Minimizing Tracer Interference to Assess in vivo Hepatic Metabolism with Glutamine-generated Mass Isotopomers

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Abstract

Minimizing Tracer Interference to Assess in vivo Hepatic Metabolism with Glutamine-generated Mass Isotopomers

Stephan Siebel
2021

Stable isotope tracers are widely used to study the in vivo kinetics of central carbon metabolism in diseases such as obesity, type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (NAFLD). However, despite their common use, $^{13}$C-labeled acetate, lactate, and propionate have led to large incongruities and inconsistencies in their in vivo measurement of hepatic metabolism. To resolve major discrepancies and recent controversies of hepatic metabolism we developed and validated $[1,2^{-13}C_2]$-L-glutamine as a novel tracer strategy for measuring hepatic central carbon metabolism. In our rodent studies $[1,2^{-13}C_2]$-L-glutamine generated a straightforward labeling pattern of key metabolites suitable for the interpretation of LC-MS/MS spectra using Mass Isotopomer Multi Ordnate Spectral Analysis (MIMOSA) principles and thereby enabled an unencumbered independent assessment of Krebs cycle fluxes. In a head-to-head comparison, we found that $[1,2^{-13}C_2]$-L-glutamine had many favorable qualities and few liabilities compared to the other tracer strategies. Additionally, given that in vivo NMR studies of mitochondrial metabolism often depend on the signal for glutamate and other abundant metabolites that act as label-trapping pools for Krebs Cycle intermediates, we were able to directly measure $\alpha$-Ketoglutarate-Glutamate exchange rate ($V_x$) and show evidence for the assumption that the intramitochondrial metabolite pool is in rapid exchange with NMR observable cytosolic metabolites. Here we report the development
and in vivo kinetic and steady state validation of [1,2-^{13}C_2]-L-glutamine as an independent tracer of hepatic metabolism.
Minimizing Tracer Interference to Assess *in vivo* Hepatic Metabolism with Glutamine-generated Mass Isotopomers

A Dissertation

Presented to the Faculty of the Graduate School

Of

Yale University

In Candidacy for the Degree of

Doctor of Philosophy

By

Stephan Siebel

Dissertation Director: Richard Glenn Kibbey, IV

December 2021
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Dedicated to the original MuskeSiebels

Isabel Ingrid Siebel

&

William Stephan Siebel

“One For All And All For One”

Zugeeignet

meinen Eltern

Joachim und Brigitte Siebel

als auch

meinen Bruedern

Christian und Michael Siebel
“Measure what is measurable, and make measurable what is not so”

Galileo Galilei
CHAPTER I: INTRODUCTION

Hepatic Krebs cycle in health and disease

The liver is an essential metabolic organ which plays a central role not only in liver specific metabolism, but it also governs the entire body’s energy metabolism by providing important substrates, such as glucose and lipids, among others, to all vital tissues. Central to the liver’s role in carbon metabolism are mitochondria. Housed within the cytoplasm of each hepatocyte these organelles maintain glucose, and lipid homeostasis through the Tricarboxylic acid cycle (TCA for short) also commonly referred to as the Krebs cycle. By coordinating the delicate balance between degradation and biosynthesis of these macronutrients the hepatic Krebs cycle ensures normoglycemia, and normolipidemia. Considering its central role in macronutrient homeostasis Sir Hans Krebs predicted during his Nobel banquet address in 1953, “I am convinced that an understanding of the process of energy production will eventually help us in solving some of the practical problems of medicine”. Since Krebs' pioneering work in the discovery and investigation of the Krebs cycle, a plethora of research has shown that aberrant central carbon metabolism plays a crucial role in the pathogenesis and pathophysiology of many disorders [26]. The most prominent metabolic disorders include non-alcoholic fatty liver (NAFLD), obesity, insulin resistance (IR) and type 2 diabetes mellitus (T2DM) [27-39], all of which promote morbidity and mortality in our society [27, 40-43]. To date Diabetes affects close to 10% of the US population and more than ~300 million people worldwide, with over 5000 children and adolescents being diagnosed with T2DM annually in the US alone[44]. Short- and long-term
complications of T2DM are grave, affect almost every organ system, and predispose patients to serious cardiovascular, renal and liver disease which can ultimately lead to premature deaths [45]. The majority of patients with T2DM also suffer from obesity and NAFLD, which affects 10% of children and 68% of adults in the U.S. NAFLD is now the most common liver disease in the obese and diabetic adult and pediatric population where it is not only a comorbidity but also a critical predisposing factor to T2DM [46, 47]. Additionally, mitochondrial oxidative, reductive and biosynthetic dysregulation have been discovered to be part of the development and progression of a variety of cancers, such as renal cell carcinoma, hereditary pheochromocytoma and paraganglioma syndrome, and gastrointestinal stromal tumors, and hepatocellular carcinoma among others [29, 37, 43, 47-50]. All of these disorders which together or by themselves affect millions of people in the US and many more worldwide, often times lead to devastating morbidity and ultimately to higher mortality. Therefore, in our pursuit of effective diagnostic, therapeutic, and curative targets for these disorders it is of utmost importance to develop a solid understanding of the fundamentals of hepatic metabolism.

Krebs cycle and hepatic metabolism

Metabolism refers to all chemical reactions that constitute networks of biochemical pathways within cells or organisms that are necessary to maintain life. Metabolic pathways consist of a series of coupled chemical reactions that are catalyzed by enzymes. Each of the consecutive enzymatic steps facilitates the chemical processing of a precursor into an intermediate molecule which is eventually converted into its final
product. Metabolism exists in one of two phases: 1. anabolism or catabolism. During catabolism larger organic nutrient molecule, such as carbohydrates, lipids, or proteins, are broken down into smaller, simpler products, such as pyruvate, lactate, CO$_2$, NH$_3$, and others. Catabolic reactions are crucial for energy production in form of reducing equivalents, such as FADH$_2$, NADH, NADPH, or high energy molecules, such as ATP and GTP. In anabolic pathways, on the other hand, smaller precursors, such as PEP and acetyl CoA, are used as building blocks for larger, more complex products, such as polysaccharides, lipids, proteins and nucleic acids. In contrast to catabolic reaction, anabolism consumes energy in the form of ATP, GTP, NADH, NADPH, and FADH$_2$. Amphibolic systems can perform both anabolism and catabolism. However, to avoid wasting energy by running both simultaneously, anabolism and catabolism are usually reciprocally regulated and tightly controlled [1] (Figure 1). The major regulatory connecting hub for glucose, lipid and protein ana- and catabolism is the Citric Acid (CAC) or Tricarboxylic Acid (TCA) cycle [9]. Although, it is commonly referred to as the Krebs cycle, named after Sir Hans Krebs, whose pioneering work laid the foundation for our modern understanding of central carbon metabolism [2]. After oxidation of their respective carbon backbones in either glycolysis, lipolysis, or proteolysis, glucose, lipid and protein metabolism converges in the Krebs cycle through their common end product acetate, which is then activated by acetyl CoA Synthetase (ACS) to acetyl CoA. The cyclic sequence of Krebs cycle reactions begins when acetyl-CoA undergoes an aldol condensation with oxaloacetate (OAA) to form citrate. This first committed step of the Krebs cycle is catalyzed by Citrate Synthase (CS). After its formation from acetyl CoA and OAA citrate is converted to isocitrate via aconitase which is then oxidized by isocitrate.
dehydrogenase (IDH) to α-ketoglutarate (αKG). Subsequently, succinate CoA is formed from the decarboxylation reaction of aKG catalyzed by aKG Dehydrogenase (OGDH). succinate CoA synthetase then gives rise to succinate. Succinate is a symmetric intramitochondrial metabolite which is exclusively formed within the Krebs Cycle and metabolized to fumarate via succinate dehydrogenase (SDH). Fumarase catalyzes the conversion of fumarate to malate (M) which is then oxidized to oxaloacetate (OAA) by malate dehydrogenase (MDH) thus completing one full turn of the Krebs cycle. Through this series of cyclic enzymatic reactions, acetyl CoA is completely oxidized to CO₂ during one full turn of the cycle. During some of the chemical processes of the Krebs Cycle reducing equivalents, such as NADH and FADH₂, are formed. These are crucial components of the mitochondrial electron transfer chain and oxidative phosphorylation allowing the mitochondrion to produce and conserve large amounts of energy in the form of ATP, necessary for all vital cellular processes. The four major oxidative reactions of the Krebs Cycle are catalyzed by isocitrate dehydrogenase (forward reaction), αKG dehydrogenase, succinate dehydrogenase and malate dehydrogenase.
Figure 1. Krebs cycle metabolism.
Top Panel: The Krebs is an amphibolic connecting hub which mediates the two phases of metabolism, anabolism and catabolism. Depending on the metabolic state of the cell reducing equivalences and high energy molecules are either synthesized or utilized. Bottom Panel: A depiction of all major metabolic reactions associated with the Krebs cycle. Oxidative fluxes are depicted in blue, exchange reactions in orange, anaplerotic fluxes in red and cataplerotic fluxes in green. ACLy = ATP citrate Lyase, CS =citrate synthase, GDH= glutamate dehydrogenase, IDHnet= isocitrate dehydrogenase (net), MDH= malate dehydrogenase, ME= malic enzyme, OGDH= α ketoglutarate dehydrogenase, PC= pyruvate carboxylase, PCC=propionate CoA carboxylase, PDH= pyruvate dehydrogenase, PEPCK = phosphoenolpyruvate carboxykinase, SDH= succinate dehydrogenase.
Anaplerosis and cataplerosis

The concentrations of Krebs cycle intermediates are relatively constant irrespective of whether the hepatocyte is in a state of anabolic or catabolic metabolism. During anabolism, when the Krebs cycles assumes its biosynthetic function, it channels its designated intermediates, such as OAA or citrate, into specific metabolic pathways, where they are used as precursors for gluconeogenesis (liver and kidney), lipogenesis (liver) and glyceroneogenesis (adipose tissue) (Figure 2) [3]. The removal of intermediates from the Krebs cycle for biosynthetic processes is commonly termed cataplerosis. The two main cataplerotic fluxes are mediated by phosphoenolpyruvate carboxykinase.

Figure 2. Anaplerosis and Cataplerosis.
Anaplerotic fluxes are shown in red, cataplerotic fluxes and their biosynthetic products in green. Anaplerosis is balanced by cataplerosis to maintain mass balance. Cataplerotic fluxes are important for biosynthesis of many metabolites (green) to maintain glucose and lipid homeostasis as well as maintenance of Redox Potentials.
(PEPCK), and malic enzyme (ME), and to a lesser extent ATP citrate lyase (ACLy). Phosphoenolpyruvate carboxykinase (PEPCK), the main cataplerotic reaction, catalyzes the formation of phosphoenolpyruvate (PEP) from OAA whereas malic enzyme (ME) facilitates the conversion of malate to pyruvate. Both cataplerotic fluxes partake in gluconeogenesis. However, flux through ME is negligible under physiologic conditions compared to PEPCK [4]. To maintain cycle intermediates at almost constant concentrations, every carbon efflux from the Krebs cycle needs to replenished by an equal carbon influx [1, 3]. There are three major carbon entry points into the Krebs cycle. These three anaplerotic fluxes are mediated by pyruvate carboxylase (PC), glutamate dehydrogenase (GDH), and propionate CoA carboxylase (PCC). The latter is often considered to be the major anaplerotic reactions in the liver. PCC catalyzes the carboxylation of pyruvate into OAA (Figure 2). As illustrated by their different fluxes, both, anaplerosis and cataplerosis, play important roles in the function of the Krebs cycle and thus in the regulation of amino acid, glucose, and fatty acid metabolism[3].
Reductive carboxylation and ATP citrate lyase

Reductive carboxylation catalyzed by isocitrate dehydrogenase is an important process for maintaining the intramitochondrial citrate pool which can then be used as a substrate for fatty acid and cholesterol biosynthesis mediated by ATP citrate lyase. Isocitrate dehydrogenase catalyzes a reversible step comprised of two biochemical reactions (Figure 3): 1. A decarboxylation (forward) reaction (IDHf) that converts isocitrate into aKG and 2. A reductive (reverse) carboxylation (IDHr) step that converts aKG back into isocitrate. Besides its role as an intermediate of the Krebs cycle, Citrate can be transported from the mitochondrial matrix into the cytosol via mitochondrial citrate carrier (CiC). In the cytosol, ATP citrate lyase (ACLy) catalyzes the breakdown of Citrate into cytosolic OAA and acetyl-CoA. The cytosolic OAA is transformed to malate by cytosolic malate Dehydrogenase (MDH), which is transported back into the mitochondrion via CiC in exchange for citrate. After ligation with a CoA species acetate can be converted to malonyl CoA by

![Diagram](image)

Figure 3. Reverse carboxylation and ATP Citrate Lyase reaction. The Mitochondrial Citrate Carrier (CiC) transports Citrate out of the mitochondrial matrix into the cytosol in exchange for Malate. In the cytosol ACLy facilitates the production of acetyl CoA and Malate from citrate. The reverse isocitrate dehydrogenase (IDHr) reaction catalyzes the reductive carboxylation of aKG to citrate.
acetyl CoA carboxylase (ACC) and can then be utilized as a building block in fatty acid synthesis via de novo lipogenesis (DNL) [5-7]. Another fate of acetyl-CoA is histone and non-histone acetylation through which acetyl CoA can epigenetically modify gene expression as well as regulate enzyme function [8, 9].

Amino Transferase Exchange rate (Vx)

Aminotransferases transfer an α-amino group from an amino acid to an α-carbon of a keto acid without any net carbon conversion or net deamination. Exchange reactions (Vx) (Figure 4) commonly occur in reversible systems and their directionality depends on the availability of the reactants. They play an integral role in biosynthetic processes by supplying intermediates for the Krebs cycle. Another important function of exchange reactions is to mediate the disposal of toxic nitrogen waste can eventually be detoxified in the Urea (Ornithine) cycle [9]. The two main aminotransferase reactions that facilitate exchange between intramitochondrial and cytosolic metabolite pools catalyze the interconversion of glutamate to αKG as well as OAA to aspartate and vice versa [10]. In contrast to exchange
reactions, deamination processes lead to net carbon conversion and release of ammonia when an amino acid is deaminated to a keto acid.

Substrate cycling

Originally deemed futile, substrate cycling is now considered an important aspect of amphibolic systems. Substrate cycling pathways replenish and maintain metabolite concentrations and redox potentials and contribute to thermogenesis [11-13]. During substrate cycling two naturally distinct enzymatic reactions of opposing metabolic pathways operate simultaneously by circumventing an otherwise thermodynamic unfavorable or irreversible biochemical step [14]. One important example of substrate cycling is the PEP-pyruvate cycle (Figure 5) at the intersection of glycolysis and gluconeogenesis. Here, pyruvate is converted to OAA catalyzed by PC, while OAA is transformed to PEP via PEPCK and back into pyruvate through pyruvate kinase (PK) [11, 12, 15].
Regulation of Krebs cycle flux

The major rate limiting steps in the Krebs cycle are the fluxes catalyzed by citrate synthase, isocitrate dehydrogenase and αKG dehydrogenase. Changes in substrate availability, and/or molar ratios of NAD+/NADH, ATP/ADP or CoA species, as well as negative feedback from their respective products, can all result in a shift of their enzymatic equilibria. For example, accumulation of citrate, succinyl CoA and/or ATP inhibits citrate synthase, whereas αKG dehydrogenase is only inhibited by succinyl CoA.

Increased NADH/NAD⁺ or ATP/ADP ratios inhibit all of the dehydrogenase reactions, including PDH. Additionally, acetyl CoA is a potent allosteric activator of PC [16-22] while indirectly inhibiting PDH through activation of pyruvate dehydrogenase complex kinase. Pyruvate, on the other hand, activates PDH through inhibition of pyruvate dehydrogenase kinase [51]. Endocrine hormones also play a crucial role in controlling enzymatic functions.
and thus impacting metabolic flux. Insulin suppresses expression of PEPCK while stimulating pyruvate dehydrogenase phosphatase and thereby indirectly activating PDH flux, all of which promotes glucose metabolism towards ATP production while it suppresses endogenous glucose production [23, 24]. Conversely, glucagon stimulates expression of cytosolic PEPCK [25], thus promoting cataplerotic fluxes for gluconeogenesis, especially during states of limited exogenous glucose supply, such as fasting and starvation.

**Stable isotope tracer methods**

In recent years, increasing emphasis has been placed on studying potential molecular mechanisms and genetic variations underlying metabolic dysfunction. Whole genome and exome sequencing, mRNA expression profiling and SNP arrays, to name a few, have been used to identify genetic targets for early clinical diagnosis and intervention. However, despite the undisputable place of molecular tests in clinical and scientific investigation of metabolic and endocrine disorders, they come with their own limitations. Genetic variants, and expression profiles, similar to measuring metabolite concentrations, are only a static snapshot of a highly dynamic system. Kinetic investigation of metabolic fluxes *in vivo* using stable isotope tracers, on the other hand, give valuable insights into the dynamic workings of a metabolic system as a whole and how it may change over time. Thus, metabolic flux analyses (MFA) can serve as a functional readout of metabolism and add important phenotypical characterization to existing genotypical information by tracing directionality of chemical reactions within a metabolic pathway and
by identifying deviation from a physiologic baseline before and during the development and progression of disease. Stable isotope tracers are a safe method to study metabolism as a whole—at a single or over the course of multiple timepoints—rather than focusing on individual, static aspects, such as metabolite concentrations and gene expression profiles [51, 52]. Due to their non-radioactivity, stable isotope based tracer strategies have gained wide popularity in research studies, especially of the *in vivo* kinetics of human and rodent central carbon metabolism since the mid 1980’s [53, 54].

Atoms are made up of three main particles: positively charged protons, uncharged neutrons and negatively charged electrons. A stable Isotope is a non-radioactive element that differs in number of neutrons but has the same number of protons as their lighter counterpart [55]. For instance, carbon 12 is the most abundant carbon atom in nature. However, up to 1.11% of all carbons have one additional neutron and occur as carbon 13 (atom mass unit [amu] of 13 compared to an amu of 12 of carbon 12). Table 1 shows examples of atoms and their natural distribution patterns of naturally occurring stable isotopes. In tracers one or more of the lighter (more abundant) atoms in the chemical structure of a metabolite is/are replaced by its respective stable isotopes (label) and therefore differ in mass from its non-labeled counterpart, which is referred to as a tracee. A prominent tracer strategy is the carbon 13 (\(^{13}\)C) tracer method. Herein, \(^{13}\)C

<table>
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<tr>
<th>Element</th>
<th>Isotope</th>
<th>Occurrence (%)</th>
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<tr>
<td>Carbon</td>
<td>(^{12})C</td>
<td>98.89</td>
</tr>
<tr>
<td></td>
<td>(^{13})C</td>
<td>1.11</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>(^{1})H</td>
<td>99.9885</td>
</tr>
<tr>
<td></td>
<td>(^{2})H</td>
<td>0.115</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>(^{14})N</td>
<td>99.632</td>
</tr>
<tr>
<td></td>
<td>(^{15})N</td>
<td>0.368</td>
</tr>
<tr>
<td>Oxygen</td>
<td>(^{16})O</td>
<td>99.757</td>
</tr>
<tr>
<td></td>
<td>(^{17})O</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>(^{18})O</td>
<td>0.205</td>
</tr>
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Table 1: Examples of common atoms and their stable isotopes and their natural abundance.
label is introduced into a metabolic system by exchanging one or more of the commonly occurring $^{12}\text{C}$ atom(s) of a metabolite’s carbon backbone with its natural isotope $^{13}\text{C}$ (Figure 7).

In this thesis we are following the terminology and nomenclature of Seeman et al and the conventional nomenclature of the International Union of Pure and Applied Chemistry (IUPAC) [56]: The term isotopologue, indicated by M, describes a molecule that carries n number of labels (heavy (stable) isotopes) within its structure: $M+n$ ($n = 0, 1, 2, 3, \ldots n$).

Mass isotopologues differ in molecular weight due to difference in content of heavy isotopes but not in difference in molecular structure. This nomenclature is position non-specific meaning that it indicates that the molecule is carrying one or more stable isotope(s) but it does not indicate its position within the carbon chain of the molecule. The term isotopomer on the other hand represents the exact position of the heavy molecule (isotope) within a molecule’s carbon backbone, i.e., $6^{13}\text{C}_1$-citrate vs $1^{13}\text{C}_1$-citrate [56]. Numbers preceding the atom indicate the position of the stable isotope within the molecular structure, while the following superscript indicates the stable isotope and the subscript following the letter name for the atom indicates the quantity of heavy isotopes.

![Figure 7. Stable isotope tracers. Examples of carbon numbering and isotope labeling. Citrate is a 6-carbon molecule, numbered C1 through C6. The solid circle denotes a stable isotope carbon 13 whereas the open circles indicate carbon 12. An Isotopologue refers only to the total number of stable isotopes within a molecule with non-position specific label information, e.g., citrate M+0 (no stable isotope) or citrate M+1 (one stable isotope). Isotopomer: position specific label information, e.g., $6^{13}\text{C}_1$-citrate which carries one stable isotope in carbon position six.](image)
Fractional enrichment of stable isotope tracers

The relative occurrence of a tracer (stable isotope labeled metabolite) to its tracee (unlabeled metabolite) within a defined metabolite pool, e.g. plasma, is described as the fractional isotopic enrichment of a metabolite, also referred to as relative abundance of a stable isotope tracer. This fractional enrichment of a metabolite pool with tracer can be expressed in many different ways, such as Tracer to Tracee ratio, Molar Percent Enrichment and Atom Percent Excess (APE%) (Figure 8) [65, 66]. Going forward we will use the ratio of labeled metabolite (tracer) to its total metabolite pool (unlabeled and labeled metabolite) expressed as Atom Percent Excess (APE%) [55]:

\[ \text{APE\%} = \left( \frac{\text{Tracer}}{\text{Tracer} + \text{Tracee}} \right) \times 100 \]
Measuring tracer signal

Even though stable isotope labeled metabolites (tracers) undergo the exact same physiological processes as their unlabeled counterpart (tracee), the difference in mass and in quantum-magnetic properties of the heavier isotope can be used to differentiate it from its lighter naturally more abundant atoms by Mass Spectrometry and Nuclear Magnetic Resonance spectroscopy, respectively [55, 57].
Nuclear Magnetic Resonance spectroscopy (NMR)

(for a detailed description see Supplemental Information)

Nuclear Magnetic Resonance spectroscopy (NMR) is a magnetic resonance spectroscopy (MRS) based, non-invasive analytical technique that allows for dynamic and static investigation of biochemical composition of tissues and body fluids in vivo and in vitro. Today, two common types of NMR used in metabolic research are proton- and carbon-NMR (1H-NMR and 13C-NMR) [58, 59]. In 1H-NMR the observed nucleus is hydrogen atom (1H). 1H-NMR is a popular method due to the high isotopic abundance of 1H (~99% of all hydrogen atoms) and the comparably easier acquisition of NMR spectra in comparison to 13C spectra. In 13C-NMR the observed nucleus is 13C, the only MR visible carbon isotope. Carbon NMR is a useful method to obtain position specific information of carbon label within an organic molecule. Both, 13C-NMR and 1H-NMR, come with some considerable disadvantages and limitations for in vivo studies [60]. For instance 1H-NMR spectra may be dominated by resonances of highly abundant molecules, such as water or lipids, and thereby overlap and obscure important peaks of lower abundant metabolites making exact spectral assignment difficult [61-65]. On the other hand, 13C-NMR suffers from limited sensitivity due to low signal to noise ratios, significantly lower magnetogyric ratio of 13C compared to 1H, a broad chemical shift and an isotopic abundance of only 1.1% [66]. In addition to its low sensitivity, 13C-NMR is a fairly laborious method requiring long scan times and metabolite concentrations in the millimolar range [61-65].
Some of these limitations have been overcome by higher localization accuracy and improved sensitivity of Proton observed, carbon decoupled/edited NMR spectroscopy (POCE) [64]. POCE is a $^1$H-$^{13}$C heteronuclear editing method which consists of the acquisition of two spin-echo measurements, one with a broad-band inversion pulse applied at the $^{13}$C frequency, and the other without the inversion pulse. The difference between the spectra represents protons bound to $^{13}$C at twice the true intensity, while the sub-spectrum without the inversion pulse represents the protons for $^{13}$C-labeled and the unlabeled compounds, i.e., the total concentrations. $^{13}$C decoupling is applied during the acquisition of the free-induction decays to collapse $^{13}$C-$^1$H couplings, reducing spectral complexity while increasing the sensitive of $^{13}$C-NMR more than tenfold [64]. However, its overall application is still limited in vivo by low spectral resolution [65, 99].

