in response to loss of the transmembrane potential. This can be initiated by several stimuli including Bax, which accumulates in the outer mitochondrial membrane where it oligomerizes and mediates permeabilization. tBid can also directly mediate mitochondrial permeabilization or it can interact with and activate Bax. tBid is produced following proteolysis of Bid by caspase 8. Cytochrome C release activates caspase 9, which subsequently activates the executioner caspases 3 and 7. Caspase 8 is also involved in the extrinsic apoptotic pathway where it directly activates caspases 3 and 7. In addition, caspase 8 negatively regulates necroptosis by cleavage of substrates such as RIPK3. NAT1 is required for multiple steps in the apoptotic pathways including Bax expression, cytochrome C release, caspase 9 activation and caspase 8 activation. The loss of caspase 8 in NAT1 deficiency may inhibit the intrinsic apoptotic pathway by reducing tBid formation and activate the necroptosis pathway by relieving inhibition of RIPK3.

**Fig. 4. Expression of NAT1 and NAT2 in blood cells from patients with amyotrophic lateral sclerosis.**
(A) In ALS, muscle cells switch from using glucose to fatty acids for energy production. The increase in fatty acid uptake up-regulates PDK4, which phosphorylates pyruvate dehydrogenase, part of the pyruvate dehydrogenase complex (PDC), and inhibits pyruvate entry into mitochondria. Adapted from Palamuic et al (Palamuic et al., 2015). Sites where NAT1 (PDC) and NAT2 (glucose/fatty acid uptake) have been reported to influence fuel usage are also shown. (B) There is no correlation between NAT1 and NAT2 expression in
ALS patients (Spearman’s rho = 0.14, p>0.05). The dotted lines divide patients into quadrants using the respective median expression values. (C) Kaplan-Meier survival curves for NAT1 expression in ALS patients (p = 0.02, Log-Rank test). Patients (n = 397) were divided into low and high NAT1 expression based on the median value of mRNA levels. Median survival was 5.3 years in high NAT1 expressing patients versus 7.5 years in low NAT1 expressing patients. (D) Kaplan-Meier survival curves for NAT2 expression in ALS patients (p=0.08, Log-Rank test). Median survival was 5.2 years in high NAT1 expressing patients versus 6.6 years in low NAT1 expressing patients. (E) Kaplan-Meier survival curves for ALS patients with low NAT1 and low NAT2 (n = 86) versus patients with high NAT1 and high NAT2 (n = 76; p=0.01, Log-Rank test). Median survival was 5.1 years in high/high NAT expressing patients versus 8.2 years in low/low NAT expressing patients.