Mass Spectrometry (MS)

The first mass analyzer, then called parabola spectrograph, was developed in 1912 by the English physicist and Nobel Laureate J. J. Thomson, who discovered that the flow of charged particles can be deflected when exposed to an electromagnetic field. Based on his discovery Thomson built an instrument that could separate charged particles based on their mass number and subsequently detected on photographic plates[67]. This invention made it possible to show evidence of the existence of stable isotopes of an element by Thomson after their discovery by Frederick Soddy in 1913 [68]. Since its first development over a century ago Mass analyzers have evolved and today many different types exist. One of the most prominent Mass analyzers in use is the quadrupole mass
analyzer (Figure 9), which consists of four parallel circular or hyperbolic rods. Two opposing rods form a pair and are electronically connected. To generate an electrodynamic field a direct current (DC) and a radiofrequency (RF) are applied to each pair. While a negative DC is applied to one pair of rods, a positive DC voltage is applied to the other. This causes a shift in RF phase by 180 degrees between the two pairs. The “magnitude of the electrodynamic field” is determined by its RF and DC voltage. Only ions of a certain m/z value or a specific m/z range will resonate with any given combination of DC and RF voltages and thus have a stable trajectory within the magnetic field to pass through to the detector. Ions, whose m/z value does not resonate with the electrodymanic field will hit the rods, be neutralized and eventually removed from the mass analyzer. Mass analyzers can be run in one of two modes: 1. Scan Mode (SM) and 2. Selective Ion Monitoring Mode (SIM). In Scan Mode, both, the applied DC and RF voltage are changed in rapid succession to sequentially identify the constituent ions of a mixture of analytes with varying m/z ratios. Additionally, quadrupole systems can rapidly switch polarity and thereby monitor various molecular ions of different polarity with each scan. In SIM mode, the electrokinetic field is
preset to only allow ions of a preselected m/z ratio through to the detector. This method is used to look for specific analytes of interest within a sample with very high sensitivity [55, 69, 70].

Tandem Mass Spectrometry (MS/MS)

An evolution of MS is Tandem Mass Spectrometry, abbreviated as MS/MS, which provides much higher analytical specificity compared to single MS. In MS/MS two separated mass analyzers are set up in tandem to perform two independent consecutive mass analyzer steps back-to-back. The triple quadrupole system is a common configuration of Tandem Mass Spectrometry (Figure 10). Herein, a total of three quadrupoles are coupled serially, two of which function as a mass analyzer and one as a collision cell. The first quadrupole denoted as Q1 is a mass analyzer which moves precursor ions of a specific m/z range or value towards the second quadrupole, Q2, which functions as a collision cell and not as a mass analyzer. In this intermediate region of the tandem mass spectrometer, preselected ions from Q1 are submitted to an inert collision gas which facilitates collision induced dissociation, or fragmentation, of the precursor or parent ion into their respective fragments, or daughter ions, which then move on to the third quadrupole, Q3. This second mass analyzer, like Q1, is set to filter out product (or daughter) ions of a specific m/z so that they can progress to the detector. The high analytical specificity of MS/MS stems from its ability distinguish analytes of similar retention time and m/z ratio by their fragmentation pattern. Fragmentation patterns are
very unique to individual compounds and highly reproducible thus adding an additional dimension to accurate ion identification.

Similar to single MS, tandem MS can be run in different modes. In selective reaction monitoring (SRM) Q1 and Q2 are set to scan for very specific parent to daughter ion fragments, whereas in multiple reaction monitoring Q1 filters out a specific parent ion while Q3 is set to scan for multiple product ions generated from the fragmentation of its respective parent ion in Q2 [70, 71]. Mass spectrometry is a popular tool in metabolic research due to its high sensitivity, high reproducibility and relatively fast sample processing time. However, sample analysis can only be performed on in vitro and ex vivo specimens, making it an invasive method and the sample liable to post-mortem
degradation if not handled and processed properly. Also, even though Mass spectrometry can be used to identify isotopologues of different metabolites, it cannot provide position specific (isotopomeric) label information.

Mass Isotopomer Multi-Ordinate Spectral Analysis (MIMOSA)[71]

As mentioned above, one of the major shortcomings of Mass Spectrometry compared to Nuclear Magnetic Resonance Spectroscopy is its inability to obtain position specific (isotopomeric) labeling information of metabolites. This limitation was partly overcome by the development of Mass Isotopomer Multi-Ordinate Spectral Analysis (MIMOSA) which expands MS/MS analysis beyond mere measurement of isotopologues. MIMOSA is a flux analysis platform that allows for stepwise computation of key metabolic flux rates. The “mass isotopomer” aspect of MIMOSA utilizes the unique precursor to product ion relationships in MS/MS-based ion fragmentation to identify carbon-specific position of label. The “multi-ordinate” aspect directly interprets the kinetic transfer of isotope label as it traverses a metabolic pathway by grouping measurable metabolites, such as citrate, glutamate and succinate, into isotopomer families according to the metabolic reaction they originated from (atom fate map). The stoichiometric relationship between those isotopomer families can then be solved algebraically and the isotopomeric information deduced [71]. A full description of MIMOSA is exemplified in CHAPTER III.

A comparison of individual strengths and weaknesses of NMR and Mass is shown in Table (2)
Current controversies in stable isotope tracing

Despite being a staple of metabolic research for nearly three and a half decades, stable isotope tracer methods come with their own liabilities and pitfalls. Three commonly used tracer methods employed to measure and quantify hepatic metabolism in vivo, $^{13}$C-labeled acetate, lactate, and propionate, have led to highly divergent concepts of basal hepatic metabolism and have raised concerns about the validity of the data generated using these tracer methods [72-76]. For instance, depending on the design of the study, up to a 5-fold difference has been reported in the literature for key basal metabolic fluxes.
including pyruvate cycling ($V_{\text{PEPCK}}$ and $V_{\text{PK}}$) and anaplerosis ($V_{\text{PC}}$). These differing measurements of basal metabolic state have made it impossible to pinpoint the exact targetable aberrations of hepatic mitochondrial metabolism that play a role in the development and progression of T2DM, IR and NAFLD [4, 29, 41, 75, 77-79].

The reason for these stark differences in metabolic rates has to do with the complexity and interrelatedness of metabolism. Due to which metabolic flux analyses require simplifications derived from multiple assumptions if any conclusions are to be made about a metabolic system. The degree to which these assumptions are accurate determines how reliably a metabolic modeling platform can compute metabolic fluxes. Such assumptions may have been measured or may have been made a priori based on in silico simulations, educated guesses and mathematical inferences. Oftentimes, assumptions remain subjective since they have not been experimentally validated with direct measurements and hence they are susceptible to compounding and analytical errors [74, 75, 77]. Consequently, calculated metabolic rates are highly platform and model dependent and can vary significantly [80].
In general, the debate regarding the validity of tracer studies surrounds the choice of tracer, its primary tissue of uptake and oxidation, as well as possible zonation of its metabolism within the target tissue and its possible impact on metabolism. Interpretation and analysis of stable isotope tracer studies are performed under the assumption that a tracer, regardless of its route of administration, does not alter metabolism, is predominantly and homogenously taken up and metabolized (oxidized) by the primary target tissue (liver) without confounding label contribution from secondary tracers. Table (3) shows a list of common pitfalls associated with tracer studies which are explained in more detail in the section below.

<table>
<thead>
<tr>
<th>Common Pitfalls of Tracer Studies</th>
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<tbody>
<tr>
<td>1. Tracer modulates metabolism (Tracer Assumption)</td>
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<td>2. Failure to reach steady-state, yet using steady state assumption for metabolic modeling</td>
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<tr>
<td>3. Insensitivity to positional enrichment</td>
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<td>4. Ambiguity in isotopomers</td>
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<td>5. Reversible metabolic exchange reactions</td>
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<td>6. Absent or distant relationship between measured precursor and product enrichments</td>
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<tr>
<td>7. Post-isolation/post-mortem analyte degradation</td>
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<tr>
<td>8. Insufficient signal-to-noise</td>
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<tr>
<td>9. Metabolic compartmentation and zonation</td>
</tr>
<tr>
<td>10. Label dilution</td>
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<tr>
<td>11. Incorrect natural abundance and background correction</td>
</tr>
<tr>
<td>12. The requirement for high level technological and analytical expertise</td>
</tr>
</tbody>
</table>

*Table 3. Potential shortcomings and pitfalls of in vivo tracer experiments. [2, 5, 14-19]*
Tracer Assumption

By definition a tracer is expected to trace a metabolic system without altering it. However, when a tracer is infused close to its natural turnover rate, the tracer can become a substrate for the metabolic reactions it is supposed to track, such that by providing mass to the system, it can impact metabolism. Violation of this tracer assumption, therefore, can generate widely spurious results. This was clearly demonstrated for glycerol as a tracer of hepatic gluconeogenesis [91] where endogenous glucose production rates increased linearly with the rate of glycerol infusion was increased. A similar concern has arisen in human and rodent studies where $^{13}$C-labeled propionate was used to assess hepatic metabolism but demonstrated substantial modification of, hepatic pyruvate cycling, anaplerotic fluxes and mitochondrial metabolism [4, 29, 77, 92, 93]. Likewise, concerns about $^{13}$C-acetate as a tracer method have been raised. For instance, metabolic models based on labeled acetate use acetate flux ($V_{AC}$) to calculate Krebs cycle flux ($V_{TCA}$). However, acetate infusion contributed up to 25% to overall acetate flux and thus artificially increased $V_{TCA}$ which in turn led to an erroneously low relative anaplerotic flux ($V_{ANA}/V_{TCA}$) which was considered an “investigator- or tracer-perturbed” flux calculations[29, 74].

Steady State Assumption

Many in vivo studies are designed to determine metabolic fluxes at steady state. These calculations assume that metabolite concentrations are constant and isotope enrichment has reached a plateau across all key metabolites by the time samples are taken. At steady state, mass and isotope balance equations can be derived and solved from the fractional enrichment data for metabolic flux analysis. If a system is using the steady state
assumptions but is not at or near steady state the metabolic flux analysis will generate erroneous flux rates. Therefore it is essential to experimentally confirm steady state in such cases [76].

Reversible metabolic exchange reactions and insensitivity to positional enrichment

Exchange reactions catalyze the reversible interconversion of amino and keto acids without any net carbon loss or gain. Thus, as part of reversible systems exchange reaction can lead to significant isotopic label dilution within a metabolic pool without contributing any mass to a metabolic network. When not accounted for in metabolic flux analysis label dilution from exchange reaction can be mistaken for a net metabolic flux and thereby overestimate metabolic rates. For example, in vitro studies demonstrated that the dilution of aKG by the exchange with its reciprocal unlabeled glutamate pool led to subsequent dilution of citrate through a diluting exchange catalyzed by IDH. When using isotopologue information only in metabolic flux analysis this dilutional exchange is missed and leads to an overestimation of reverse carboxylation reaction of IDHr flux [71]. Therefore it is necessary to obtain position specific labeling information of citrate to measure its dilutional exchange from aKG directly and correct for it when calculating flux rates of IDH based on fractional enrichments. Unfortunately, studies that cannot the position specific labeling information of Krebs cycle intermediates, such as citrate, can easily miss these fine but important nuances, and hence are liable to spurious flux analysis.
Post-isolation and post-mortem analyte degradation/metabolism

As an invasive method Mass Spectrometry cannot measure metabolites in vivo and therefore requires ex vivo tissue samples for analysis following an in vivo tracer study. However, tissues and their volatile, low abundant metabolites, such as acetyl CoA, OAA, etc, are highly sensitive to postmortem changes and cell degradation if not processed rapidly and efficiently. Therefore, ex vivo tissue and plasma samples require rapid in situ freeze clamping with aluminum tongs pre-chilled in liquid nitrogen prior to further analyses to avoid spuriously low measurements of enrichments and concentrations these already low abundant and labile metabolites. For example, studies have shown that hepatic acetyl-CoA degrades within less than 30 s after collecting liver tissue [81]. Therefore extreme diligence need to go into post study tissue isolation to avoid postmortem degradation and spurious flux calculations.

Fast Vx Assumption

Another longstanding concern/debate[94-98] pertains to the amino transferase exchange rate (Vx) which underlies an assumption used for NMR-based in vivo metabolic flux calculations. 13C-NMR cannot measure Krebs cycle intermediates directly in vivo because of their low concentrations. Instead, 13C-NMR can detect the strong 13C signal of aspartate and glutamate. These amino acids are assumed to be in rapid exchange with Krebs cycle intermediates, OAA and αKG, respectively. Due to their higher signal they can act as NMR-observable trapping pools for 13C label in Krebs cycle intermediates if and only if the exchange rate of these pools is fast relative to the Krebs cycle flux. A common
assumption in modeling oxidative metabolism, then, is that Vx is very rapid exchange of isotope label between the mitochondrial and cytosolic metabolite pools and thereby makes glutamate and aspartate labeling a reliable readout of αKG and OAA, respectively. If Vx exchange is actually slow it would result in marked errors in $V_{TCA}$ and other related rates [94, 97, 99, 100].

### Homogeneity of tracer uptake

*In vivo* tracer studies generally assume that there is uniform utilization of a tracer by all cells in a tissue such that an enrichment of the tissue as a whole is the same as the individual cells. Metabolic zonation pertains to the preferential uptake of a metabolite by one cell type versus another across a tissue bed. For instance, some tracers are of sufficiently low concentration and are taken up so avidly by the liver, that the cells on the periportal side have much higher exposure to tracer than those on the perivenous side. This results in different enrichments across the hepatic bed that are ‘averaged’ by tissue homogenization or spectral analysis. Propionate, glycerol and acetate are all subject to metabolic zonation [73, 101-104]. In the case of propionate, periportal hepatocytes were shown to take up significantly more tracer than perivenous hepatocytes [101-103]. The heterogeneity of tracer uptake is particularly problematic when flux estimates are based on the assumption that a tracer distributes its label evenly across all hepatocytes. By averaging out high and low labeling signal overall Krebs cycle activity will not only underestimate metabolic flux analysis but it will also be significantly skewed when any changes in zonation occur [73, 101, 102, 104]. Another source of a skewed label signal
has been reported for acetate as a tracer. $[^{13}\text{C}]$acetate undergoes significant dilution in the splanchnic region before entering the hepatic Krebs cycle, resulting in a heterogeneous tracer pool being taken up into liver metabolism. This has reportedly led to lower enrichments of Krebs cycle intermediates and therefore to an underestimation of Krebs cycle flux [105, 106].

**Dilutional influx into the Krebs cycle**

At metabolic and isotopic steady state, the relative contribution of a labeled precursor to product is determined by the ratio of their $^{13}$C enrichment ratios. When all of the substrate is converted into its product the ratio should be 1. However, at intersecting pathways when more than one substrate contributes to the product, the entry of carbon from an unlabeled source will dilute (reduce) the precursor to product enrichment ratio proportionately to its relative contribution to the overall product formation. Therefore this precursor to product ratio is particularly helpful for determining if significant dilutional fluxes, e.g., anaplerosis, or exchange reactions, at metabolic intersections exist. Moreover, knowledge about the dilution of Krebs cycle intermediates is crucial for accurate metabolic flux calculation as it directly impacts metabolic flux analysis and can lead to significant errors in flux calculations when not properly factored into the metabolic model. For instance, due to the restricted information about individual Krebs cycle fluxes that can be gleaned from the labeling pattern of NMR observable aspartate and glutamate pool, dilutional fluxes into the Krebs cycle from anaplerotic sources other than PC or exchange reactions have not been measured before and rather assumed to be negligible. Yet, recent *in vitro* studies of Krebs cycle metabolism have shown reported a significant
label dilution of Krebs cycle intermediates coming from anaplerotic glutamate influx as well as from exchange reactions between the Krebs cycle intermediate αKG and the intracellular glutamate pool [71]. Consequently, omitting dilution of mitochondrial metabolite enrichments has a significant impact on critical Krebs cycle rates, inasmuch that it will lead to an underestimation of overall Krebs cycle rate which in turn will result in an overestimation of reductive carboxylation, PC mediated anaplerosis and substrate cycling relative to overall Krebs cycle flux.

**Label recycling, secondary tracers and isotopomer/isotopologue ambiguity**

When a tracer, such as $[^{13}\text{C}]$acetate, is infused systemically, it not only enters the primary tissue of interest, such as liver, but it will also be taken up by peripheral, extrahepatic organs, e.g. muscle, where its label can be metabolized and recycled to generate a secondary tracer, such as $[^{13}\text{C}]$glutamine. The labeled glutamine from muscle can then enter the systemic circulation and introduce $^{13}\text{C}$ label into the hepatic Krebs cycle. As a consequence, the intramitochondrial $[^{13}\text{C}]$ glutamate/glutamine pool no longer reflects just the label originating from $[^{13}\text{C}]$acetate but it is a mixed pool of intra- and extrahepatic labeling distribution [74]. Thereby leading to overlapping, ambiguous labeling patterns of key hepatic metabolites confounding metabolic flux analyses (Figure 11). Similar to label recycling resulting in mixed pools of primary and secondary tracers, another concern pertaining to tracer studies are ambiguous, non-distinct labeling patterns. The concern raised about $[1-^{13}\text{C}]$-acetate was its inability to generate a unique enough labeling pattern to properly assess anaplerosis *in vivo* when using the ratio of $[1-^{13}\text{C}]$- to $[5-^{13}\text{C}]$-labeled
glutamate. For example, [1-\textsuperscript{13}C]\textsuperscript{}acetate generates [5-\textsuperscript{13}C]\textsuperscript{}glutamate with each turn of the Krebs cycle and the resultant enrichment remains constant at steady state. However, [1-\textsuperscript{13}C]-glutamate on the other hand is not equally generated to C5 labeled glutamate as it needs an additional turn of the Krebs cycle to be made. Moreover, \textsuperscript{13}C label in C1 position of glutamate is liable to dilution from anaplerotic PC flux at the level of OAA. Thereby, erroneously underestimating anaplerosis \textit{in vivo} [77, 107]. Another examples is [1,2,3-\textsuperscript{13}C\textsubscript{3}]lactate. This tracer has been speculated to not fully undergo randomization of label across fumarase, resulting in lower than expected mass isotopomer distribution in glucose and hence in underestimation of relative pyruvate cycling and in overestimation of gluconeogenesis [107]. Table (4) shows a list of primary tracers and their assumed secondary tracers.

<table>
<thead>
<tr>
<th>Primary tracer</th>
<th>Secondary Tracer (Tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textsuperscript{13}Cacetate</td>
<td>\textsuperscript{13}C\textsuperscript{}glutamine (muscle)</td>
</tr>
<tr>
<td>\textsuperscript{13}Cglucose</td>
<td>\textsuperscript{13}C\textsuperscript{}lactate, \textsuperscript{13}C CO\textsubscript{2} (liver, muscle)</td>
</tr>
<tr>
<td>\textsuperscript{13}C\textsuperscript{}lactate</td>
<td>\textsuperscript{13}C CO\textsubscript{2}, \textsuperscript{13}C\textsuperscript{}glucose (liver)</td>
</tr>
<tr>
<td>\textsuperscript{13}Cpropionate</td>
<td>\textsuperscript{13}C\textsuperscript{}glucose (kidney)</td>
</tr>
</tbody>
</table>

Table 4: Primary tracers and their potential secondary tracers and tissue of origin.
Incorrect natural abundance and background correction

Since stable isotopes of exist naturally, any metabolite has a certain probability of carrying one or more stable isotopes in its molecular structure. The heavier isotopes of some atoms occur at a significant abundance. For instance, in the case of carbon atoms, 1.11% of all carbons are $^{13}$C with the remainder being $^{12}$C [53, 55]. Therefore, for a metabolite with $n$ number of carbons, there is a $n \times 1.1\%$ chance of carrying a naturally occurring $^{13}$C label in its carbon backbone, with $2^n$ possible patterns of positional $^{13}$C distribution across
the entire (asymmetrical) molecule. For example, a six carbon molecule has a probability of 6.66% of having any one of its $^{12}\text{C}$ carbons naturally being replaced by a $^{13}\text{C}$.

Since metabolic flux analysis is made based on the relative distribution of isotopic enrichment from an exogenous tracer to subsequent metabolites throughout a metabolic pathway [55, 66] the naturally occurring label needs to be taken into consideration and corrected for. Otherwise, the non-negligible contribution of NA of $^{13}\text{C}$ will lead to spurious quantitative and qualitative flux estimates of metabolic networks [82-84]. To account for naturally occurring label, natural abundance (NA) correction need to precede any APE% based flux calculations. Many different approaches have been developed to account for NA, such as computation of NA contribution to labeled samples by measuring unlabeled standards to mathematical NA correction matrices and binomial and multinomial probability calculations [82]. Regardless of how the NA is computed, it is ultimately subtracted from the overall measured enrichment of each atom [55, 85, 86]. However, there are many factors that can impact the accuracy of NA corrections techniques, such as tracer impurity, imperfect mass spectra with missing peaks and poor signal to noise intensity, or simply not considering that once an atom is labeled by a tracer it can no longer carry a natural abundant stable isotope. Especially, in the two latter cases simple correction for natural abundance by subtraction can lead to overcorrecting and thereby to negative enrichment data [85, 87]. In addition to naturally occurring isotopes, background noise in the analyzed sample can confound and artificially raise isotope signal. Therefore it also needs to be accounted for. To adjust for both, NA and background noise, a common practice is to use a baseline background (control) sample without any tracer infusion for background correction and then perform NA correction on the background corrected
samples by correcting the total tracer enrichment by the natural abundance of the respective atom. Of note, this approach does not take into account changes in the metabolic system during which the background enrichment might change. Therefore, this approach is inadequate for studies in which the system under investigation undergoes major changes, e.g. during drug intervention, clamp studies or with different diets. In those cases, control samples that undergo the same systemic change without tracer infusion need to be obtained simultaneously with the tracer study. Incorrect attribution of label incorporation into a metabolite either from omitting to correct for background and NA or from overly correcting for NA will lead to biased qualitative and quantitative flux analysis [82, 83, 85, 88-90].

Expertise and technological limitations

Both, NMR and MS analysis require high level of expertise for day to day operation, proper calibration, adjustment of instrumentation settings are vital for accurate analytical measurements and troubleshooting. Otherwise, false peak detection and assignment can lead to misinterpretation of data and thus to spurious flux analysis. Technical limitations of NMR for in vivo studies stem from low sensitivity of $^{13}$C-NMR and overlapping $^1$H-NMR spectra. For example, in $^1$H-NMR highly abundant molecules, such as water or lipids, can overlap and obscure important peaks of lower abundant metabolites making exact spectral assignment difficult [60-65]. On the other hand, $^{13}$C-NMR suffers from limited sensitivity due to the low natural abundance of $^{13}$C (1.1%). Therefore, metabolites must be highly concentrated to detect a measurable signal and to overcome the low signal to noise ratio of $^{13}$C-NMR [66]. Thus necessitating metabolite concentrations in the millimolar
range [55, 65]. Some of these limitations have been overcome by higher localization
genuity and improved sensitivity of POCE. However, its overall application is still limited
_in vivo_ by low spectral resolution [65, 99]. MS spectrometry is more sensitive than $^{13}$C-
NMR, but especially single MS cannot provide position specific label information. This
limitation for MS can be -at least in part- be overcome by MS/MS. Through strategic
fragmentation and the resultant parent daughter fragment relationships of specific
metabolites isotopomeric information can be deduced. However, thus far, many of these
techniques have been restricted to major centers and researchers with expertise in the
area of metabolic studies.

To summarize, misrepresentation of metabolic flux can originate from many different
potential flaws in tracer study design, experimentation and analysis. Each of the here
proposed shortcomings and potential pitfalls of stable isotope tracer studies, individually
or in any combination, can result -or have resulted- in biased flux analysis and thus in
spurious flux calculations. Therefore, every tracer method warrants careful validation and
diligence in data processing and analysis to establish a “ground truth” of hepatic
metabolism [74, 75, 77, 94-96, 100, 108, 109].
CHAPTER II: SYSTEMATIC DEVELOPMENT AND VALIDATION
OF GLUTAMINE AS A TRACER OF HEPATIC METABOLISM

Development of $[1,2^{13}C_2]$-L-Glutamine for \textit{in vivo} metabolic flux analysis

Many considerations go into the design of an \textit{in vivo} tracer study for metabolic flux calculations. A step-wise process was developed to validate $[1,2^{13}C_2]$-L-glutamine as a tracer. We systematically and empirically assessed potential fatal pitfalls of glutamine as tracer that could significantly bias the flux analysis including: 1) Identification of an infusion rate that does not alter metabolism, 2) Definition of input functions for calculations and screening for complex secondary labeling patterns, 3) Confirmation of isotopic steady state, 4) identification of relevant isotopologues and 5) kinetic and steady-state mass isotopomer analysis of citrate, glutamate, succinate.

Identification of an infusion rate that does not alter metabolism

By definition, a tracer should trace metabolism without altering it (the ‘tracer assumption’). Hence, the tracer infusion should not significantly impact downstream chemical reactions or the constituent tracee pool of the metabolic system that is being assessed [74]. An ideal tracer rate is one that not only leaves metabolic homeostasis unperturbed but still provides sufficiently detectable label signal across all key metabolites necessary for metabolic flux analysis (MFA) by MS or NMR [53, 55, 110]. Commonly a tracer infusion rate is chosen that is a fraction of the endogenous natural turnover rate of its tracee. At metabolic and isotopic steady state, the turnover rate is a balance between the rate of appearance and rate of removal of a tracee into and from its designated tracee pool, e.g.,
glucose in plasma. [53, 55, 110]. Tracer infusion rates can be optimized by first determining the endogenous turnover rate and then empirically testing different infusion rates while monitoring the system for changes in metabolic homeostasis, e.g., metabolite concentrations, hormone secretion, etc. In this manner, the requirement for adequate signal can be maximized while metabolic impact is minimized.

First, we empirically determined the tracer infusion rates that maximized glutamine enrichment but minimized the impact on systemic metabolism. Basal glutamine turnover rates were calculated from primed continuous intravenous infusion of $[1,2^{13}\text{C}_2]$-L-glutamine in overnight fasted, male Sprague Dawley rats (Figure 12).

![Figure 12. Determining endogenous glutamine turnover.](image-url)
To identify an infusion rate for L-glutamine that minimally perturbed glucose homeostasis, we infused “cold” (unlabeled) L-glutamine at three increasing rates, that is, 3-, 6-, and 12-\(\mu\)mol/kg/min in 30 minute increments along with a primed continuous infusion [6,6-\(^2\)H\(_2\)]-glucose to trace the endogenous glucose turnover. The glutamine infusion was started during and metabolic isotopic steady state of the glucose tracer. We measured plasma glucose, glycerol and insulin concentrations before and after each 30 mins glutamine infusion increment (Figure 13).

Figure 13. Identifying a tracer infusion rate for glutamine.
To identify an infusion rate for L-glutamine that does not perturb glucose homeostasis we infused “cold” (unlabeled) L-glutamine at three increasing rates, that is, 3-, 6-, and 12-\(\mu\)mol/kg/min along with [6,6-\(^2\)H\(_2\)]-glucose to trace endogenous glucose turnover and measured plasma glucose, glycerol and insulin concentrations before and after each 30 mins glutamine infusion increment.
At 3 µmol/kg/min of tracer, the calculated glutamine rate of appearance (Ra) was 28±2.5 µmol/kg/min which was in keeping with prior reports \[111, 112\] (Figure 14). Increasing the infusion rate as high as 12 µmol/kg/min (40% of the endogenous rate) did not impact the glutamine Ra (30±1.4 µmol/kg/min) but roughly doubled the plasma and hepatic glutamine concentration (Figure 15 D, E). The 12 µmol/kg/min infusion also did not change the steady state concentration of other key metabolites. Importantly, glutamine infusion did not perturb the cytosolic redox state ([NAD+]/[NADH] ratio), which can be assessed by the pyruvate to lactate ratio because of the rapid equilibrium across LDH (Figure 15 F)[113]. In a separate experiment, a primed-continuous infusion of [6,6 \(^2\)H\(_2\)]-D-glucose (for measurement of glucose Ra) was followed by the concomitant step-wise infusion of unlabeled glutamine vs. ½ NS as a control at the same three rates[55, 110, 114, 115]. Importantly, endogenous glucose Ra as well as plasma glucose and insulin concentrations were unchanged compared to control (Figure 15 A-C). Together these data demonstrate that, as a tracer, glutamine infusion as high as 40% of the endogenous glutamine Ra, does not significantly change whole body glucose metabolism or redox.

![Glutamine Turnover](image-url)
Glutamine infusion rates do not have significant impact on (A) plasma glucose concentration (B) Endogenous glucose production (C) Plasma insulin concentration (D) Plasma metabolites (E) Liver metabolites (F) Plasma RedOx state. Even at an infusion of 12μmol/kg/min all metabolites remained at steady state. N=6-8

Figure 15. Developing glutamine as a tracer.
Defining input functions and screening for complex secondary labeling patterns

At steady state, systemic infusion of a labeled tracer, such as glutamine, does not interact with any singular tissue but could enter into the metabolic pathways of any tissue capable of taking it up. Any tissue that takes up the metabolite then has the possibility to generate other $^{13}$C-labeled products that can reenter the circulation. These ‘secondary tracers’ have the potential impact metabolite labeling patterns within the tissue of interest. To investigate if infused $^{13}$C label from glutamine was transferred to other metabolites we assessed for high likelihood secondary tracers in lactate, pyruvate, lactate, glycerol, alanine, bicarbonate and short chain fatty acids (SCFA). Importantly, because all of these metabolites have high turnover rates, they could potentially circulate to other tissues and be incorporated in metabolic processes that might confound metabolic analysis. As an extreme case, $[1,2^{-13}C_2]$glutamine was continuously infused for 4 hours at 12 µmol/kg/min and then plasma, liver, kidney, muscle and WAT (freeze clamped *in situ*) were collected and screened for label distribution (Figure 16).

*Figure 16. Screening for label recycling.*
Figure 17a: POCE Spectrum of liver: Top trace: Total spectrum and Bottom Trace: difference spectrum from liver following 4 hours of labeled glutamine infusion.

Figure 17b: Detail of POCE lactate C3 difference spectrum. A small enrichment was seen in C3 lactate.

Figure 17c: Detail of POCE glutamine/glutamate C3 and C4 difference spectrum. There was no discernable enrichment in glutamine/glutamate C3 and C4 above background, natural abundance and overlapping spectra.

Figure 17d: Detail of POCE glutamine/glutamate C2 difference spectrum. Enrichment of C2 of glutamine and glutamate.

Figure 17. POCE Spectrum of liver.
In the tissue and plasma extracts, heteronuclear Proton Observed Carbon Edited (POCE) $^{13}$C/$^1$H NMR acquisitions were performed for unbiased detection of significant secondary tracers developed during the 4h infusion (Figure 17a-e) and Table 5). As expected, a large NMR doublet was observed for glutamine C2 (Figure) as well as singlets for 3,4 glucose but no other significant secondary labeling was observed with the exception of an unexpected enrichment of lactate and alanine in the C3 resonance that was confirmed by LC/MS. GC-MS did not identify other unexpected gluconeogenic substrates including short chain fatty acids and glycerol (Figure 18). This indicates, that in addition to [1,2-$^{13}$C$_2$] glutamine,
[\textsuperscript{3-\textsuperscript{13}C}] lactate must be considered in the model. The spectra for glutamine/glutamate C3 and C4 showed no discernable enrichment with heavy overlap from other metabolites, such as β-hydroxy butyrate.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>\textsuperscript{13}C Label Position</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>C3</td>
<td>Liver</td>
</tr>
<tr>
<td>Lactate</td>
<td>C3</td>
<td>Liver</td>
</tr>
<tr>
<td>Glu/Gln</td>
<td>C2</td>
<td>Liver, Plasma</td>
</tr>
<tr>
<td>Glycerol</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Propionate</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>SCFA</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>BOHB</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>

Table 5. Label distribution.
Label distribution after a 4 hour infusion of [1,2-\textsuperscript{13}C\textsubscript{2}]-L-glutamine. No complex secondary labeling patterns were generated by [1,2 \textsuperscript{13}C\textsubscript{2}]-L-glutamine. Glu/Gln = glutamate, glutamine; SCFA = short chain fatty acids; BOHB = beta hydroxy butyrate.
Determination of isotopic steady state

One of the goals of *in vivo* kinetic studies is to infuse a tracer until a constant isotope enrichment is reached. This is also referred to as plateau enrichment (Ep), isotopic equilibrium or isotopic steady state and is crucial for metabolic modeling. At steady state net flux is zero, meaning that all influxes equal all effluxes and thus the pool sizes are constant. At isotopic and metabolic steady state mass balance and isotope balance equations can be solved for metabolic flux analysis (MFA) and the *in vivo* kinetics, such as turnover rates, of metabolites can be assessed (Figure 19).

*Figure 19. Steady States.*
Panels show infusion (F) of $^{13}$C label (tracer) into a $^{12}$C metabolite pool (tracee). The overall concentration of metabolites does not change with the tracer infusion. At steady state the rate of appearance (Ra) equals the rate of disappearance (Rd) and a plateau enrichment (Ep) of isotope label is achieved within the metabolite pool. During a primed continuous infusion the Ep approximates a square wave function. Left Panel: A shows the metabolic pool before and during the primed continuous tracer infusion D-F. A: no tracer infusion; B-C: primed bolus; D-E: continuous infusion. Metabolic Steady State: A-E; Isotopic Steady State (Ep): E. Right Panel shows the measurement of metabolite concentrations before (A) and during tracer infusion (B-D) and at steady state (D).
After confirming that at a glutamine infusion rate of 12 μmol/kg/min plasma and liver metabolite concentration did not change (we administered [1,2-\(^{13}\)C\(_2\)]-L-glutamine as a primed continuous infusion to ensure detectable tracer signal within key metabolites and to characterize their plasma and tissue isotope build up curves and plateau enrichments. The ability to track evolution of fractional enrichments of plasma metabolites during a tracer infusion by serial blood draws is relatively straightforward. In order to assess temporal build up of label in liver metabolites, hepatic tissue was isolated from timed cohorts of different animals (Figure 20).

![Diagram](image)

*Figure 20. Determining isotopic build up curves and steady states for [1,2-\(^{13}\)C\(_2\)] L-glutamine.*

Plasma samples following the primed-continuous infusion of [1,2-\(^{13}\)C\(_2\)]-L-glutamine displayed an approximately square wave pattern for M+2 Glutamine that was at steady state by 15 minutes with an enrichment of 25%. This plateau enrichment remained stable for the duration of the 2h experiment (Figure 21 top panel).
Plasma and hepatic Glutamine enrichments were fully equilibrated within minutes of

Figure 21.Isotopic steady state.

[1,2 $^{13}$C$_2$]-L-Glutamine label reaches steady state within less than 60 mins across all metabolites in plasma (top) and liver (bottom). Glutamine was infused at a primed continuous rate of 12 umol/kg/min. N=6-8.

Plasma and hepatic Glutamine enrichments were fully equilibrated within minutes of
starting the infusion (Figure 21). Glucose was a dominant secondary metabolite and its M+1 isotopologue exponentially increased to a plateau within one hour to a value of 2%. No significant M+2 glucose was detected. M+1 pyruvate, lactate, alanine, and glutamate all trailed the glucose enrichments at a lower percentage. Similarly, extra- and intramitochondrial metabolites/intermediates downstream from glutamine reached their stable fractional enrichment plateaus within less than 60 mins of the glutamine tracer infusion (Figure 21). The fast equilibration of $^{13}$C label with whole body Krebs cycle metabolism was indicative of a prompt succession of consecutive reactions where isotope label traversed the entire Krebs cycle to reach equilibrium rapidly.

Identification of relevant isotopologues

The M+2 glutamine tracer is deaminated to M+2 glutamate that enters the Krebs Cycle as M+2 αKG via the GDH reaction. Doubly labeled αKG was then decarboxylated to M+1 succinate via OGDH and further metabolized to M+1 malate, citrate, and αKG. Then, through aminotransferase exchange M+1 αKG produced M+1 glutamate. Any remaining $^{13}$C label that had traversed the oxidative Krebs cycle was then lost to CO$_2$ during the decarboxylation reaction of OGDH. Thus, after one full traverse of the Krebs cycle $^{13}$C-label from doubly labeled glutamine did not contribute to subsequent turns past αKG. The other fate for M+2 αKG was reductive carboxylation to M+2 citrate catalyzed by IDHr. Next, M+2 citrate is metabolized to M+0 acetyl CoA and M+2 malate by the ACLy reaction. No higher isotopologue species, such as M+3 citrate or malate were observed. Table 6 shows the complete list of measured isotopologues.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Isotopologue</th>
<th>APE% SEM (+/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td>M1</td>
<td>1.12 (0.13)</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>25.18 (1)</td>
</tr>
<tr>
<td>Glutamate</td>
<td>M1</td>
<td>2.32 (0.28)</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>6.76 (0.63)</td>
</tr>
<tr>
<td>Succinate</td>
<td>M1</td>
<td>5.24 (0.46)</td>
</tr>
<tr>
<td>Malate</td>
<td>M1</td>
<td>2.95 (0.19)</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>0.24 (0.05)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>M1</td>
<td>2.6 (0.36)</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>0.13 (0.07)</td>
</tr>
<tr>
<td>Citrate</td>
<td>M1</td>
<td>2.2 (0.24)</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>3.13 (0.45)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>M1</td>
<td>0.58 (0.11)</td>
</tr>
<tr>
<td>PEP</td>
<td>M1</td>
<td>1.15 (0.56)</td>
</tr>
<tr>
<td>Lactate</td>
<td>M1</td>
<td>0.48 (0.09)</td>
</tr>
</tbody>
</table>

*Table 6. Steady state enrichments.*

Steady State enrichments (APE%) of all LC/MS observed isotopologues. Data are the mean (SEM) of n=8
Mass isotopomer analysis of citrate, glutamate, and succinate

Some mitochondrial metabolites, such as OAA, succinyl CoA and acetyl CoA are difficult to measure directly due to various reasons, including their overall low abundance, compartmentalization (cytosol vs mitochondrial matrix), as well as analytical difficulties stemming from high volatility, instability and fast degradation during processing or complex mass spectrometry fragmentation patterns [71, 116]. Others can be measured but because they are in rapid equilibrium (e.g., oxaloacetate<->malate<->fumarate, citrate<->aconitate<->isocitrate), they provide limited additional information. Due to the resultant distinct $^{13}$C labeling pattern of Krebs cycle intermediates at steady state, key intermediates can be grouped into unique isotopologue and isotopomer families based on their precursor/product fragment relationships as described by Alves et al. using the MIMOSA strategy. Figure (22) shows the complete initial theoretical fate map of all possible isotopologues and isotopomers of all key Krebs cycle and constituent metabolites.

Through stepwise elimination of superfluous isotopologue and isotopomer species along with positional deconvolvement of citrate, glutamate, and succinate the theoretical fate map could be simplified to an actual “observed” fate map (Figure 24) and the information could then be used as key inputs for specific metabolic flux analyses (CHAPTER III).
Figure 22. Theoretical carbon fate map.
Theoretical carbon fate map expected from $[1,2,^{13}C_2]$glutamine tracer strategy. V = metabolic flux. Isotologues/isotopomers are grouped based on the enzymatic reaction it originated from indicated by subscript after metabolite name. ACLY = ATP citrate lyase, CS = citrate synthase, IDHr/f = isocitrate dehydrogenase (reverse/forward), OGDH = alpha ketoglutarate dehydrogenase, PC = pyruvate carboxylase, PDH = pyruvate dehydrogenase, SDH = succinate dehydrogenase.
1.1 Deconvolution of citrate

Citrate isotopologues are grouped into distinct families based on stoichiometric labeling patterns and their inherent precursor/product relationships mediated by specific enzymatic reactions. For instance, citrate isotopomers can be grouped into stoichiometric families based on their generation via CS (e.g., condensation of labeled or unlabeled OAA with labeled or unlabeled acetyl-CoA). Similarly, ligation of labeled or unlabeled αKG with labeled or unlabeled CO₂ can batch affect labeling patterns via the IDHr reaction. Citrate has a prochiral center which is recognizable by enzymes but not by the mass spectrometer. Fragmentation of a citrate parent ion can generate a pro-S and pro-R daughters. During 191/111 citrate fragmentation, carbons C1 and C5 are lost with equal probability. The probabilities citrate families occupancy can be determined from the stoichiometry of isotopologues generated from enzymatic racemization of precursors. Likewise, known probabilities of parent-daughter fragmentation patterns can be used to compute the occupancy of each isotopomer family. Table 7 shows the predicted fragmentation patterns based on the initial theoretical fate map (Figure 22). Obviously, this is too complicated to find a unique distribution of isotopologues into families. Fortunately, using information from initial NMR and MS analysis of the samples as well
Table 7. Theoretical parent daughter.
Parent daughter fragmentation patterns of citrate predicted from the theoretical fate map. IDHr: reverse Isocitrate Dehydrogenase, PDH: Pyruvate Dehydrogenase, CS: Citrate Synthase, CSPC: Citrate Synthase with label originating from Pyruvate Decarboxylase, CSACLy: Citrate Synthase with label originating from Pyruvate Decarboxylase

as following the kinetics of labeling, many of the theoretically possible families can be
eliminated to provide a much simpler distribution of family occupancies (Figure 24, at the end of this chapter).

For instance, from the absence of observed citrate isotopologues m/z >193, any families containing members with M+3, M+4, M+5 and M+6 citrate can be eliminated due to the fact that we did not measure any citrate 194 or higher m/z parent ions. Since M+2 citrate was the most enriched of the citrate isotopologues, then in the absence of measured M+3 citrate, labeled CO₂ is unlikely to interfere with rate calculations in the liver. A measured citrate 193/112 to 193/113 ratio of ~1 ruled out significant family e (which has a ratio of 1/3) that would occur from the recycling of M+2 malate from ACLy back into citrate via CS. Lack of detectable succinate M+2 also ruled out significant [3,6-¹³C₂]-citrate or significant combinations M+1 acetyl CoA with M+1 OAA. Regarding the latter, this should not be surprising since the highest predicted combination based on the measured isotopologues is less than 0.02% enriched.

From the remaining parent daughter fragments, only one M+2 family (family a) and three citrate M+1 families (families b, c and d) were observed (Table 8).
With these limited possibilities, practical information for flux determinations can be obtained from the fragment deconvolution which is discussed below.

<table>
<thead>
<tr>
<th>Citrate Family</th>
<th>192/111</th>
<th>192/112</th>
<th>193/112</th>
<th>193/113</th>
<th>194/113</th>
<th>194/114</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-IDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b-PDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-CS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-CS&lt;sub&gt;PC&lt;/sub&gt;</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 8. Observed citrate isotopologues and their respective.
Citrate Isotopomer Families grouped based on their original enzymatic reaction and eliminating other family members based on precursor/product enrichment. IDH<sub>r</sub>: reverse Isocitrate Dehydrogenase, PDH: Pyruvate Dehydrogenase, CS: Citrate Synthase, CS<sub>PC</sub> Citrate Synthase with label originating from Pyruvate Decarboxylase

Citrate M+3 to M+6

Citrate 194 or higher m/z ions were not detected and eliminated any citrate families containing these isotopologues from the carbon fate map. Therefore, $^{13}$C enrichment of the CO<sub>2</sub> pool and combinatorial labeling was negligible and did not contribute significantly to labeling patterns through IDH<sub>r</sub> or PC.

Citrate M+2

[1,2-$^{13}$C<sub>2</sub>]citrate which originates from reverse IDH. IDH<sub>r</sub> catalyzes the production of [(1,2)-$^{13}$C<sub>2</sub>]citrate from [1,2-$^{13}$C<sub>2</sub>]αKG coming from [(1,2)-$^{13}$C<sub>2</sub>]glutamate. The malate/OAA M+2 isotopomers (denoted as [(1,2)(3,4)-$^{13}$C<sub>2</sub>]OAA) originated only from citrate M+2 through the ACLy reaction. However, due to its low abundance, malate/OAA M+2 did not contribute any measurable label to citrate through CS as indicated by the 1:1 ratio of
193/112 to 193/113. An excessive deviation from this ratio would come from [(3,4)-
\textsuperscript{13}C_2]malate being converted to [(3,6)-\textsuperscript{13}C_2]citrate that would otherwise increase the pool
of citrate 193/113 relative to 193/112. Mal M+2 enrichment was slightly higher than Asp
M+2 indicating equilibrium across OAA. Citrate isotopologue family $cit_a$ was the only
observed citrate M+2 family.

\[ cit_a = 2\times193/112 = 2\times193/113 = \text{citrate M+2} \]

\textbf{Citrate M+1}

Isotopologue family $cit_c$ originates from the condensation of oxaloacetate M+1 with
unlabeled acetyl CoA through CS. Isotopologue family $cit_b$ originates from condensation
of unlabeled oxaloacetate with labeled acetyl CoA through PDH. Isotopologue family $cit_d$
originates from anaplerotic flux through PC. Using the 111 daughter, family $cit_{b+d}$ are
computationally indistinguishable using the 111 fragment and despite having different
positional probabilities collectively denoted as [(2)(3)(4)-\textsuperscript{13}C_1]citrate without assignment
of a probability to any individual position. The contribution of the $cit$ families to the 192/112
species are:

- \[ 192/112 = cit_b + \frac{3}{4} cit_c + cit_d \]

Since $cit_{b+d}$ are indistinguishable then:

- \[ cit_{b+d} = 192/112 - \frac{3}{4} cit_c \]
Isotopologue Family \( cit_c \) originates from labeled OAA generated by the oxidative Krebs cycle and unlabeled acetyl CoA and represents \([(1)(6)-^{13}C_1]\)citrate with equal probability of C1 and C6. We take advantage of the fact that only family c generates the M+1 fragment 192/111.

- \( cit_c = 4*192/111 \)

With that, the size of family \( cit_{b+d} \) can be solved by substituting \( cit_c \) with \( 4*192/111 \) since there are 3 192/112 generated for every 192/111:

- \( cit_{b+d} = 192/112 - 3*192/111 = Cit_{M+1} - 4*192/111 \)

Given the low predicted flux of \( V_{PDH} \) relative to \( V_{PC} \) in the liver, \( cit_{b+d} \) is expected to dominate the \( cit_{b+d} \) signal. Family \( cit_c \) represents the first turn of the TCA cycle and its apparent size is anticipated to be large, but it will be further impacted by reductive exchange reactions as well as any unexpected C5 label. In the liver, there was an undetectably low amount of labeled bicarbonate (see above), therefore reductive exchange across IDH will dilute (rather than augment) the M+1 enrichment with from loss of label in \([6^{13}C_1]\)citrate to bicarbonate to form citrate M+0. Since by comparing the precursor \([1,2^{13}C_2]\)glutamate enrichment relative to its unique product \([1,2^{13}C_2]\)citrate we can directly measure the amount of reverse exchange flux (defined below as \( \Phi_{Glu->Cit} \)), this information can be used to correct the size of the \([(1),(6)^{13}C_1]\)citrate pool before solving for the corrected \( cit_{c^*} \) family size.

- \( cit_{c^*} = cit_c \ast (1 + \Phi_{Glu->Cit}) \)
In contrast, any C5 label coming from PDH will over-estimate this pool size by contributing to the generation of the 192/111 parent/daughter. In this case, there will be an expansion of the M+1 pool size. Because of the overall enrichment of acetyl CoA is low (and C2 lactate/pyruvate/alanine enrichments demonstrated to be low by NMR) and the PDH is generally small relative to CS, this is expected to be small and left uncorrected in this study.

Thus, the equations for citrate devolvement used in this study are:

- \( cit_a = 2 \times \text{193/112} = 2 \times \text{193/113} = \text{citrate}_{M+2} \)
- \( cit_{b+d} = \text{cit}_{M+1} - cit_{c^*} \)
- \( cit_{c^*} = cit_c \times (1 + \varphi_{\text{Glu} \rightarrow \text{Cit}}) \)

For the purpose of calculating sequential precursor product relationships (see \( \Phi \) calculations below), when there are different numbers of carbons, it is valuable to assess the same carbons in a precursor relative to its product. For instance, citrate has 6 carbons but loses C6 following decarboxylation to aKG/glutamate. So, the relevant comparison in the oxidative direction is \([ (1)(2)(3)(4)(5) \text{ } ^{13}\text{C}_1\text{citrate} \) to glutamate\(_{M+1}\) (since glutamate only has 5 carbons). Since after correcting for reductive exchanges across IDH, for \( cit_{c^*} = C1 + C6 \), then it possible to calculate \([ (1)(2)(3)(4)(5) \text{ } ^{13}\text{C}_1\text{citrate} \) as follows:

- \( \frac{1}{2}cit_{c^*} = [1 \text{ } ^{13}\text{C}_1\text{citrate} = [6 \text{ } ^{13}\text{C}_1\text{citrate} \)
- \( cit_{b+d} = [(2),(3),(4) \text{ } ^{13}\text{C}_1\text{citrate} \)

since \([5 \text{ } ^{13}\text{C}_1\text{citrate} \) is small then:
\[
\text{[(1),(2),(3),(4),(5) } \text{^{13}C}\text{]}\text{citrate} = \text{cit}_{b+d} + \frac{1}{2}\text{cit}_c
\]

5.2 Deconvolution of Glutamate Isotopomers

If it is made by the Krebs cycle, the carbon backbone of glutamate originates from intramitochondrial OAA (C2-4) and pyruvate-derived acetyl-CoA (C4,5). During fragmentation in the mass spectrometer, the 41 Da daughter fragment specifically isolates the carbons from the acetyl CoA moiety of glutamate (Alves et al.). Any \(^{13}\text{C}\) label entry via PDH into the Krebs cycle will result in either the daughter fragment of 42 [(4)(5) \(^{13}\text{C}\)] or 43 [(4,5) \(^{13}\text{C}\)]-glutamine. On the other hand, label generated within the Krebs cycle passing through OAA will yield any of the following parent/daughter pairs: \(147/41\), \(148/41\), \(149/41\) (considering only cases where acetyl CoA is unlabeled). The M+1 glutamate species that originate within the Krebs cycle are [(1)(2)(3)-\(^{13}\text{C}\)]-L-glutamate whose label came from [(2)(3)(4)-\(^{13}\text{C}\)]OAA, and [4-\(^{13}\text{C}\)]-L-glutamate which formed from the condensation of [2-\(^{13}\text{C}\)]acetyl CoA with M+0 OAA. This acetyl CoA species originated from peripheral [3-\(^{13}\text{C}\)]lactate which was confirmed by NMR analysis. [5-\(^{13}\text{C}\)]glutamate was not observed by NMR, nor [2-\(^{13}\text{C}\)]alanine, pyruvate or lactate which ruled out that there was substantial [5-\(^{13}\text{C}\)]glutamate. Moreover, significant label in C5 of glutamate would have skewed the overall citrate 193/112 to 193/113 ratio in favor for 193/112 (but the ratio approximated unity). The glutamate parent-daughter ion pairs measured to evaluate these labeling patterns are as follows (of the ones screened only those in black were observed:

- Glutamate C4,5 isotopologues (PDH flux):
Glu\textsubscript{4,5}\textsuperscript{0} = [(4),(5) \textsuperscript{13}C\textsubscript{0}]glutamate = $\Sigma(146/41, 147/41, 148/41, 149/41)$

Glu\textsubscript{4,5}\textsuperscript{1} = [(4),(5) \textsuperscript{13}C\textsubscript{1}]glutamate = $\Sigma(147/42, 148/42, 149/42, 150/42)$

Glu\textsubscript{4,5}\textsuperscript{2} = [(4,5) \textsuperscript{13}C\textsubscript{2}]glutamate = $\Sigma(148/43, 149/43, 150/43, 151/43)$

- Glutamate C1-3 isotopologues (CS flux):

Glu\textsubscript{1,2,3}\textsuperscript{0} = [(1),(2),(3)-\textsuperscript{13}C\textsubscript{0}]glutamate = $\Sigma(146/41, 147/42, 148/43)$

Glu\textsubscript{1,2,3}\textsuperscript{1} = [(1),(2),(3)-\textsuperscript{13}C\textsubscript{1}]glutamate = $\Sigma(147/41, 148/42, 149/43)$

Glu\textsubscript{1,2,3}\textsuperscript{2} = [(1,2), (1,3), (2,3)-\textsuperscript{13}C\textsubscript{2}]glutamate = $\Sigma(148/41, 149/42, 150/43)$

Glu\textsubscript{1,2,3}\textsuperscript{3} = [(1,2,3)-\textsuperscript{13}C\textsubscript{3}]glutamate = $\Sigma(149/41, 150/42, 151/43)$

Based on the observed distribution of citrate isotopomer families reflecting OAA enrichment, only Glu\textsubscript{1,2,3}\textsuperscript{2} = [(1,2)-\textsuperscript{13}C\textsubscript{2}]glutamate was observed. Similarly, only Glu\textsubscript{4,5}\textsuperscript{1} = [(4) \textsuperscript{13}C\textsubscript{1}]glutamate reflecting AcCoA enrichment were observed
5.3 Deconvolvement of Succinate Isotopomers

Succinate is a symmetric mitochondrial metabolite. Unlike some other Krebs cycle intermediates, under most conditions it is almost exclusively formed within the Krebs cycle. $^{13}$C-label traversing the Krebs cycle is evenly reflected across the symmetry plane of succinate. Taking advantage of its fragmentation pattern, succinate loses either C1 or C4 with equal probability resulting in the 117/73 fragment. In general, if there is an even distribution of $^{13}$C labels with equal probability of M+1 enrichment in all four carbons of succinate we expect a 3:1 118/74 to 118/73 fragment ratio. Similarly, if label is only found in the C1 or C4 position we would expect a 1:1 ratio of the 118/74 to 118/73 fragments. Any deviation from these ratios (along with the isotopomeric information of citrate and glutamate) can be used to deduce the proportional contribution of different sources of label, for example, as an indirect readout of PC carbon entry. Since we cannot obtain position-specific label information from the malate fragment, deconvolvement of succinate is needed to calculate PC flux. As depicted in Figure 23, two succinate M+1 families exist, that is $[[1(4)-^{13}\text{C}_1]\text{succinate}$ and $[[2(3)-^{13}\text{C}_1]\text{succinate}$. The only source of $[[1(4)-^{13}\text{C}_1]\text{succinate}$ is through OGDH whereas $[[2(3)-^{13}\text{C}_1]\text{succinate}$ originates from $[[2(3)-^{13}\text{C}_1]\text{malate/OAA}$ during sequential forward reactions of the Krebs cycle: anaplerotic PC-derived $^{13}$C carbons give rise to malate/OAA M+1 (equally as $[2-^{13}\text{C}_1]$- or $[3-^{13}\text{C}_1]$OAA/malate due to the racemic scrambling of the $[3-^{13}\text{C}_1]$OAA label across the irreversible reactions of fumarase and SDH). While the label of $[2-^{13}\text{C}_1]$OAA will become C1 or C4 of succinate, the label of $[3-^{13}\text{C}_1]$-OAA will be C2 or C3 of succinate. These OAA labeling patterns can give rise to the $[[1(2)(3)(4)-^{13}\text{C}_1]\text{succinate}$ labeling family. There was virtually no succinate M+2 generated from $[(1,2)-^{13}\text{C}_2]\text{glutamine}$ within the forward
turn of the Krebs cycle which aided in ruling out higher isotopomeric glutamate and thereby citrate species, such as [(2,3)(2,4)(2,5)(3,4)(3,5)(4,5)-\textsuperscript{13}C\textsubscript{2}]glutamine.

Succinate M+1

\[(1)(4)-\textsuperscript{13}C\textsubscript{1}]\text{succinate} = 2\times118.0 / 73.0

\[(2)(3)-\textsuperscript{13}C\textsubscript{1}]\text{succinate} = 118 / 74-118 / 73

5.4 Deconvolvement of malate isotopomers

Since the fragmentation of malate results in the loss of H\textsubscript{2}O leaving the carbon backbone intact, only isotopologue and not isotopomeric information can be gleaned from the fragment analysis of malate. The isotopomers, however, can be deduced from the deconvolution of glutamate and citrate considering that SDH is irreversible. Given that succinate M+2 was not observed, then all M+2 malate originates from citrate M+2 through

\begin{figure}[h]
\begin{center}
\includegraphics[width=\textwidth]{succinate_families.png}
\end{center}
\caption{Succinate families. Organizing succinate isotopomers into succinate families based on their origin within the Krebs cycle.}
\end{figure}
ACLy. The following malate isotopologues/isotopomers are reported with positional distributions not distinguishable except the M+2 isotopomers which are racemic:

\[(1)(2)(3)(4)^{13}C_1\text{malate} = 134/116\]
\[(1,2)(3,4)^{13}C_2\text{malate} = 135/117\]

5.5 Deconvolution of aspartate isotopomers

During the fragmentation of aspartate the $H_2O$ is lost and thereby does not provide positional information. At isotopic equilibrium with malate and fumarate the carbons of OAA are racemized between positions 1 and 4 as well as carbon 2 and 3. Aspartate and malate share OAA as a common intermediate. Therefore, the similar isotopic build up curves of M+1 Malate and Aspartate as well as M+2 malate and aspartate suggest isotopic equilibrium between these two intermediates across OAA.

In summary, MIMOSA allowed for a straightforward interpretation of label information generated by $[1,2^{-13}C_2]$glutamine (Figure 24) and by grouping key metabolites into distinct isotopomer families based on the enzymatic reaction that catalyzed their
production (Table 9) generated more refined input functions for metabolic flux analysis (CHAPTER III) than would be possible by isotopologue information alone. As expected, all of the isotopomer families reached steady state within less than one hour (Figure 25) and therefore measurements from our 60 mins time points were used for metabolic flux analysis in the following chapter.

Figure 24. Observed carbon fate map.
Observed carbon fate map from [1,2-13C2]-L-glutamine tracer strategy after stepwise elimination of isotopologues and isotopomers of citrate, glutamate, malate by MIMOSA deconvolution. V = metabolic flux. Isotopologues/isotopomers families are based on the enzymatic reaction they originated from (indicated by subscript after metabolite name). ACLy = ATP citrate lyase, CS = citrate synthase, IDHr/f = isocitrate dehydrogenase (reverse/forward), OGDH = alpha ketoglutarate dehydrogenase, PC = pyruvate carboxylase, PDH = pyruvate dehydrogenase, SDH = succinate dehydrogenase.
<table>
<thead>
<tr>
<th>Isotopologue Family</th>
<th>Isotopologue</th>
<th>Isotopomer</th>
<th>Calculation (Precursor/Product Fragments)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Malate</strong></td>
<td>Mal\textsubscript{a+b}</td>
<td>M+1</td>
<td>[(1)(2)(3)(4)\textsuperscript{13}C\textsubscript{1}]malate</td>
</tr>
<tr>
<td></td>
<td>Mal\textsubscript{c}</td>
<td>M+2</td>
<td>[(1,2)(3,4)\textsuperscript{13}C\textsubscript{2}]malate</td>
</tr>
<tr>
<td><strong>Citrate</strong></td>
<td>Cit\textsubscript{a}</td>
<td>M+2</td>
<td>[(1,2)\textsuperscript{13}C\textsubscript{2}]citrate</td>
</tr>
<tr>
<td></td>
<td>Cit\textsubscript{c}</td>
<td>M+1</td>
<td>[(1),(6)\textsuperscript{13}C\textsubscript{1}]citrate</td>
</tr>
<tr>
<td></td>
<td>Cit\textsubscript{b+d}</td>
<td>M+1</td>
<td>[(2)(3)(4)(6)\textsuperscript{13}C\textsubscript{1}]citrate</td>
</tr>
<tr>
<td></td>
<td>Cit\textsubscript{b+c+d}</td>
<td>M+1</td>
<td>[(1)(2)(3)(4)(5)\textsuperscript{13}C\textsubscript{1}]citrate</td>
</tr>
<tr>
<td></td>
<td>Cit\textsubscript{c}</td>
<td>M+1</td>
<td>[(1)(6)\textsuperscript{13}C\textsubscript{1}]citrate</td>
</tr>
<tr>
<td><strong>Glutamate</strong></td>
<td>Glu\textsubscript{a}</td>
<td>M+2</td>
<td>[(1,2)\textsuperscript{13}C\textsubscript{2}]glutamate</td>
</tr>
<tr>
<td></td>
<td>Glu\textsubscript{c+d}</td>
<td>M+1</td>
<td>[(1)(2)(3)\textsuperscript{13}C\textsubscript{1}]glutamate</td>
</tr>
<tr>
<td></td>
<td>Glu\textsubscript{b}</td>
<td>M+1</td>
<td>[(4)\textsuperscript{13}C\textsubscript{1}]glutamate</td>
</tr>
<tr>
<td><strong>Succinate</strong></td>
<td>Suc\textsubscript{a}</td>
<td>M+1</td>
<td>[(1)(4)\textsuperscript{13}C\textsubscript{1}]succinate</td>
</tr>
<tr>
<td></td>
<td>Suc\textsubscript{b}</td>
<td>M+1</td>
<td>[(2)(3)\textsuperscript{13}C\textsubscript{1}]succinate</td>
</tr>
</tbody>
</table>

*Table 9. Summary of all isotopomers.*

A summary of all isotopomic deconvolvement of the observed isotopologues by MIMOSA as described in the text.
The time dependent isotopic build up curves for all isotopomer families shows that they all reach steady state within less than 60 mins of the primed continuous infusion of [(1,2)\(^{13}\)C\(_2\)]glutamine.

Figure 25. Build up curves of isotopomorphic families.
CHAPTER III: CHARACTERIZING BASAL HEPATIC KREBS CYCLE METABOLISM AND ASSESSING ASSUMPTIONS AND CONTROVERSIES

Assumptions in metabolic modeling

Stable isotope based tracer methods are a safe and popular method to study metabolism. However, current tracer methods used for metabolic flux analysis of *in vivo* hepatic metabolism have led to highly controversial concepts of key basal metabolic fluxes, such as pyruvate cycling ($V_{\text{PEPCK}}$ and $V_{\text{PK}}$) and anaplerosis ($V_{\text{PC}}$) [4, 29, 41, 75, 77-79]. These controversies stem from the fact, that all *in vivo* tracer-based metabolic modeling techniques rely on assumptions. Since not all parameters of a metabolic system are directly measurable/observable *in vivo* due to technical or analytical limitations, and given the complexity and interrelatedness of metabolic networks, metabolic flux analyses require simplifications derived from multiple assumptions. The most common assumptions are that the system is in metabolic and isotopic steady state (for an endpoint study), tracer uptake is homogenous across an entire tissue without being diluted or impacting metabolism or generating complex secondary tracer signals.

When these assumptions remain unvalidated, inaccuracies can introduce significant biases into the final metabolic flux analysis. Given the importance of accurate measurements of hepatic metabolism, in order to identify its role in the development and progression of insulin resistance, T2DM, obesity and NAFLD as well as to discover novel
therapeutic targets then the resolution of uncertainties surrounding key assumptions is crucial for future in vivo studies of hepatic metabolism.

Experimental design to measure relative and absolute Krebs cycle rates

To experimentally assess individual Krebs cycle fluxes and to validate the assumptions listed in Table (3) with direct measurements we performed primed continuous infusions of $[1,2^{-13}C_2]$glutamine and measured the steady state fractional enrichment of key Krebs cycle intermediates. The isotopomeric enrichment data was used to numerically solve mass and isotope balance equations written for metabolic flux analysis (Figure 26). The position-specific labeling information obtained from our mass isotopomer deconvolvement of citrate, glutamate and succinate by MIMOSA (described in CHAPTER II) were used for direct measurement of previously assumed isotope exchange rates ($V_x$) and Krebs cycle dilutions ($\Phi_s$) along with individual rates for oxidative, anaplerotic and cataplerotic fluxes.
Positional carbon transfer

The evaluation of the positional transfer of labeled carbons across sequential metabolites within the Krebs cycle and adjacent pathways is fundamental for our metabolic flux analysis. Citrate is a symmetric molecule but it contains a prochiral center which is stereochemically distinguishable by enzymes. The pro-R arm contains carbons 1, 2, 3 and 6 from OAA while the pro-S arm consists of carbons 4 and 5 contributed by acetyl-CoA generated either via β-oxidation or PDH reaction. Subsequently the pro-S arm will become C4 and C5 of glutamate during the IDH reaction. Thus, based on the prochirality of citrate, isotopomers generated by PDH, PC or CS flux can be distinguished.
Metabolism of $[1,2^{-13}C_2]$glutamine leads to the formation of $[1,2^{-13}C_2]$glutamate which enters the Krebs cycle via GDH as $[1,2^{-13}C_2]aKG$. Doubly labeled $aKG$ undergoes two fates: decarboxylation of its C1 to $[(1)(4)^{-13}C_1]$succinate via OGDH or $[1,2^{-13}C_2]$citrate catalyzed by IDHr. $[(1)(4)^{-13}C_1]$succinate generates $[(1)(4)^{-13}C_1]$OAA/malate which condenses with acetyl CoA to form citrate. Label in C1 position of pyruvate is lost during the decarboxylation reaction of PDH or re-introduced into C2 and C3 position of OAA through PC. Additionally, any ligation of labeled CO$_2$ catalyzed by PC can generate any of the following OAA species: $[2,4^{-13}C_2]$OAA, $[3,4^{-13}C_2]$OAA, $[1,2^{-13}C_2]$OAA or $[1,3^{-13}C_2]$OAA along with their respective citrate isotopologues. Additional contribution to the overall malate isotopologue pool (M+2) comes from citrate that is metabolized by ACLy to cytosolic malate (C1,2,3,6) and acetyl CoA (C4,5).

**Assessment of dilutional fluxes**

At metabolic and isotopic steady state, the relative contribution of a pathway to a specific product can be determined by the $^{13}$C enrichment ($\phi$) ratio of precursor A to product B ($\phi_{AB}$). For instance, in a unidirectional reaction if a labeled precursor is the sole substrate converted to a specific product, the enrichment ratio between product and precursor will approach 1 at steady state. If additional substrates from other pathways, contribute to the same product, then the relative contribution of the labeled precursor and the additional unlabeled substrate(s) will be less than or equal to the total enrichment of the product and result in an enrichment of product to labeled precursor ratio of less than 1. The discrete
entry of unlabeled carbon from confluent, intersecting pathways can be described as follows:

\[ \varphi_A \rightarrow B = \frac{A}{B} \leq 1 \]

and the relative contribution from unlabeled metabolites can then be defined as 1-\(\varphi_{AB}\). This is particularly helpful to determine if significant dilutional fluxes, e.g., anaplerosis, and/or exchange reactions, exist.

**Results**

As shown in Figure (27), there was significant serial dilution across consecutive metabolites as the \(^{13}\text{C}\) label traversed the Krebs cycle (Figure 27, top panel) with a remarkable total drop of 70% in the plateau enrichment after one full turn at 60 mins (Figure 27, bottom panel). Because C6 is lost during the IDH reaction, then this indicates a ~35% dilution of label by unlabeled metabolites in equilibrium with different steps of the cycle. This sequential dilution indicated significant influx of unlabeled carbons from multiple confluent, intersecting pathways into the Krebs Cycle. The time to half max enrichment was calculated for each by fitting to a single exponential. The difference in t\(_{1/2}\) for for glutamate M+2 and glutamate M+1 was about 10 minutes, indicating that >5 full turns of the cycle by 60 minutes consistent with being at isotopic steady state.
At isotopic steady state there was serial label dilution across all Krebs cycle intermediates (top panel). The evolution of glutamate M+2 into M+1 glutamate in vivo during a full turn of the Krebs cycle (bottom panel). Curves were fit to a single exponential and $t_{1/2}$ shown by vertical dashed lines.

Figure 27. Sequential Hepatic Metabolite.
In addition to anaplerotic fluxes another source of label dilution in reversible systems are “isotopic exchange reactions” which transfer isotopic label between metabolites without net carbon conversion leading to label dilution. Aminotransferase exchange reactions associated with the Krebs cycle catalyze the interconversion of glutamate and αKG as well as OAA and aspartate [10]. The kinetic measurements of the aminotransferase exchange reaction \( V_x \) in vivo were made by comparing the temporal build up curves of glutamate vs succinate positional isotopomers. Unfortunately, the deconvolvement does not provide exact carbon matching between glutamate and succinate. Since the observed equilibration between glutamate and succinate was fast relative to the TCA cycle, \([C(1,2)\-^{13}C_2]glutamate\) was compared to succinate \(M+1([1\(\-^{13}C_1\]succinate = 2\*118/73).\)

During the early time points, C2 enrichment of the succinate C1 and C4 isotopomers by coming from tracer glutamine and then glutamate. Here the contributions of \([5^{13}C_1\]glutamate\) were small (as shown above) and \([2^{13}C_1\]glutamate\) derived from PC flux would be small and late making the comparison a reasonable surrogate for the transfer of glutamate enrichment into the Krebs cycle. The temporal isotopic build up curves between these two isotopologues were superimposable and not significantly different, confirming a fast exchange of label from the cytosolic glutamate into the mitochondrial matrix pool of αKG (Figure 28).
Aminotransferase Exchange Rate ($V_x$)

Figure 28. Amino Transferase Exchange reaction ($V_x$).

Amino Transferase Exchange reaction ($V_x$) catalyzes a rapid exchange between cytosolic and intramitochondrial metabolites. Isotope build up curves of Glutamate M+2 and Succinate M+1. No statistical significant difference between both lines.
Metabolic flux analysis (MFA)

In metabolic modeling studies, the labeling rate of each intermediate depends on the precursor enrichments, enzyme kinetics, the metabolic flux through the system and its respective pool size [67]. Atom fate maps provide information about individual reactions within a pathway but do not give information about flux. To determine relative fluxes a steady state a metabolic flux analysis is performed using the measured fractional tracer enrichments. The concept of metabolic flux analysis and modeling is based on mathematical formalisms[71, 117-121]. Herein, from steady state relationships differential equations can be derived to describe the rate of change of enrichment of a labeled metabolite arising from a metabolic inflow/influx minus the outflow/efflux of the product. This is described in the following reaction:

\[ A \xrightarrow{E_1} B \xrightarrow{E_2} C \]

where A is the substrate that is converted into product B and then into C. The reactions are catalyzed by enzyme E1 and then E2 respectively. The general mass and isotope balance equations describing the change in the concentration of B is:

Mass Balance: \[ \frac{d[B]}{dt} = V_{E1} - V_{E2} \]

If A is an enriched tracer (A*), then the change in the isotope balance of the fractional enrichment of B* over time is described by:

Isotope Balance: \[ \frac{d[FE_{B*}]}{dt} = FE_{A*} \cdot v_{E_1} - FE_{B*} \cdot v_{E_2} \]

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Of note, exchange reactions do not contribute to or consume mass (i.e., no net carbon conversion) and therefore do not need to be accounted for in mass balance equations. However, they contribute label and therefore need to be factored into isotope balance equations.

**Phi (φ) ratios**

To determine the relative carbon contribution to a metabolic reaction we derived first order differential equations describing the transfer of label from a $^{13}$C enriched substrate to its product as described above.

At metabolic and isotopic steady-state, the fractional enrichment of $^{13}$C label is constant and therefore the relative contribution of E1 to E2 can be described as the phi (φ) ratio of precursor to product $φA \to B$ where the relative contribution of the input relative to the output is equal to the enrichment of the product to its precursor and therefore can be solved for by

$$φA \to B = \frac{V_{E1}}{V_{E2}} = \frac{F_{E_B^*}}{F_{E_A^*}}$$
Based on the above principle preliminary phi (φ) ratio relationships were established for our metabolic flux analysis as follows:

**Mass Balance of anaplerosis and cataplerosis**

\[
\frac{d\text{OAA}}{dt} = V_{SDH} + V_{PC} + V_{ACly} - (V_{CS} + V_{PEPCK})
\]

At steady state:

\[
V_{SDH} + V_{PC} + V_{ACly} = V_{CS} + V_{PEPCK}
\]

Thus:

\[
\frac{V_{SDH} + V_{PC} + V_{ACly}}{V_{CS} + V_{PEPCK}} = 1
\]

We make the following definitions of fractional fluxes:

- \( F_{SDH} \): \( \frac{V_{SDH}}{V_{CS} + V_{PEPCK}} = f_s \)
- \( F_{ACly} \): \( \frac{V_{ACly}}{V_{CS} + V_{PEPCK}} = f_c \)
- \( F_{PC} \): \( \frac{V_{PC}}{V_{CS} + V_{PEPCK}} = f_p \)

The sum of the fractional fluxes then equals 1.

\[
f_s + f_p + f_c = 1
\]

Rearranging to solve for \( f_p \)

\[
f_p = 1 - f_s - f_c
\]

Solving for \( f_c \)

Since malate and OAA are in isotopic equilibrium across all carbons, we can substitute malate for OAA.

Then isotope balance for malate M+2 is:
\[
\frac{d(MalM+2)}{dt} = V_{SDH} (\text{Succ M + 2}) + V_{PC} (\text{Pyr M + 2}) + V_{ACLy} (\text{Cit M + 2}) - \\
(Mal M + 2)(V_{CS} + V_{PEPCK+ME})
\]

At steady state:

\[
V_{SDH} (\text{Succ M + 2}) + V_{PC} (\text{Pyr M + 2}) + V_{ACLy} (\text{Cit M + 2}) = (Mal M + 2)(V_{CS} + V_{PEPCK+ME})
\]

Since there was no detectable M+2 succinate or pyruvate, this simplifies to:

\[
V_{ACLy} (\text{Cit M + 2}) = (V_{CS} + V_{PEPCK+ME})(Mal M + 2)
\]

Rearranging:

\[
\frac{V_{ACLy}}{V_{CS} + V_{PEPCK+ME}} = \frac{Malate_{M+2}}{Citrate_{[1,2]}}
\]

Substituting previously defined variables and relationships

\[
f_c = \frac{Malate_{M+2}}{Citrate_{[1,2]}} = \frac{Malate_c}{Citrate_a}
\]
Solving for $f_s$

Isotope balance for malate M+1:

$$\frac{d(Mal_{M+1})}{dt} = V_{SDH}(Succ_{M+1}) + V_{PC}(Pyr_{M+1}) + V_{ACLY} \left( [(1)(2)(3)(6)C_1]Cit \right) - (Mal_{M+1})(V_{CS} + V_{PEPCK+ME})$$

At steady state:

$$V_{SDH}(Succ_{M+1}) + V_{PC}(Pyr_{M+1}) + V_{ACLY} \left( [(1)(2)(3)(6)C_1]Cit \right) = (Mal_{M+1})(V_{CS} + V_{PEPCK+ME})$$

Divide by $V_{CS} + V_{PEPCK+ME}$

$$\frac{(Succ_{M+1})V_{SDH}}{(V_{CS} + V_{PEPCK+ME})} + \frac{(Pyr_{M+1})V_{PC}}{(V_{CS} + V_{PEPCK+ME})} + \frac{[(1)(2)(3)(6)C_1]Cit V_{ACLY}}{(V_{CS} + V_{PEPCK+ME})} = (Mal_{M+1})$$

Substitute fraction relations

$$f_s(Succ_{M+1}) + (1 - f_c - f_s)(Pyr_{M+1}) + f_c([(1)(2)(3)(6)C_1]Cit) = Mal_{M+1}$$

Expand

$$f_s(Succ_{M+1}) + Pyr_{M+1} - f_c(Pyr_{M+1}) - f_s(Pyr_{M+1}) + f_c([(1)(2)(3)(6)C_1]Cit) = Mal_{M+1}$$

Combine terms

$$f_s(Succ_{M+1} - Pyr_{M+1}) + f_c([(1)(2)(3)(6)C_1]Cit - Pyr_{M+1}) = Mal_{M+1} - Pyr_{M+1}$$
Solve for $f_s$

$$f_s = ((\text{Mal}_{M+1} \cdot \text{Pyr}_{M+1}) + f_c(\text{Pyr}_{M+1} - [(1)(2)(3)(6)^{13}C_1]Cit)) / (\text{Succ}_{M+1} - \text{Pyr}_{M+1})$$

[Note: New isotopomer definition assuming C5 enrichment is negligible]

$$[(1)(2)(3)^{13}C_1]Cit = \frac{(1 - [(4)^{13}C_1]\text{Glu})}{[(1)(2)(3)(4)(5)^{13}C_4]\text{Glu}} \cdot [(1)(2)(3)(5)^{13}C_1]Cit$$

$$[(1)(2)(3)^{13}C_1]Cit + \frac{1}{2} \text{Cit}_c = [(1)(2)(3)(6)^{13}C_1]Cit$$

$$[(1)(2)(3)(6)^{13}C_1]Cit = \frac{(1 - (147/42 + 148/42))}{\text{Glu}_{M+1}} \cdot (192/112 - 192/111) + 2 \cdot 192/111$$

The derivation of all the equations used to generate mass isotopomer measurements for each label are based on the study by Alves et al. [71] and are presented in part below (for all fluxes relative to CS). The full derivations are shown in more detail in the Supplemental Information.

Establishing PDH relative to CS

$$\frac{V_{PDH}}{V_{CS}} = \frac{\text{Gl}u_{M+1}PDH}{\frac{\varphi_{Cit-Glu}}{\varphi_{Cit-Glu}}} \div \text{Pyr}_{M+1}$$

Establishing IDHf relative to CS
Mass Balance: \( \frac{d\text{Cit}}{dt} = V_{CS} + V_{IDHr} - V_{ACL} - V_{IDHf} \)

Rearrange: \( V_{CS} + V_{IDHr} = V_{ACL} + V_{IDHf} \)

Solve:

\[
\frac{V_{CS}}{V_{CS}} + \frac{V_{IDHr}}{V_{CS}} = \frac{V_{ACL}}{V_{CS}} + \frac{V_{IDHf}}{V_{CS}}
\]

\[
1 + \frac{V_{IDHr}}{V_{CS}} = \frac{V_{ACL}}{V_{CS}} + \frac{V_{IDHf}}{V_{CS}}
\]

\[
\frac{V_{IDHf}}{V_{CS}} = 1 - \frac{V_{ACL}}{V_{CS}} + \frac{V_{IDHr}}{V_{CS}} = 1 - \frac{(\text{Mal } M+2 - \text{Cit } M+2)}{\text{(Cit } M+2 - \text{Glu } M+2)}
\]

\[
\frac{V_{IDHr}}{V_{CS}} = \frac{(\text{Mal } M+2 - \text{Cit } M+2)}{\text{(Cit } M+2 - \text{Glu } M+2)}
\]

Mass Balance: \( V_{IDHnet} = V_{IDHf} - V_{IDHr} \)

Isotope Balance: \( \frac{d\text{Cit}_{M+2}}{dt} = aK_{G}M+2*V_{IDHr} - \text{Cit}_{M+2}*V_{IDHf} \)

Because \( V_x \) is fast:: \( aK_{G}M+2 = \text{Glu}_{M+2} \)

Substitute: \( \text{Glu}_{M+2}*V_{IDHr} = \text{Cit}_{M+2}*V_{IDHf} \)

Rearrange: \( V_{IDHf} = V_{IDHf}(\text{Glu}_{M+2}/\text{Cit}_{M+2}) \)

Substitute for \( V_{IDHf} \): \( V_{IDHf} = (V_{IDHf} - V_{IDHnet})(\text{Glu}_{M+2}/\text{Cit}_{M+2}) \)
Solve: \( \frac{V_{\text{IDH}}}{V_{\text{IDHnet}}} = \frac{\text{GluM}+2/\text{CitM}+2}{(\text{GluM}+2/\text{CitM}+2 - 1)} \)

Net IDH flux is the difference between forward and reverse IDH flux:

Mass Balance \( \frac{V_{\text{IDHnet}}}{V_{CS}} = \frac{V_{\text{IDHf}}}{V_{CS}} - \frac{V_{\text{IDHr}}}{V_{CS}} \)

From the above established relationships the following relative fluxes can be established:

\( \frac{V_{\text{OGDH}}}{V_{CS}} = \frac{V_{\text{OGDH}}}{V_{SDH}} - \frac{V_{CS}}{V_{SDH}} \)

Establishing SDH relative CS

From the above fluxes relative to \( V_{\text{SDH}} \) fluxes relative to CS can be established as

\[ V_{\text{ACL}_{\text{V}}} = \frac{f_c}{f_s} = \left( \frac{\text{Malate}_{M+2}}{\text{Citrate}_{[1,2]}} - \frac{(\text{MalM}+1 - \text{PyrM}+1 + f_c(\text{PyrM}+1 - (1)(2)(3)(6)^{13}C_1\text{Cit}))}{(S_{M+1} - P_{M+1})} \right) \]
\[
\frac{V_{PC}}{V_{SDH}} = \frac{f_p}{f_s} = \left(1 - \frac{(M_{M+1} - f_c + \text{Cit}_{[2][3][6]} + P_{M+1}) - P_{M+1}}{(S_{M+1} - P_{M+1})} - \frac{\text{Malate}_{M+2}}{\text{Citrate}_{[1,2]}}\right) \div \left(\frac{(\text{MalM+1} - \text{PyrM+1}) + fc(\text{PyrM+1} - [(1)(2)(3)(6)_{13C1}\text{Cit}])}{(S_{M+1} - P_{M+1})}\right)
\]

\[
\frac{V_{GDH}}{V_{SDH}} = \left(1 - \frac{\text{Glu} M+2\text{AA} - \text{Gln} M+2}{(\text{Citrate}_{[1,2]} - \text{Gln} M+2)}\right) \ast \left(\frac{\text{Succ}_{a}}{\text{Glu}_{a} + \text{Glu}_{b} + \text{Glu}_{c+d}}\right)
\]

\[
\frac{V_{CS}}{V_{SDH}} = \left(\frac{\text{Malate}_{M+2}}{\text{Citrate}_{[1,2]}}\right) \div \left(\frac{(\text{MalM+1} - \text{PyrM+1}) + fc(\text{PyrM+1} - [(1)(2)(3)(6)_{13C1}\text{Cit}])}{(S_{M+1} - P_{M+1})}\right) + \left(1 - \frac{(\text{Glu} M+2\text{AA} - \text{Gln} M+2)}{(\text{Citrate}_{[1,2]} - \text{Gln} M+2)}\right) \ast \left(\frac{\text{Glu} M+2\text{AA} - \text{Gln} M+2}{(\text{Citrate}_{[1,2]} - \text{Gln} M+2)}\right)
\]

\[
\frac{V_{OGDH}}{V_{SDH}} = 1 - \frac{\text{Glu} M+2\text{AA} - \text{Gln} M+2}{(\text{Citrate}_{[1,2]} - \text{Gln} M+2)}
\]

\[
\frac{V_{Prop}}{V_{SDH}} = 1 - \frac{V_{OGDH}}{V_{SDH}} = 1 - \frac{\text{Succ} (1)(4)}{\text{GluM+2\text{AA}+GluM+1}}
\]
Establishing SDH relative to CS

Equation: \[ \frac{V_{SDH}}{V_{CS}} = \frac{1}{\frac{V_{CS}}{V_{SDH}}} \]

Solve:

\[
\frac{V_{SDH}}{V_{CS}} = 1 \div \left( \frac{(\text{Malate}_{M+2})}{\text{Citrate}_{[1,2]}} \div \left( \frac{((\text{Malate}_{M+1} - \text{Pyr}_{M+1}) + \text{fc}((\text{Pyr}_{M+1} - \left[ (1)(2)(3)(6)^{13}C_1 \right] \text{Cit}))}{(S_{M+1} - P_{M+1})} \right) + \right)

\left( 1 - \frac{(\text{Glu} M + 2\text{OAA} - \text{Gln} M + 2)}{(\text{Citrate}_{[1,2]} - \text{Gln} M + 2)} \right) \right) \cdot \left( 1 - \frac{(\text{Glu} M + 2\text{OAA} - \text{Gln} M + 2)}{(\text{Citrate}_{[1,2]} - \text{Gln} M + 2)} \right)

Establishing PC relative to CS

Equation: \[ \frac{V_{PC}}{V_{CS}} = \frac{V_{PC}}{V_{SDH}} \div \frac{V_{CS}}{V_{SDH}} \]

Solve:

\[
\frac{V_{PC}}{V_{CS}} = \left( 1 - \frac{((\text{Malate}_{M+1} - \text{Pyr}_{M+1}) + \text{fc}((\text{Pyr}_{M+1} - \left[ (1)(2)(3)(6)^{13}C_1 \right] \text{Cit}))}{(S_{M+1} - P_{M+1})} \right) \div \frac{\text{Malate}_{M+2}}{\text{Citrate}_{[1,2]}}
\]
Establishing Prop relative to CS

Equation: \[
\frac{V_{Prop}}{V_{CS}} = \frac{V_{Prop}}{V_{SDH}} + \frac{V_{CS}}{V_{SDH}}
\]

Solve:

\[
\frac{V_{Prop}}{V_{CS}} = \left(1 - \frac{\text{Succ}^{(1)(4)}}{\text{Glu}+2\text{OAA}+\text{Glu}+1}\right) \div \left(\frac{\text{Malate}^{M+2}}{\text{Citrate}_{[1,2]}}\right) \div 
\]

\[
\left(\frac{\text{MalM}+1 - \text{PyrM}+1 + fc(\text{PyrM}+1 - [(1)(2)(3)(6)C_{13}C\text{t}])}{(S_{M+1} - P_{M+1})}\right) + 
\]

\[
\left(1 - \frac{(\text{Glu M}+2\text{OAA} - \text{Gln M+2})}{(\text{Citrate}_{[1,2]} - \text{Gln M+2})}\right) \times \left(\frac{(\text{Glu M}+2\text{OAA} - \text{Gln M+2})}{(\text{Citrate}_{[1,2]} - \text{Gln M+2})}\right)
\]
Establishing GDH relative to CS

Equation: \[
\frac{V_{GDH}}{V_{CS}} = \frac{V_{GDH}}{V_{SDH}} + \frac{V_{CS}}{V_{SDH}}
\]

Solve: \[
\frac{V_{GDH}}{V_{CS}} = \left(1 - \frac{(Glu + 2OAD - Gln M+2)}{(Citrate_{1,2} - Gln M+2)}\right) \cdot \left(\frac{\text{Succ} a}{\text{Glu} + \text{Glu} + \text{Succ} d}\right) \div \\
\left(\frac{(\text{MalM}+1 - \text{PyrM}+1) + fc(\text{PyrM}+1 - \left[(1)(2)(3)(6)_{13} C_1][c(l)]\right)}{(S_{M+1} - P_{M+1})}\right) + \\
\left(1 - \frac{(Glu + 2OAD - Gln M+2)}{(Citrate_{1,2} - Gln M+2)}\right) \cdot \frac{(Glu + 2OAD - Gln M+2)}{(Citrate_{1,2} - Gln M+2)}
\]

Since at steady state anaplerosis is equal to cataplerosis

\[
\frac{V_{PPECK+ME}}{V_{CS}} = \frac{V_{ANA}}{V_{CS}} = \frac{V_{ANA}}{V_{SDH}} + \frac{V_{CS}}{V_{SDH}}
\]

Establishing ACLy relative to CS
Equation: \[ \frac{V_{ACL_y}}{V_{CS}} = \frac{V_{ACL_y}}{V_{SDH}} \times \frac{V_{CS}}{V_{SDH}} \]

Solve:

\[
\frac{V_{ACL_y}}{V_{CS}} = \left( \frac{Malate_{M+2}}{Citrate_{[1,2]}} \right) \div \left( \frac{(Malate_{M+1} - PyrM+1 + fc(PyrM+1 - [1(2)(3)(6)\text{C}]Cit))}{(S_{M+1} - P_{M+1})} \right) \div \\
\left( \frac{Malate_{M+2}}{Citrate_{[1,2]}} \right) \div \left( \frac{(Malate_{M+1} - PyrM+1 + fc(PyrM+1 - [1(2)(3)(6)\text{C}]Cit))}{(S_{M+1} - P_{M+1})} \right) + \\
\left( 1 - \frac{(Glu M+2\_OAA - Gln M+2)}{Citrate_{[1,2]} - Gln M+2} \right) \times \left( \frac{(Glu M+2\_OAA - Gln M+2)}{Citrate_{[1,2]} - Gln M+2} \right)
\]

After mass and isotope balance equations were derived for the glutamine labeling strategy for key reactions of the Krebs cycle we were able to numerically solve these equations from the measured enrichment data to calculate specific relative fluxes. From the high-resolution position-specific label analysis provided by POCE, and \(^{13}\)C NMR, along with the direct deconvolution of citrate, glutamate and succinate isotopomers by our analytical platform MIMOSA, tracing of isotopomer distributions along metabolic networks was made possible [71, 80, 122] (see CHAPTER II). Incorporation of position-specific isotopomeric information into the analysis (in addition to the more general position non-specific isotopologue data), in principle, improves the estimations of metabolic fluxes than possible with isotopologue data alone [71, 118]. We calculated relevant mitochondrial oxidative (IDH, OGDH, SDH), anaplerotic (PC, PCC, GDH), cataplerotic
Relative Krebs cycle fluxes

The cyclic sequence of Krebs cycle reactions begins when acetyl-CoA undergoes an aldol condensation with oxaloacetate (OAA) to form citrate. This first committed step of the Krebs cycle is catalyzed by citrate synthase (CS). Three major oxidative reactions of the Krebs cycle are catalyzed by isocitrate dehydrogenase (forward reaction, IDHf), αKG dehydrogenase, and succinate dehydrogenase. All of these reactions have previously not been measured individually in vivo due to sensitivity limitations of in vivo $^{13}$CNMR which made position specific labeling information inaccessible for direct measurements. Therefore, the individual oxidative reactions of the Krebs cycle have been grouped with CS and reported as one overarching rate, that is, $V_{TCA}$. With position specific labeling information of key Krebs cycle intermediates it was possible to calculate rates for each of these oxidation reactions relative to CS. Furthermore, our isotopomer MFA enabled us to estimate rates for all three key anaplerotic (PC, GDH, PCC) and the major cataplerotic flux through PEPCK in addition to the ACLy reaction.
Figure 29. Relative Krebs cycle fluxes.

(a) Overview over Krebs cycle fluxes (b) oxidative fluxes, (c, d) anaplerotic fluxes, (e) cataplerotic fluxes, including ACLy flux (f). Glutamine was infused at a primed continuous rate 12 umol/kg/min for 60 mins. N=8.
Dilutional influx into the Krebs cycle is considered anaplerotic when balanced by a cataplerotic efflux. The progressive dilution of $^{13}$C signal across sequential Krebs cycle intermediates described above was consistent with significant carbon contribution coming from anaplerosis. The three anaplerotic fluxes stemmed from 1) propionate ($V_{PCC}$) at approximately 20%, 2) pyruvate ($V_{PC}$), which accounted for 75% and 3) glutamate ($V_{GDH}$) contributing the remaining 5% of total anaplerotic fluxes (Figure 29 c,d). All of which were balanced by cataplerotic flux through PEPCK+ME (29 e), that plays an important role in gluconeogenesis and pyruvate cycling. Substrate cycling is mediated by the simultaneous occurrence of two opposing enzymatic reactions leading to the net hydrolysis of ATP. Pyruvate cycling begins with the conversion of pyruvate to OAA by PC (anaplerosis). OAA undergoes metabolism through PEPCK into PEP which subsequently cycles back into pyruvate through pyruvate kinase [15]. Pyruvate cycling is the difference between cataplerosis through PEPCK and anaplerosis mediated by PC. The rate of PEPCK sets the maximum rate of pyruvate re-cycling. Figure shows that the relative rate of pyruvate carboxylase is close to the same rate as CS whereas PEPCK is only 1.5 or 1.2 times the rate of CS (Figure 29 e) which indicates that the pyruvate cycling rate is substantially smaller than measured using the propionate tracer strategy and more consistent with previously reported rates using lactate as a tracer [4]. Additionally, our metabolic flux analysis demonstrated that each of the three measured oxidative steps, IDH, ODGH and SDH, are work at distinct rates (Figure 29 b) and adjust to adjacent anaplerotic and/or cataplerotic pathways.

Isocitrate dehydrogenase catalyzes two half reactions where decarboxylation converts isocitrate into aKG (referred to as IDHf) as well as reductive carboxylation to
convert aKG back into isocitrate (IDHr). The latter reaction has been suggested to be an important step in de novo lipogenesis (DNL) where ATP Citrate Lyase (ACLy) catalyzes citrate conversion into cytosolic malate and acetyl CoA (though this suggestion to date has only considered the unidirectional reactions). ACLy contributes significantly to basal hepatic metabolism (Figure 29 e). IDH is commonly treated as one reversible reaction. However, distinct rates for IDHf and IDHr lead to an overall net flow (IDHnet) towards the oxidative reaction (Figure 29 b, 30).

Figure 30. Reductive carboxylation.
Isotope build up curves of glutamate M+2 → citrate M+1 → malate M+2, reflect flux through reductive carboxylation and ATP citrate lyase, respectively.
Absolute Krebs cycle rates ("Bootstrapping")

From the relative rates the absolute Krebs cycle rates can be calculated if there is a known flux rate to link it to. Here this was done by bootstrapping the relative fluxes onto the externally measured absolute rate of endogenous glucose production (EGP, glucose $R_a$) [15, 123]. Under the assumption that during fasting all gluconeogenic flux comes from PEPCK absolute rates and that glycerol and fructose made small or fixed contributions, rates relative to CS were determined by "strapping" steady-state relative fluxes of $V_{PEPCK+ME}/V_{CS}$ onto the direct measurements. All other absolute fluxes were then calculated in relation to $V_{CS}$ (Figure 31)[4, 15].
## Absolute Fluxes

<table>
<thead>
<tr>
<th></th>
<th>Formula</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>( V_{CS} = EGP \times \frac{V_{PEPCK}}{V_{CS}} ) = ( EGP \times \frac{V_{CS}}{V_{PEPCK}} ) = ( V_{PEPCK} \times \frac{V_{CS}}{V_{PEPCK}} )</td>
</tr>
<tr>
<td>2</td>
<td>( V_{IDHf} = V_{CS} \times \frac{V_{IDHf}}{V_{CS}} )</td>
</tr>
<tr>
<td>3</td>
<td>( V_{IDHr} = V_{CS} \times \frac{V_{IDHr}}{V_{CS}} )</td>
</tr>
<tr>
<td>4</td>
<td>( V_{IDHnet} = V_{CS} \times \frac{V_{IDHnet}}{V_{CS}} )</td>
</tr>
<tr>
<td>5</td>
<td>( V_{SDH} = V_{CS} \times \frac{V_{SDH}}{V_{CS}} )</td>
</tr>
<tr>
<td>6</td>
<td>( V_{SDH} = V_{CS} \times \frac{V_{SDH}}{V_{CS}} )</td>
</tr>
<tr>
<td>7</td>
<td>( V_{PEPCK} = V_{CS} \times \frac{V_{PEPCK}}{V_{CS}} )</td>
</tr>
<tr>
<td>8</td>
<td>( V_{PCC} = V_{CS} \times \frac{V_{PCC}}{V_{CS}} )</td>
</tr>
<tr>
<td>9</td>
<td>( V_{GDH} = V_{CS} \times \frac{V_{GDH}}{V_{CS}} )</td>
</tr>
<tr>
<td>10</td>
<td>( V_{PC} = V_{CS} \times \frac{V_{PC}}{V_{CS}} )</td>
</tr>
<tr>
<td>11</td>
<td>( V_{ACLY} = V_{CS} \times \frac{V_{ACLY}}{V_{CS}} )</td>
</tr>
</tbody>
</table>

Lastly, we not only determined absolute rates for each key step of the Krebs cycle, from the dynamic data points we also calculated the time it takes to complete one full turn of the cycle which is accomplished when the [1-\(^{13}\)C]glutamate isotopomer is made from [1,2-\(^{13}\)C\(_2\)] glutamate with half the enrichment lost from C6 citrate at the IDH step. The difference in the half max time for each of those two isotopomers reflects the full circuit
which was actually completed in only approximately 13 minutes (Figure 32). Therefore, within one hour the Krebs cycle completes five full turns which further corroborates that under proper tracer condition full label equilibration across all key intermediates can be achieved in approximately 60 minutes.
Discussion

Just as Hans Krebs predicted almost seven decades ago, the Krebs cycle stands in the center of attention of the scientific community in an attempt to explain the origin and
progression of many diseases in mankind. To date numerous research studies have demonstrated that mitochondrial oxidative and biosynthetic metabolism play an integral role in the pathogenesis and development of insulin resistance, type 2 diabetes mellitus, NAFLD and obesity [27-39]. To accurately identify how and to what extent mitochondrial oxidative and biosynthetic fluxes are involved in these common disorders accurate measurements of absolute and relative Krebs cycle fluxes in health and disease are of utmost importance. Recent stable isotope research, both in rodents and humans, of hepatic metabolism at basal state and in disease models has led to vastly inconsistent/incongruent and controversial results. For instance, anaplerosis through PC has been reported to be approximately 1.4 times the rate of the Krebs cycle by one group while another measured anaplerotic flux through PC at approximately 5 times the Krebs cycle rate, yet with minimal overall impact on flux into gluconeogenesis [41, 107, 124, 125]. Similarly, substrate cycling has been in the center of the controversial debate regarding basal hepatic metabolism [109]. There has been a four to five-fold difference between published rates pyruvate cycling depending on the study [74-77, 116, 125]. If anaplerosis and substrate cycling were truly manifold faster than the Krebs cycle turns, these fluxes would put the metabolic system into a precarious thermodynamic state/situation. For example, PEP-pyruvate cycle at the intersection of glycolysis and gluconeogenesis. Here, pyruvate is converted to OAA catalyzed by PC, while OAA is transformed to PEP via PEPCK and back into pyruvate through pyruvate kinase (PK) with an overall consumption of 1 molecules of ATP which at a fast pace would overwhelm the direct ATP production of the Krebs cycle [11, 12, 15]. Moreover, the change in RedOx state would likely expose to the cell to oxidative stresses and eventually cell damage and apoptosis/death.
Therefore, previously reported rates of anaplerosis being approximately 1.4 times the rate of the Krebs cycle and pyruvate cycling making up only a small fraction of anaplerosis makes physiologic sense [4, 116, 126]. Recent in vitro studies have challenged the paradigm of excessively fast anaplerotic and cycling fluxes by showing that the overestimation of pyruvate cycling in metabolic modeling is related to limited isotopomeric that leads to substantial miscalculations of key metabolic fluxes, such as anaplerosis and $V_{TCA}$ [71]. Here, in our study, we show that the rates for anaplerosis in vivo are much closer to the overall rate of CS and that pyruvate cycling is just a fraction of Krebs cycle metabolism. By obtaining direct isotopomeric information of all key Krebs cycle intermediates we were able to develop a much more constraint metabolic model of all oxidative and biosynthetic fluxes and thereby estimate fluxes much closer to their physiologic rates.

Moreover, we could show that there is significant label dilution into Krebs cycle from two additional sources, besides PC, which are glutamate and propionate. Previously not measured in vivo and deemed metabolically negligible, these dilutional fluxes, when not considered in the metabolic model, will lead to significant underestimation of the $V_{TCA}$.

An uncorrected, falsely low, rate of $V_{TCA}$ will artificially increase all fluxes relative to it, e.g., anaplerosis and pyruvate cycling. While in turn, as part of a modeling vicious cycle, overestimation of pyruvate cycling further contributes to the underestimation of Krebs cycle flux, as mentioned before. An additional important finding of our flux analysis is that summarizing all oxidative reactions as $V_{TCA}$ is an oversimplification of and misses fine nuances of Krebs cycle metabolism. Our position-specific tracking of kinetic label transfer demonstrates that actually each oxidative step within the Krebs cycle is a series
of individual enzymatic reactions that adjusts to its adjacent anaplerotic fluxes rather than one steady circular sequence of metabolic steps.

Finally, from the isotopomeric information obtained with MIMOSA, we were able to directly assess aminotransferase exchange, Vx, which has been in the center of longstanding debates pertaining to NMR-based \textit{in vivo} metabolic flux calculations [94-98]. While $^{13}$CNMR is a method for non-invasive measurements of hepatic metabolism, $^{13}$C labeled tracers have limited sensitivity given their overall low concentrations and makes direct assessment of multiple Krebs cycle intermediates a challenge \textit{in vivo}. Instead, $^{13}$C NMR relies on the highly abundant $^{13}$C signal of aspartate and glutamate. These amino acids are assumed to be in rapid exchange through aminotransferase exchange reactions (Vx) with the Krebs cycle intermediates OAA and αKG, respectively. Due to their higher abundance glutamate’s and aspartate’s can act as NMR-observable trapping pools for $^{13}$C label in Krebs cycle intermediates. Since Vx has never been measured \textit{in vivo} before, all of $^{13}$CNMR metabolic flux calculations and modeling is based on the underlying assumption that the isotopic exchange rate between glutamate and αKG as well as OAA and aspartate mediated is fast relative to the Krebs cycle flux and thus makes glutamate and aspartate a kinetic component of the Krebs cycle [94-98]. If Vx exchange were slow and unadjusted for, it would result in marked errors in calculation of $V_{\text{TCA}}$, and other related rates, such as anaplerosis [94, 97, 99, 100]. Taking advantage of our direct measurement of Krebs cycle intermediates and their positional labeling information we were able to directly assess the isotope exchange rate and thereby validate this key assumption which forms the basis of all \textit{in vivo} $^{13}$C NMR flux calculations and show that
glutamate and aKG are in rapid exchange. As such label information of glutamate is a reliable readout of Krebs cycle metabolism.

CHAPTER IV: CROSS VALIDATION OF MIMOSA FLUX ANALYSIS WITH KINETIC MODELING

Due to experimental and methodological limitations (some of which are described above), not all flux parameters of a metabolic system can always be measured directly and solved manually as described above. To derive additional pertinent fluxes that capture otherwise experimentally unobservable fluxes, more comprehensive metabolic flux models were constructed mathematically with the help of metabolic modeling software [127]. A metabolic flux model was constructed mathematically with the modeling software CWave [128] whose computational modeling process consists of a syntactic approach employing a series of coupled differential equations written to describe the metabolic system under investigation[99, 129-132]. CWave derived algebraic equations for all metabolic reactions and then solved them numerically from experimentally determined isotope patterns (see Supplemental Information). These measured enrichment data serve as input functions to stochastically compute kinetic and steady state metabolic flow and simulate the unknown (unmeasured) parameters of the atom fate map [110, 117, 133, 134]. Figure (33) shows the metabolic model of the Krebs cycle and its constituent pathways in its entirety including all enzymatic reactions that CWave computed from the isotopologue and isotopomeric data. The position specific information generated by our MIMOSA analysis
provided important constraints to the dynamic model which allowed for precise relative flux analysis with tight standard deviation (Table 10).

The computational model of kinetic and steady state fluxes modeled by CWave provided an opportunity to not only expand but also to cross-validate the metabolic flux analysis performed with MIMOSA. Comparing relative metabolic fluxes both models showed strong congruency between their respective analysis and confirmed that 1) $V_{ICDHr}$, the reductive component of IDH is slower than the oxidative reaction ($V_{ICDHf}$) of IDH leading to a net IDH flux (IDHnet) of approximately 18 umol/kg/min, 2) $V_{ACLy}$ relative to $V_{CS}$, contributes significantly to basal hepatic metabolism. 3) pyruvate cycling was much slower than previously reported with PC much closer to the rate of CS and PEPCK only

Figure 33. Kinetic metabolic model (CWave).
Schematic of the reactions modeled by CWave. From the isotopomeric and isotopologue data generated by MIMOSA, CWave modeled all metabolic rates of the Krebs cycle and adjacent pathways with high confidence. A description of all reactions and abbreviations is provided in detail in the Method Chapter.

The computational model of kinetic and steady state fluxes modeled by CWave provided an opportunity to not only expand but also to cross-validate the metabolic flux analysis performed with MIMOSA. Comparing relative metabolic fluxes both models showed strong congruency between their respective analysis and confirmed that 1) $V_{ICDHr}$, the reductive component of IDH is slower than the oxidative reaction ($V_{ICDHf}$) of IDH leading to a net IDH flux (IDHnet) of approximately 18 umol/kg/min, 2) $V_{ACLy}$ relative to $V_{CS}$, contributes significantly to basal hepatic metabolism. 3) pyruvate cycling was much slower than previously reported with PC much closer to the rate of CS and PEPCK only
1.2 times the rate of CS, 4) the Amino Transferase Exchange rate (Vx) converting glutamate to αKG is extremely fast and thereby makes the cytosolic glutamate pool a kinetic component of the intramitochondrial Krebs cycle whereas 5) the reaction catalyzed by GDH designated as VGDH is much slower as Vx (Table 10).

The only difference between the MIMOSA generated steady state model and the kinetic model created by CWave was the relative rate for PDH (VPDH/VCS). In contrast to MIMOSA CWave uses enrichment data from pyruvate to calculate PDH flux. However, direct measurements of pyruvate and its fractional enrichment are difficult due to pyruvate’s poor signal to noise ratios in the mass spectra. Therefore, relative flux calculations of PDH by CWave suffer from the imprecise data input of pyruvate enrichment.

MIMOSA flux calculations, on the other hand, takes advantage of the fact that the acetyl-Coa moiety of glutamate (C4, C5) originates from pyruvate C2 and C3: pyruvate C2, C3 → acetyl-CoA C1, C2 → glutamate C4, C5. Through strategic fragmentation of glutamate in the tandem MS glutamate’s m/z 41 daughter fragment consists of its C4, C5 of the glutamate carbon backbone which represents the acetyl-CoA moiety coming from pyruvate. Any fractional enrichment of pyruvate can be deduced from the enrichment in the m/z 41 daughter fragment:

**Glutamate C4,5 isotopologues (PDH flux):**

\[
\text{GluC4,5}_0 = [C4,5^{13\text{C}0}\text{Glutamate} = \Sigma(146/41, 147/41, 148/41, 149/41)
\]

\[
\text{GluC4,5}_1 = [C4,5^{13\text{C}1}\text{Glutamate} = \Sigma(147/42, 148/42, 149/42,150/42)
\]

\[
\text{GluC4,5}_2 = [C4,5^{13\text{C}2}\text{Glutamate} = \Sigma(148/43, 149/43, 150/43, 151/43)
\]
Since glutamate spectra have an excellent signal to noise ratio its measurement is much more precise and reliable than pyruvate. Hence, using the enrichment of C4,C5 glutamate as a surrogate input function for metabolic flux analysis results in much more robust rate estimates.
## Comparison of Relative Rates

<table>
<thead>
<tr>
<th>Relative Rate</th>
<th>Steady State Model</th>
<th>CWave Kinetic Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD ( +/- )</td>
</tr>
<tr>
<td>$V_{PC/VCS}$</td>
<td>115.35</td>
<td>(42.3)</td>
</tr>
<tr>
<td>$V_{Prop/VCS}$</td>
<td>32.72</td>
<td>(8.89)</td>
</tr>
<tr>
<td>$V_{(PEPCK+ME)/VCS}$</td>
<td>157.20</td>
<td>(45.22)</td>
</tr>
<tr>
<td>$V_{PDH/VCS}$</td>
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<td>(22.91)</td>
</tr>
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<td>(23.78)</td>
</tr>
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<td>$V_{IDHf/VCS}$</td>
<td>163.69</td>
<td>(32.96)</td>
</tr>
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<td>$V_{OGDH/VCS}$</td>
<td>82.39</td>
<td>(11.52)</td>
</tr>
<tr>
<td>$V_{SDH/VCS}$</td>
<td>115.12</td>
<td>(18.76)</td>
</tr>
<tr>
<td>$V_{ACLy/VCS}$</td>
<td>17.6</td>
<td>(11.5)</td>
</tr>
</tbody>
</table>

Table 10. Comparison of steady state and kinetic model.

Comparison of relative rates calculated by MIMOSA Metabolic Model vs CWave Kinetic. Both models showed high congruence with no significant differences between flux rates except for PDH.
CHAPTER V: ASSESSING PITFALLS OF COMMON TRACER STRATEGIES

Existing tracer strategies have led to highly divergent concepts of hepatic metabolism. To better understand the origins of the differences in reported metabolic flux rates we systematically evaluated three of the common tracer strategies for potential liabilities and shortcomings in tracing hepatic metabolism. To this end we performed primed continuous infusion of each "unlabeled/cold" tracer at their respective previously reported infusion rates: L-glutamine (12 μmol/kg/min), L-lactate (40 μmol/kg/min), L-propionate (55 μmol/kg/min) [29, 74, 92, 107, 116, 135]. Di-deuterated-Glucose was co-infused as a tracer for glucose metabolism along with each unlabeled "cold" tracer. Plasma samples were taken at 0, 30, 60, and 120 min for measurement of plasma glucose, alanine, pyruvate, and insulin concentrations as well as glucose enrichments for turnover analysis. At the end of the study, freeze-clamped livers were obtained to measure hepatic metabolites. Results were compared to our glutamine tracer strategy (Figure 34, top panel). We then performed separate experiments where we co-infused [1,2-13C2]-L-glutamine as a tracer of mitochondrial metabolism along with either cold acetate, lactate or propionate to assess whether these existing tracer strategies have an impact on metabolism (Figure 34, bottom panel).
As shown in Figure 35 (top panels) compared to glutamine, which did not perturb glucose homeostasis, acetate, lactate and propionate all significantly increased endogenous glucose production and plasma insulin levels. Furthermore, lactate and propionate also raised glucose concentration significantly from baseline. Additionally, all three tracers
All commonly used tracers impacted glucose homeostasis and liver metabolism. Acetate, glutamine, lactate and propionate were infused as a primed continuous infusion and (a) plasma glucose concentration, (b) endogenous glucose production, (c) plasma insulin concentration, (d) plasma metabolites, (e) liver metabolites were measured. Control group was infused with 1/2NS instead of a cold tracer. N=7-10.

*Figure 35. Contemporary tracers impact glucose metabolism.*
compared to glutamine and thereby perturbed metabolic steady state. Their impact on metabolism was further corroborated by their impact on absolute and relative fluxes in the liver (Figure 36 and 37). Compared to glutamine studies acetate increased anaplerosis and cataplerosis, respectively (\( V_{PC} \) and \( V_{PEPCK+ME} \)), whereas lactate predominantly increased PDH flux. Propionate on the other hand mainly pushed oxidation in the distal half of the Krebs cycle thereby increasing cataplerotic fluxes (PEPCK+ME).
Acetate, Lactate and Propionate alter relative (a) oxidative fluxes, (b) anaplerotic fluxes, (c) cataplerotic fluxes, including (d) ACLy fluxes. N=6-10.

Figure 36. Commonly used tracers impact relative metabolic fluxes.
Acetate, Lactate and Propionate alter absolute (a) oxidative fluxes, (b) anaplerotic fluxes, (c) cataplerotic fluxes, including (d) ACLy fluxes. N=6-10.

*Figure 37. Commonly used tracers impact absolute metabolic fluxes.*
CHAPTER VI: DISCUSSION AND FUTURE APPLICATIONS

Over the past decades, stable isotope based metabolic flux analysis has given us a unprecedented glimpse into the system-wide dynamics of in vivo mitochondrial metabolism that often expanded well beyond the static and isolated snapshots of metabolism yielded by molecular studies, such as metabolomics, RNAseq, and whole genome sequencing [51, 136]. For instance, metabolic flux analyses demonstrated that dysregulated hepatic oxidative and anaplerotic Krebs Cycle fluxes along with increased cataplerotic flux into biosynthetic pathways contribute significantly to hyperglycemia and dyslipidemia manifesting in NAFLD, insulin resistance, and type 2 diabetes mellitus [27, 40-43]. However, at the same time stable isotope-based tracer methods have led to highly divergent and controversial conclusions and concepts of these hepatic Krebs Cycle fluxes [4, 29, 41, 75, 77-79]. It is impossible that minor differences in experimental setup – such as potential differences in the microbiome between animals studied at two different universities – could lead to an up to 5-fold difference in pyruvate cycling measured with $^{13}$C-propionate and lactate as a tracer[4, 41, 42]. The reason for the discrepancies is based in erroneous/spurious flux calculations that occur due to problematic tracer study design and implementation [10, 71, 72, 74, 84, 86, 107, 137]. Unbiased measurements of basal metabolic fluxes through the Krebs cycle and adjacent pathways are crucial for our understanding and investigation of the etiology and progression of obesity, NAFLD, insulin resistance, and diabetes mellitus.

Stable isotope metabolic flux analyses provide a safe and elegant method to study minute changes of highly dynamic metabolic systems and can serve as a critical means of assessing target engagement when identifying novel therapeutic and curative targets.
[60]. Even though a “one size fits all” tracer method may not exist, herein, we reported the systematic development of [1,2-\textsuperscript{13}C\textsubscript{2}]-L-glutamine as a novel tracer strategy that has been systematically validated to provide an unencumbered interrogation of basal hepatic Krebs cycle metabolism and to help solving several of its existing controversies by identifying many of the challenges and shortcomings of existing tracer methods.

[1,2-\textsuperscript{13}C\textsubscript{2}]-L-glutamine generated mass isotopomers interrogate \textit{in vivo} hepatic metabolism with minimal tracer interference

A metabolic isotope tracer is a molecule which is subject to the same physiological processes as its respective tracee but does not interfere with metabolism. One of the most common concerns regarding stable isotope tracer studies pertains to potential violations of this “Tracer assumption”: Stable isotope-based tracers are not completely mass-less compared to radioisotopes whose radioactive signal can be readily detected even at minuscule concentrations. Signal sensitivity for stable isotopes, on the other hand, is much lower resulting in poor signal to noise ratios. To overcome this insensitivity issue, oftentimes high and rapid infusion rates are necessary to achieve measurable fractional enrichments and thus discernable signal to noise ratios [55]. When infused at rates close to the natural turnover of its tracee, a tracer can add significant mass to the system and shift the equilibrium of the reactions it is supposed to trace. Thereby it can violate tracer assumptions and invalidate its putative tracer function. This change in
enzyme kinetics will ultimately impact metabolic homeostasis. Therefore, it is prudent to find an infusion rate for a stable isotope tracer that does not interfere with its endogenous turnover to avoid perturbing the metabolic system under investigation, yet is high enough to introduce sufficient fractional enrichment of the tracer into the Krebs Cycle and its constituent pathways. We empirically tested multiple infusion rates for \([1,2\text{-}^{13}\text{C}_2]\)-L-glutamine until we identified a rate which allowed for a robust transfer of label from our tracer to downstream traces. Thus, providing measurable signal intensity of key metabolic intermediates, yet without pushing glucose or glutamine metabolism, as demonstrated by a lack of differences in plasma glucose, glycerol, insulin concentrations and endogenous glucose and glutamine turnover rates. Additionally, at our infusion rate (12 mmol/kg/min), tracer signal reached isotopic steady state across all key plasma and liver metabolites quickly and reliably without sacrificing labeling signal. Due to the high costs of isotope tracers and the possible effects of extended infusion times on subjects’ comfort and metabolism, a shorter duration of infusion, as is possible with \([1,2\text{-}^{13}\text{C}_2]\)-L-glutamine, is preferred by many investigators.

\([1,2\text{-}^{13}\text{C}_2]\)-L-glutamine provides distinct mass spectra amenable to MIMOSA

An inherent limitation of stable isotope-based approaches has been the restricted number of metabolites and isotopomers that can be measured by either NMR or Mass Spectrometry alone. Scarcity of directly measurable fractional enrichment of key metabolites make a metabolic flux model reliant on the accuracy of its underlying assumptions and \textit{in silico} approximations of the unknown parameters. However, making the right assumptions can be complicated by complex and at times overlapping labeling
patterns leading to ambiguity of the data used for in silico simulation and subsequent stable isotope-based flux analysis. Complex and ambiguous mass spectra commonly stem from choice of tracer, position of label within the tracer’s carbon backbone, distribution of label across one or multiple turns of the Krebs cycle and label recycling in the form of “secondary tracers,” thereby leading to complex, labile axiomatic dependencies on higher order algebraic equations needed to approximate flux through a metabolic system. For example, the more complex the mathematical analysis the more liable it is to perpetuating and compounding experimental and analytical errors which in turn will result in erroneous flux calculations. [1,2-\textsuperscript{13}C\textsubscript{2}]-L-glutamine generated distinguishable isotopomer and isotopologue species of key metabolites that were free of any overlapping, convoluted primary or secondary labeling schemes and thus amenable for novel application of our in vivo analytical platform MIMOSA [71]. Due to the lack of any ambiguity of quantitative and qualitative tracer and tracee measurements at isotopic dynamic and steady state, we were able to directly interpret the carbon-specific kinetic flow of isotope label across sequential key metabolites and along intersecting metabolic pathways. The unambiguous fractional enrichment patterns provided the necessary input functions to directly solve and calculate all key metabolic fluxes through first order differential equations.
Not all Krebs cycle fluxes are equal

Due to its low sensitivity, in vivo $^{13}$C-NMR cannot detect label signal from Krebs cycle intermediates directly. Instead, it relies on measuring signal from higher abundant, cytosolic metabolites, such as Aspartate and Glutamate, that are in direct exchange with Krebs cycle intermediates (OAA and αKG, respectively). Since the actual Krebs cycle intermediates are “invisible” to NMR, all oxidative reactions of the Krebs cycle have traditionally been reported and for metabolic modeling purposes simplified as one circular flux pathway, the rate of which is often denoted as $V_{TCA}$. Combining the glutamine tracer approach with our MS/MS based MIMOSA, we were able to trace individual steps of the Krebs cycle and calculate relative rates for all of its major metabolic reactions. We found that all oxidative Krebs cycle fluxes are not equal and collapsing them into one singular rate is an oversimplification. For instance, succinate dehydrogenase flux ($V_{SDH}$) is about 15% larger than citrate synthase flux ($V_{CS}$) whereas $V_{OGDH}$, and isocitrate dehydrogenase net ($V_{IDHnet}$) are only $\frac{3}{4}$ of CS. These different oxidative rates exemplify the fact that the Krebs cycle must be viewed as a series of individual reactions that adjust to their adjacent anaplerotic and cataplerotic fluxes accordingly to maintain mass balance. This is of particular importance when tracer studies are designed to interrogate only parts of the Krebs Cycle, but the data are used to make estimates about the Krebs Cycle rate as a whole or about proximal flux rates, such as $V_{CS}$. 
There are three major anaplerotic entry points into the Krebs cycle

Another crucial finding pertains to anaplerotic (dilutional) fluxes. The importance of assessing and accounting for all anaplerotic fluxes properly is underlined by the successive dilution of label across Krebs cycle intermediates with one turn of the Krebs cycle. One implicit assumption that goes into the calculations of most metabolic models of hepatic metabolism is that there is no dilution of label as the cycle turns. However, our data demonstrated that there is significant sequential dilution of label through one turn of the Krebs cycle. As a matter of fact, tracer enrichment was reduced by approximately 70% through one full turn of the Krebs cycle. In addition to the commonly reported anaplerotic flux through PC we found significant anaplerotic contribution from two other sources, that is, glutamate and propionate. These anaplerotic fluxes have previously been regarded as negligible. However, while PC still accounted for 75% of all anaplerotic fluxes, anaplerosis from propionate constituted approximately 20% and glutamate approximately 5%. The unexpectedly large anaplerotic flux from propionate underlines that there is significant contribution from endogenous propionate, most likely coming from gut fermentation, to hepatic metabolism in the basal state. Assuming minimal dilution of label as it traverses the Krebs cycle and omitting to factor it into flux calculations, especially when using surrogate metabolites as input functions for metabolic modeling, will result in spuriously low $V_{TCA}$ rates and spuriously high cataplerotic fluxes, such as PEPCK, by falsely attributing a reduction in the tracer to downstream tracee ratio to low activity of oxidative reactions rather than to dilutional flux.
Isocitrate consists of two distinct reactions and ACLy contributes significantly to basal hepatic metabolism

Isocitrate dehydrogenase catalyzes the step in the Krebs cycle between Isocitrate and αKG. IDH’s enzymatic function is often simply described as one reversible exchange reaction. However, our data demonstrated that this simplification does not hold true. IDH catalyzes rather two very distinct, independent enzymatic steps: an oxidative (forward) reaction (IDHf) converts Isocitrate to αKG and a reductive carboxylation (reverse) reaction (IDHr) which carboxylates αKG to Isocitrate. Since IDHf flux exceeds IDHr, there is an overall net flux (IDHnet) towards αKG. However, the significant reverse carbon flow from αKG to Isocitrate and ultimately to Citrate at baseline is of tremendous importance for two reasons: First, IDHr is a crucial step in one of the anabolic functions of the Krebs cycle because it provides Citrate for ATP Citrate Lyase. ACLy catalyzes the cleavage of citrate into cytosolic malate and acetate which after activation to acetyl CoA is used as a building block for the biosynthesis of lipids in the process of de novo lipogenesis (DNL) and cholesterol in the mevalonate pathway. Previously thought to predominantly occur in the fed state [5, 6] we are showing here that flux through ACLy makes up almost 20% of basal hepatic metabolism in the fasted state. While DNL might mainly occur in the fed state during a surplus of fuel substrates, hepatic cholesterologenesis is reciprocal to exogenous cholesterol intake and highest at night during fasting with the majority of basal endogenous cholesterol production coming from the liver [138{Schroor, 2019 #822}]. Thus, explaining significant ACLy contribution at baseline. Moreover, acetyl-CoA is not only an important substrate for lipid and cholesterol production but a vital building block in ketogenesis and an allosteric activator of PC, a key step in gluconeogenesis [31-37].
In the prolonged fasted state, the brain relies heavily not only on endogenous glucose production but on alternative fuels such as ketone bodies when exogenous glucose supplies diminish. Ketones can easily cross the blood-brain barrier and can be used as energy sources in the central nervous system. Besides its direct role as a metabolite in energy metabolism, AcCoA is also an important substrate for histone acetylation and thus an vital epigenetic regulator of DNA [139]. Secondly, acknowledging ACLy’s active role during basal hepatic mitochondrial metabolism is important because ACLy flux adds significantly to the overall pool of malate and therefore of OAA, which serves as something of a metabolic hub that links the Krebs cycle to gluconeogenesis. Depending on the tracer strategy and analytical platform, significant flux through ACLy can bias the interpretation of relative fluxes when only measuring malate or its trapping pool (aspartate). Falsely attributing all of malate’s fractional enrichment to V_{TCA} and not correcting for V_{ACLy} will result in spurious oxidative rates. Overall, under fasting conditions, reductive carboxylation and ACLy appear to play

Figure 38. ACLy’s role in basal metabolism. The role of ACLy in de novo lipogenesis, cholesterologenesis, epigenetic histone and protein modulation.
a much more important role in cell homeostasis than previously assumed and flux through these mitochondrial nodes needs to be factored into accurate flux analysis.

**Glutamate is a kinetic component of the Krebs cycle due to rapid exchange reaction Vx**

An important base assumption of $^{13}$CNMR based metabolic modeling pertains to the aminotransferase exchange rate ($V_X$) between the cytosolic glutamate and the intramitochondrial $\alpha$KG pool. Due to its low sensitivity, NMR cannot measure Krebs cycle intermediates directly and relies on measurements of high abundance surrogate metabolites that are trapping pools for low abundance intermediates, such as glutamate for $\alpha$KG. An inherent assumption of most NMR metabolic modeling schema is that $V_X$ is so fast that glutamate becomes a kinetic component of the Krebs cycle and an immediate readout of $\alpha$KG labeling [94, 96, 99, 100]. Inaccuracy of this assumption, i.e., if the exchange reaction is not significantly faster than the Krebs cycle or varies substantially under different conditions, would mean that $V_X$ needs to be determined separately for each experimental condition to calculate accurate Krebs cycle reactions by NMR. One reason we chose glutamine as a tracer method is its strategic entry point into Krebs cycle which allowed for an unprecedented direct kinetic measurement of $V_X$ *in vivo*. Tracing the kinetic transfer of label between specific glutamate and succinate isotopomers we were able to show that glutamate is indeed in fast exchange with the intramitochondrial metabolite pool and $^{13}$C label equilibrates within less than ~5-10 minutes. These data thereby validate the assumption that signal from labeled glutamate is a reliable readout of $\alpha$KG enrichments and thus can be used as a surrogate input function in metabolic modeling of the mitochondrial metabolism.
Glucose serves as an extramitochondrial trapping pool

An additional advantage of our tracer strategy is that glucose is a trapping pool for \([1,2^{13}C_2]-L\text{-glutamine}\) and thereby serves as a convenient systemic readout of hepatic glucose production that is easily accessible for minimally invasive clinical testing. Tracing glucose labeling would allow for assessment of changes in gluconeogenic fluxes which are often altered in fatty liver and diabetes mellitus.

\[1,2^{13}C_2\text{-L-glutamine}\] minimizes uncertainties of metabolic modeling and shows strong congruencies between MIMOSA and computational analysis

Many assumptions about unknown parameters regarding label distribution and transformation are inherent in the development of isotope fate maps and ultimately in metabolic modeling. Oftentimes elaborate series of mathematical equations are necessary to approximate individual reactions of a metabolic system. The elaborate scope of direct isotopic measurements provided by \([1,2^{13}C_2]-L\text{-glutamine}\) in conjunction with MIMOSA provided a solid foundation that could be used as inputs for mathematical modeling software CWave [128]. Thus, assumption about unknown parameters could be replaced by directly measured fractional enrichments of key metabolites. CWave was able to compute the full panoply of Krebs cycle isotopomers, calculate absolute and relative metabolic fluxes with high certainty and thereby construct a highly constrained, comprehensive metabolic flux model. The metabolic flux analysis performed with CWave
was used to cross-validate the flux calculations made with MIMOSA. We found high congruency between the data output from both modeling approaches. Most modeling software requires powerful processors and a certain level of expertise. Showing that our initial “manual” metabolic flux analysis was highly consistent with the computational output underlines the robustness of our approach and offers a “user-friendly” alternative for investigators performing isotope studies.

**Absolute Krebs cycle rates were calculated by bootstrapping relative rates on EGP**

Absolute Krebs cycle rates were determined by “bootstrapping” an externally measured absolute rate, such as endogenous glucose production (EGP, glucose Ra) onto relative metabolic rates derived from steady state equations [15, 123]. Under the assumption that during fasting all gluconeogenic flux comes from PEPCK, absolute rates for CS were determined by back-calculating from direct measurements of EGP multiplied by relative fluxes of V_{PEPCK+ME}/V_{CS}. All other absolute fluxes were then calculated using their ratio relative to V_{CS}[4, 15]. Relative metabolic fluxes give insight into the fractional contribution of one metabolic flux/reaction relative to another, e.g., V_{SDH} to V_{CS}, which is informative for determining directionality of metabolic processes and relative contribution of one pathway to another. However, when both fluxes increase or decrease proportionately, relative fluxes remain the same even though the overall metabolic flux has changed. For instance, even though flux through PEPCK might not be statistically significantly increased relative to CS in clinically latent insulin resistance because CS rate increased simultaneously, but absolute flux through gluconeogenesis might already be significantly
elevated and herald ensuing diabetes mellitus. Therefore, measuring both flux rates and ratios will complete the metabolic picture and bridge their respective shortcomings.

**Liabilities of existing tracer methods**

Applying our glutamine tracer strategy, we investigated the root cause of the controversial concepts of basal hepatic metabolism. Unlike *in vitro* systems that are closed and more controllable, *in vivo* systems are open and susceptible to systemic physiologic changes that could impact metabolism in the tissue of interest. The liver as a central organ of glucose, lipid and amino acid metabolism is specifically sensitive to minute changes in substrate concentrations and hormone levels. Therefore, accounting for a tracer’s specific liabilities and comprehensively validating it for the metabolic study at hand is extremely important to avoid spurious results. To investigate potential liabilities of current tracer methods that have led to recent metabolic controversies, we applied a doubly labeled glutamine strategy as an independent tracer method and assessed all absolute and relative Krebs cycle rates after infusion of unlabeled L-acetate, L-lactate, or L-propionate at one of their reported infusion rates [29, 74, 92, 107, 116, 135]. We found that, unlike glutamine, all three contemporary tracer methods impacted glucose homeostasis and central carbon metabolism. Acetate is activated to acetyl CoA which is an allosteric activator of PC [16-22] and pyruvate dehydrogenase complex kinase [140] consequently increasing anaplerotic flux and decreasing substrate flux into the oxidative part of the Krebs cycle. Accordingly, our absolute and relative flux analysis showed that acetate increased anaplerosis and cataplerosis ($V_{PC}$ and $V_{PEPCK+ME}$, respectively). In contrast, lactate predominantly increased PDH flux. These data stand to reason because lactate
is converted to pyruvate which in turn activates PDH through inhibition of pyruvate dehydrogenase kinase [51]. Increased PDH and decreased PEPCK activity might further be amplified by the elevated insulin levels we observed during the Lactate infusion because pyruvate dehydrogenase phosphatase is stimulated by insulin [23]. Propionate increased oxidation in the distal half of the Krebs cycle, thereby pushing cataplerotic fluxes ($V_{\text{PEPCK+ME}}$) and substrate cycling. Propionyl-CoA, the activated propionate species, has been described as a potent activator of PC and identified to play a role in increasing EGP [4, 141]. Our data along with previous [4, 142, 143] studies also demonstrated that propionate at commonly used infusion rates significantly increased concentrations of Krebs cycle intermediates. The resultant metabolic push into the distal part of the Krebs cycle leads to increased flux through malate dehydrogenase/ME which decreases the NAD+/NADH molar ratio. Reduced levels of NAD+ can lead to decreased flow through PDH and increased pyruvate cycling and gluconeogenesis. Taken together, these data shed light on possible explanations for the divergence of concepts of hepatic metabolism. Different tracers have their respective strengths tracing specific aspects of metabolism; however, here we have shown that if not carefully validated these tracers can push and alter metabolism. Thereby, it is crucial to validate that the key assumption of stable isotope tracing: the tracer must simply trace but not direct the metabolic fate of label in order to avoid spurious flux rate calculations. Moreover, some changes were not obvious from relative rates alone but became significant when absolute rates were assessed. This further highlights the importance of taking a comprehensive approach when investigating metabolism rather than relying on isolated aspects.
Potential pitfalls of glutamine as a tracer

We have not yet fully validated glutamine as a tracer of Krebs cycle flux in tissues other than the liver. Even though we did not find any unexpected secondary labeling patterns from glutamine in kidney, muscle, or WAT, its effect on mitochondrial metabolism in those tissues still needs to be assessed. The ideal infusion rate may vary based on the condition under investigation. For example, a tracer is more likely to provide substrate and violate tracer assumptions under conditions of prolonged starvation than in fed, substrate-replete animals, so ideally one should validate the infusion rates under their conditions of interest. For instance, the kidney is reported to be a major site of Glutamine metabolism, where Glutamine is avidly taken up and used as a preferred substrate for gluconeogenesis. Therefore, glutamine could potentially violate tracer assumptions when used to model renal metabolism, particularly under prolonged fasting conditions where the renal contribution to gluconeogenesis likely increases[144]. Another possible shortcoming is that glutamine has not been evaluated for multiple tracer approaches. Since stable isotope tracer are not completely massless, infusing labeled glutamine along with other labeled tracers might have a cumulative mass effect resulting in potential violation of tracer assumptions. Therefore, adjustments might need to be made to the infusion rate. Another potential pitfall pertains to the bootstrapping method. Under prolonged fasting conditions additional contribution to GNG can come from other sources, such as glycerol, in which case PEPCK flux cannot be used as the sole relative flux related to EGP. Lastly, the overall enrichment of plasma glucose coming from [1,2-13C2]glutamine was fairly low and might not be high enough to be used for future NMR studies to assess substrate contributions to glucose production when infused at our proposed infusion rate.
Concluding remarks

The Krebs cycle is a multifaceted pathway and plays a central role in the etiology and progression of some of the most common metabolic and endocrine diseases, all of which have reached epidemic proportions in our society. Previous stable isotope tracer methods meant to elucidate the underlying metabolic flux aberrations of insulin resistance, T2DM, and NAFLD have resulted in many controversies regarding central carbon metabolism and call for a critical assessment of stable isotope tracer methodology. The identified limitations ranged from interference of the tracer with metabolism, insufficient or too complicated tracer enrichments, to technical and analytical shortcomings. To address these issues, we empirically developed and comprehensively validated $[1,2^{-13}C_2]$-L-glutamine as a novel tracer method that in conjunction with our analytical platform MIMOSA expanded the scope of traceable metabolites and added isotopomeric information to the metabolic flux analysis. This approach allowed for direct calculation of previously inaccessible Krebs cycle reactions, such as individual oxidative, anaplerotic and exchange reactions, some of which have never been measured before in vivo. This study highlights that many of the current assumptions of in vivo metabolic modeling in use for metabolic modeling need to be revised and critically revisited. For instance, one novel insight gleaned from our experiments is that label entering the Krebs cycle undergoes sequential dilution at distinct metabolic intersections, such as PCC and GDH. Moreover, ACLy flux contributes significant mass to Malate even under basal conditions. When these significant fluxes are neglected in MFA because only isolated reactions are assessed and used as readouts of the entire cycle, the analysis is liable to miss important
qualitative and quantitative aspects of mitochondrial metabolism and generate misconceptions. [1,2-\textsuperscript{13}C\textsubscript{2}]-L-glutamine provided a comprehensive and holistic interrogation of Krebs Cycle metabolism \textit{in vivo} that can be employed to investigate some of medicine’s most pervasive problems, such as diabetes mellitus, NAFLD and obesity. Therefore, we anticipate it will aid in identifying novel diagnostic and therapeutic targets as well as enable us to identify the underlying metabolic predisposition and progression of NAFLD to NASH and hepatocellular cancer.

**Future Applications**

Going forwards we extend our [1,2-\textsuperscript{13}C\textsubscript{2}]-L-glutamine strategy to measure basal mitochondrial metabolism in additional tissues \textit{in vivo}, such as adipose tissue, kidney and muscle with the intention to expand our metabolic model from the liver to whole body metabolism. By studying isolated organs we get great insights about individual metabolic aspect, but might miss the bigger picture, especially considering that different organs contribute to metabolism in various ways. Understanding the metabolic cross-talk and relative contribution of each organ might hold the answers to some of the unsolved problems in medicine.

Additionally, we will apply our glutamine tracer strategy to study hepatic metabolism in disease models of obesity, NAFLD and hepatocellular cancer to identify early metabolic aberrations that can be targetable for early diagnostic, preventive and/or curative interventions.
CHAPTER VI: MATERIALS AND METHODS

Rodent studies

Sprague-Dawley rats (~250+/−25g) were purchased from Charles River Laboratories and fed normal chow ad lib (Harlan-Teklad 2018). All animal protocols were approved by the Yale University Animal Care and Use Committee (IACUC) prior to performance. Rats underwent surgery under general isoflurane anesthesia to place polyethylene catheters in the left carotid artery (advanced into the proximal aorta) and right jugular vein (PE50, PE90, and PE50 tubing, respectively, Instech Solomon). After 1 week of recovery post surgery, rats were acclimated to the infusion room the night before the infusion study and fasted overnight. Studies began at 8 A.M to avoid contributions of the diurnal cycle to the phenotypes observed. Rats were unrestrained in their cages (Figure 41). At the end of each study each rat was euthanized by intravenous pentobarbital injection. Tissues, such as livers, kidneys, white adipose tissue (WAT), and muscle, were freeze-clamped in situ using tongs pre-chilled in liquid N₂ and stored at -80°C for later analysis [133-135].
All infusion studies were done as primed-continuous infusions (unless pointed out otherwise) into the arterial catheter, either by infusion of a labeled tracer alone or as a co-infusion of a labeled and an unlabeled ("cold") tracer candidate. All isotopes were obtained from Cambridge Isotopes. "Cold" tracers were purchased from Sigma Aldrich. The prime lasted for 5 mins and was 5x the basal infusion rate, followed by the described basal infusion rate for the remainder of the study. Blood samples were drawn through the venous catheter at baseline (before tracer infusion) and at multiple time points throughout and at termination of the study [136]. Blood samples were immediately transferred to a heparin-coated tube (Beckman Coulter), centrifuged at maximum rpm for 0.5 min, and plasma stored at -80°C. Tissue samples were obtained at each endpoint. Tracer infusion rates were based on published studies. For each study, 4-8 rats were studied per time point per label based on power calculations from pilot experiments. Time points measurements included a baseline at time 0 (prior to infusion

*Figure 39. Set up of rat infusion study.*

Tracer Infusion Study Setup. Sprague-Dawley rats are unrestrained in clean cages. Tracer is infused through a central arterial catheter and plasma samples are obtained through a jugular venous catheter.
of label), followed by three to four early time points (e.g., 5, 10, 15, 30 minutes) to define the shape of the build-up curves and 3-4 late time points (e.g., 90, 120 minutes) to define isotopic steady and metabolic state. Secondary tracer generation was investigated by continuous infusion of labeled tracer for 240 mins.

**Liquid Chromatography -Mass Spectrometry/Mass Spectrometry Analysis**

For MS analysis approximately 100mg of frozen tissue was lyophilized overnight in pre-chilled 1.7mL Eppendorf tubes. The lyophilized samples were then vortexed in ice cold extraction buffer (50:50 MeOH and ddH2O) in a precooled centrifuge (4°C) and spun at maximum speed for 10 mins. The supernatant was transferred into a Nanosep filter tube and centrifuged for 1 hour at maximum speed in a precooled centrifuge. The filtrate was subsequently transferred to a new pre-chilled Eppendorf tube. 1 ml of ice cold ddH2O was added and then frozen in -80°C. Once the sample was frozen the Eppendorf was placed in the Lyophilizer overnight. The lyophilized samples were resuspended in 50 ul of 50uM D4T spiked ddH2O and then transferred into 96 Well Plates for MS analysis. Reconstituted samples were injected onto a Hypercarb column (3 μm particle size, 3x150 mm, Thermo Fisher Scientific) at a flow rate of 1 mL/min and separated isocratically. Mobile Phase consisted of an aqueous phase (5% Ammonium formate, 20 ul EDTA, 0.03% acetyl acetone) and an organic phase (60% acetonitrile, 35% IPA (HPLC grade), 5% ammonium formate). Samples ionized by electrospray into an ABSCIEX 5500 QTRAP equipped with a SelexION for differential mobility separation (DMS) was acquired using multiple reaction monitoring (MRM). Retention times are confirmed with known standards and peaks integrated using Multiquant (ABSCIEX) using the individual MRM
transition pairs (Q1/Q3) and mobile phase composition for each metabolite and fragment identities were confirmed using $^{13}$C-labeled standards. The atomic percent excess (APE%) was calculated as the quotient between each Q1/Q3 area and the sum of all Q1/Q3 areas from each metabolite multiplied by 100 after correction for background and isotopomer matrix to account for natural abundance carbons for possible parent/daughter ion combination of the positive matrix for each MRM. Isobaric signals were eliminated by DMS spectroscopy. The following settings: 1. Collision Cell Exit Potential (CXP), Collision Energy (CE), Declustering Potential (DP), Entrance Potential (EP), Separation Voltage (SV)-Compensation Voltage (CoV) were optimized for each metabolite studied and are detailed below. 2. MRM transition pairs (Q1/Q3), carbons analyzed in each fragment, mobile phase and M+n refers to the molecular weight (M) plus number of 13C (n). Validation of LC-MS measurement of positional enrichment of TCA intermediates: LC-MS measurements of glutamate, glutamine, acetate, lactate, aspartate and glucose were compared with the positional $^{13}$C labeling of these amino acids measured by POCE-NMR.

**LC-MS/MS and DMS Settings**

1. Approximate Collision Cell Exit Potential (CXP), Collision Energy (CE), Declustering Potential (DP), Entrance Potential (EP), Separation Voltage (SV)-Compensation Voltage (CoV) pair for each metabolite studied. 2. MRM transition pairs (Q1/Q3), carbons analyzed in each fragment, mobile phase and M+n refers to the molecular weight (M) plus number of 13C (n).
DMS settings:

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<th>Q3</th>
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<th>DP</th>
<th>EP</th>
<th>CE</th>
<th>CXP</th>
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Metabolite isotopologues and their parent/daughter ion m/z in MS/MS:

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Gas Chromatography-Mass Spectrometry Analysis

Whole blood was spun down and plasma was separated. After spiking with [2-\textsuperscript{13}C\textsubscript{1}]-Glycerol (100 uM) as an internal standard, 20ul of plasma was used for plasma glucose/glycerol derivatization with 1:1 acetic anhydride:pyrimidine, following Ba(OH)\textsubscript{2}/ZnSO\textsubscript{4} deproteinization. GC-MS analysis of plasma samples was performed with a Hewlett-Packard 5890 gas chromatograph (HP-1 capillary column, 12 m x 0.2 mm x 0.33 Mm film thickness) interfaced to a Hewlett-Packard 5971A Mass Selective Detector operating in the positive chemical ionization mode with methane as the reagent gas. Selected ion monitoring was used to determine glucose enrichment and glycerol
concentration. Ions with m/z 200 and 202 were monitored for glucose enrichment and m/z 145 and 146 for glycerol concentrations. The glycerol concentration was calculated from the mass-to-charge ratio (m/z) of 145/146 signal.

**Plasma glucose concentrations**

Plasma glucose concentrations were measured enzymatically using the YSI 2700 Select Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH) or Analox Instruments. The glucose measured is based on the following glucose oxidase reaction: 

\[
\beta - \text{D-Glucose} + \text{O}_2 \rightarrow \text{H}_2\text{O}_2 + \text{D-Gluconic acid}
\]

Hydrogen peroxide diffuses toward the platinum anode in the probe assembly where it gives rise to the probe signal current.

**Plasma insulin concentrations**

Insulin concentrations were measured using the ALPCO Rat Insulin ELISA, a sandwich type immunoassay. The 96-well microplate is coated with a monoclonal antibody specific for insulin. The standards, controls, and samples were added to the microplate wells with the conjugate. The microplate was then incubated at room temperature on a microplate shaker at 700-900 rpm. After completion of the first incubation, the wells were washed with Wash Buffer and blotted dry. TMB Substrate was added, and the microplate incubated a second time at room temperature on a microplate shaker at 700-900 rpm. Once the second incubation was completed, Stop Solution was added, and the optical density (OD) measured by a spectrophotometer at 450 nm. The intensity of the color generated is directly proportional to the amount of insulin in the sample.
Calculation of endogenous turnover production (Tracer Dilution Model)

Rates of basal endogenous turnover were determined as the ratio of the rate of the tracer infusion (µmol.kg-1.min-1) to the plasma APE of the tracer minus the tracer infusion rate (corrected for its enrichment) → Infusion rate (µmol/kg/min) * (Infusate APE/Sample corrected APE-1). Endogenous glucose production (EGP) was determined from steady state plasma enrichments after a primed continuous infusion of [6,6-(D2)]-D-glucose (µmol.kg-1.min-1), whereas endogenous glutamine turnover was calculated from a primed continuous infusion of [1,2-13C2]-L-glutamine (3 and 12 µmol.kg-1.min-1)[102]

NMR Analysis

Approximately 150 mg of tissue was homogenized with 0.1 M HCl/methanol (2:1 vol/wt) followed by extraction with ethanol followed by centrifugation at 20,000×g. Heavy metals were removed from the supernatant on Chelex-100 columns. Samples were then lyophilized for long-term storage at −80 °C or resuspended in 500 µL of phosphate-buffered (50 mM, pH 7.0) D2O/H2O (85/15%) solution (Cambridge Isotopes, Andover, MA) solution containing 3-trimethylsilyl[2,2,3,3-D4]-propionate (0.25 mM), as a chemical shift reference. The samples from each rat were measured individually at 11.75 T on a Bruker AVANCE vertical bore NMR spectrometer (Bruker Corp., Billerica, MA, USA) using
1H-13C heteronuclear editing[50]. Spectral fitting was performed with NMRWizard [137]. Briefly, the heteronuclear editing method consists of the acquisition of two spin-echo measurements, one with a broad-band inversion pulse applied at the $^{13}$C frequency, and the other without the inversion pulse. The difference between the spectra represents protons bound to $^{13}$C (at twice the true intensity), while the sub-spectrum without the inversion pulse represents the protons for $^{13}$C-labeled and the unlabeled compounds (i.e., the total concentrations). $^{13}$C decoupling was applied during the acquisition of the free-induction decays to collapse $^{13}$C-$^1$H couplings, reducing spectral complexity while increasing sensitivity. An 8-ms echo time, 20-s repetition time, 12-ppm sweep width, and 32 768 complex data points were used.

**Natural abundance correction of MRM fragments**

As described previously [61], natural abundance was adjusted based on the value of 1.1%. A corrected isotopomer matrix, $I'(P_m,D_n)$, was generated to account for the presence of natural abundance carbons for each possible parent/daughter ion combination of the positive matrix $I(P_m,D_n)$:

$$I'(P_m,D_n) = I(P_m,D_n) \ast (1 + k(p - m)) - I(P_{m-1},D_n) \ast k((p - d) - (m - n - 1) - I(P_{m-1},D_{n-1}) \ast k(d - (n - 1)))$$

$p$ is the total number of carbons in the parent ion

$d$ is the total number of carbons in the daughter ion
$m$ is the number of 13C in the parent ion

$n$ is the number of 13C in the daughter ion

$I$ is the peak area corresponding to parent ion with mass $P$ from $0 \rightarrow p$ and daughter ion with mass $D$ from $0 \rightarrow d$

$k$ is 0.011 (the natural abundance of 13C in the environment)

$m - n \leq p - d$

Statistical analysis

All data are reported as mean ± SEM (or ±SD as indicated). Comparisons of metabolic parameters were made by a One-Way ANOVA corrected for multiple comparisons using Dunnett’s test or Two-Way ANOVA corrected for multiple comparisons using Tukey’s multiple comparisons test. $P<0.05$ was considered significant (*$p<0.05$, **$p<0.005$, ***$p<0.0005$ and ****$p<0.00005$). Analyses were conducted with GraphPad Prism v7.0. For NMR studies Mean and standard deviations were calculated for each of the fluxes determined under basal conditions using Monte Carlo analysis [24].

Figures and Cartoons

All graphs were made with GraphPad Prism or Microsoft Excel. Cartoons were made with BioRender.
SUPPLEMENTAL INFORMATION

S1. Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear Magnetic Resonance spectroscopy (NMR) is a magnetic resonance spectroscopy (MRS) based, non-invasive analytical technique that allows for dynamic and static investigation/analysis of metabolic/biochemical composition of tissues and body fluids in vivo and in vitro. NMR takes advantage of the quantum property of spin. First described by Bloch and Purcell in 1946 [59], nuclei with an odd mass or odd atomic number can spin around their own axis and thereby create their own magnetic field, referred to as magnetic moment (magnetization). Without an orienting field the direction of the magnetic momentum is random. However, when an external, constant, homogeneous magnetic field is applied the magnetic momentum of the nuclei will align with the magnetic field. The alignment can either be parallel (α spin state) or anti-parallel (β spin state) to the external magnetic field. The α spin state, also called ground state, is a lower energy state compared to the β spin state, also called excited state. The difference in distribution between the α and β spin state generates a measurable magnetization along the z-axis. However, the difference in magnetic moments between parallel and anti-parallel nuclei is extremely small (~0.01%) which explains the overall low sensitivity of NMR. When aligned in the external magnetic field, the magnetic moment of the nucleus will tip on the xy-axes at a specific angle (angular momentum) to the external magnetic field and precess (rotate) around its spin axis. The resonance frequency at which a nucleus rotates is called Larmor frequency (LF). The LF is directly proportional to the
external magnetic field strength, $B_0$, and the intrinsic magnetic properties of the respective nucleus ($\gamma$-gyromagnetic ratio) and thus characteristic to each NMR visible nucleus. The precession of each nucleus in the magnetic field is random and therefore out of phase. This is the reason why there is naturally no observable net magnetic signal in the xy-plane. To obtain a measurable signal that identifies a specific nucleus a radiofrequency transmitter is used to apply a radiofrequency pulse sequence to the magnetic field. Nuclei in the $\alpha$ spin state that precess at a Larmor frequency identical to the frequency of the pulse sequence will transition out of their alignment with the magnetic field into the higher energy $\beta$ spin state and precess in phase. This leads to an observable magnetic signal perpendicular to the external magnetic field in the xy axis. Once the RF pulse ends the nuclei relax back to their original thermodynamic equilibrium and the amplitude of the magnetization signal slowly decreases. This process is called Free Induction Decay (FID). The relaxation time (RT) is the period over which the emitted signal decays. There are two different relaxation times, $T_1$ and $T_2$, spin-lattice (longitudinal) time and spin-spin (transverse) Time, respectively. $T_1$ describes the return of the magnetic momentum along the z axis, which is the direction of the applied magnetic field and $T_2$ describes the return of the magnetic momentum in the xy plane. Differences in relaxation times are an important factor in signal intensity. The amplitude of the emitted signal and its FID is recorded/detected by a radio frequency receiver. The decaying signal of each nucleus is an oscillation of magnetization as it attenuates over time. The oscillation signal, which is equal to the precession frequency of a nucleus, forms a unique FID curve that functions as an identifiable nuclear fingerprint. The FID curve over time is then converted into signal intensities over frequency by a process called Fourier Transformation. Important factors
that impact the precession frequency of a nucleus are the chemical and electronic properties of its molecular environment. For instance, adjacent nuclei and chemical bonds can increase or decrease the precession frequency of a nucleus. The resultant chemical shift in NMR peaks from a known reference frequency is called spin-spin (or J-) coupling. Sometimes chemical shift can lead to a multitude of peaks on the NMR spectrum, all of which reflect the different frequency components of a nucleus within its chemical environment and thus aid in identifying a molecular structure and position specific label information by NMR. The chemical shift is dimensionless and expressed in parts per million (ppm) [55, 57].

S1.1 NMR CWave kinetic modeling

The following analytical process was used in CWave: first order differential input-output equations describing each reaction of the metabolic pathway were used to calculate metabolic fluxes and transport. Metabolic model parameters were obtained from non-linear least squares fitting of the experimental data set and iteratively adjusted. With each set of iterative parameters (IP) a simulated flux model was generated. The uncertainties of the parameters were calculated by Monte Carlo simulation. Herein, simulations of the initial flux model were run iteratively while adding noise to the parameters. The mean of the noise is normalized to zero with a standard deviation that reflect the difference between the experimental data and the simulated data. Every simulated data set was least squares fitted to calculate the SD for each parameter of the metabolic model. This process was repeated thousands of times until the variation of IPs consistently reached
a narrow value [99, 127]. Thus from the time course of label appearance and the plateau enrichments, kinetic constants of transport along with absolute and relative metabolic rates of all key metabolic fluxes describing the Krebs cycle and its constituent metabolic pathway were computed [127].

Table S1. Summary table of all metabolites and reactions included in the mathematical model used for flux analysis computed by CWave.

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<td>Aspartate</td>
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<tr>
<td>bOx</td>
<td>Beta oxidation</td>
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<tr>
<td>Cit</td>
<td>Citrate</td>
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<tr>
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<td>Citrate Synthase</td>
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Table S2. Summary of all reactions computed mathematically by CWave model

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<td>Pyr</td>
<td>→</td>
<td>OAA</td>
<td>PC</td>
</tr>
<tr>
<td>Suc</td>
<td>→</td>
<td>Mal</td>
<td>SDHfor</td>
</tr>
<tr>
<td>Urea (HCO₃)</td>
<td>→</td>
<td>Cit</td>
<td>ICDHrev</td>
</tr>
<tr>
<td>Urea (HCO₃)</td>
<td>→</td>
<td>OAA</td>
<td>PC</td>
</tr>
</tbody>
</table>
S1.2 CWave equations

Mass balance equations in CWave are as follows:

**Acetyl-CoA**

\[ \frac{d\text{AcCoAm}}{dt} = PDH + bOx - CS \]

**Aspartate**

\[ \frac{d\text{Asp}}{dt} = Vx_{OAAAspm} - Vx_{AspOAAm} \]

**Citrate**

\[ \frac{d\text{Citrate}}{dt} = CS + ICDHrev - [ICDHfor + ACL] \]

**Glutamate**

\[ \frac{d\text{Glu}}{dt} = Vx_{KGlu} + Vgln - [\text{GluOut} + Vx_{GluKG}] \]
Glycerol 3 Phosphate

\[
\frac{d\text{Glyc3p}}{dt} = \text{GlcTriNeo} + \text{Vglycerol} - [\text{GlyTri} + \text{GlcNeo}]
\]

\(\alpha\)KG

\[
\frac{d\text{KG}}{dt} = \text{ICDHfor} + \text{Vx_GluKG} - [\text{Vx_KGGlu} + \text{ICDHrev} + \text{KGDH}]
\]

Lactate

\[
\frac{d\text{LacPyr}}{dt} = \text{PK} + \text{Vlac} - [\text{PDH} + \text{PC}]
\]

Malate

\[
\frac{d\text{OAAmMal}}{dt} = \text{PC} + \text{Vx_AspOAAm} + \text{ACL} + 0.5\text{SDH} + 0.5\text{SDH} - [\text{Vx_OAAAspm} + \text{PEPCK} + \text{CS}]
\]

OAA

\[
\frac{d\text{OAAc}}{dt} = \text{ACL} - \text{ACL}
\]

PEP

\[
\frac{d\text{PEP}}{dt} = + \text{GlyTri} + \text{PEPCK} - [\text{PK} + \text{GlcTriNeo}]
\]
Succinate

\[ \frac{d\text{Succ}}{dt} = + V_{\text{prop}} + \text{KGDH} - \text{SDH} \]

Isotope balance equations used in CWave are as follows:

**Acetyl CoA**

\[ \frac{d\text{AcCoAm}_2}{dt} = \text{PDH(LacPyr}_{c3}/\text{LacPyr}) + \text{bOx(IsotNA}_0/\text{IsotNA}) + \text{PDH(LacPyr}_{c1,3}/\text{LacPyr}) - \text{CS(AcCoAm}_2/\text{AcCoAm)} \]

1. \[ \frac{d\text{AcCoAm}_0}{dt} = - \text{CS(AcCoAm0/AcCoAm}\{1\}) \]

2. \[ \frac{d\text{AcCoAm}_1}{dt} = \text{PDH(LacPyr}_{r2}/\text{LacPyr}) + \text{bOx(IsotNA}_0/\text{IsotNA}) + \text{PDH(LacPyr}_{r1,2}/\text{LacPyr}) - \text{CS(AcCoAm}_1/\text{AcCoAm)} \]

3. \[ \frac{d\text{AcCoAm}_{1,2}}{dt} = \text{PDH(LacPyr}_{r2,3}/\text{LacPyr}) + \text{bOx(IsotNA}_0/\text{IsotNA}) + \text{PDH(LacPyr}_{r1,2,3}/\text{LacPyr}) - \text{CS(AcCoAm}_1,2/\text{AcCoAm)} \]
**Aspartate**

1. \( \frac{d\text{Asp}_1}{dt} = V_x_{\text{OAAAspm}(\text{OAAmMal}_1/\text{OAAmMal})} - V_x_{\text{AspOAAm}(\text{Asp}_1/\text{Asp})} \)

2. \( \frac{d\text{Asp}_2}{dt} = V_x_{\text{OAAAspm}(\text{OAAmMal}_2/\text{OAAmMal})} - V_x_{\text{AspOAAm}(\text{Asp}_2/\text{Asp})} \)

3. \( \frac{d\text{Asp}_3}{dt} = V_x_{\text{OAAAspm}(\text{OAAmMal}_3/\text{OAAmMal})} - V_x_{\text{AspOAAm}(\text{Asp}_3/\text{Asp})} \)

4. \( \frac{d\text{Asp}_4}{dt} = V_x_{\text{OAAAspm}(\text{OAAmMal}_4/\text{OAAmMal})} - V_x_{\text{AspOAAm}(\text{Asp}_4/\text{Asp})} \)

5. \( \frac{d\text{Asp}_{1,2}}{dt} = V_x_{\text{OAAAspm}(\text{OAAmMal}_{1,2}/\text{OAAmMal})} - V_x_{\text{AspOAAm}(\text{Asp}_{1,2}/\text{Asp})} \)

6. \( \frac{d\text{Asp}_{1,3}}{dt} = V_x_{\text{OAAAspm}(\text{OAAmMal}_{1,3}/\text{OAAmMal})} - V_x_{\text{AspOAAm}(\text{Asp}_{1,3}/\text{Asp})} \)

7. \( \frac{d\text{Asp}_{1,4}}{dt} = V_x_{\text{OAAAspm}(\text{OAAmMal}_{1,4}/\text{OAAmMal})} - V_x_{\text{AspOAAm}(\text{Asp}_{1,4}/\text{Asp})} \)

8. \( \frac{d\text{Asp}_{2,3}}{dt} = V_x_{\text{OAAAspm}(\text{OAAmMal}_{2,3}/\text{OAAmMal})} - V_x_{\text{AspOAAm}(\text{Asp}_{2,3}/\text{Asp})} \)

9. \( \frac{d\text{Asp}_{2,4}}{dt} = V_x_{\text{OAAAspm}(\text{OAAmMal}_{2,4}/\text{OAAmMal})} - V_x_{\text{AspOAAm}(\text{Asp}_{2,4}/\text{Asp})} \)

10. \( \frac{d\text{Asp}_{3,4}}{dt} = V_x_{\text{OAAAspm}(\text{OAAmMal}_{3,4}/\text{OAAmMal})} - V_x_{\text{AspOAAm}(\text{Asp}_{3,4}/\text{Asp})} \)

11. \( \frac{d\text{Asp}_{1,3,4}}{dt} = V_x_{\text{OAAAspm}(\text{OAAmMal}_{1,3,4}/\text{OAAmMal})} - V_x_{\text{AspOAAm}(\text{Asp}_{1,3,4}/\text{Asp})} \)
12. \( \frac{d\text{Asp}_{1,2,3}}{dt} = V_x_{OAAAspm(OAAmMal_{1,2,3}/OAAmMal)} - V_x_{\text{AspOAAm(Asp}_{1,2,3}/\text{Asp})} \)

13. \( \frac{d\text{Asp}_{1,2,4}}{dt} = V_x_{OAAAspm(OAAmMal_{1,2,4}/OAAmMal)} - V_x_{\text{AspOAAm(Asp}_{1,2,4}/\text{Asp})} \)

14. \( \frac{d\text{Asp}_{2,3,4}}{dt} = V_x_{OAAAspm(OAAmMal_{2,3,4}/OAAmMal)} - V_x_{\text{AspOAAm(Asp}_{2,3,4}/\text{Asp})} \)

15. \( \frac{d\text{Asp}_{1,2,3,4}}{dt} = V_x_{OAAAspm(OAAmMal_{1,2,3,4}/OAAmMal)} - V_x_{\text{AspOAAm(Asp}_{1,2,3,4}/\text{Asp})} \)

**Citrate**

1. \( \frac{d\text{Citrate}_1}{dt} = CS(\text{AcCoAm}_0/\text{AcCoAm})(\text{OAAmMal}_4/\text{OAAmMal}) + ICDH_{rev}(\text{KG}_1/\text{KG}_0/\text{Urea}) - [ICDH_{for} + ACL](\text{Citrate}_1/\text{Citrate}) \)

2. \( \frac{d\text{Citrate}_2}{dt} = CS(\text{AcCoAm}_0/\text{AcCoAm})(\text{OAAmMal}_3/\text{OAAmMal}) + ICDH_{rev}(\text{KG}_2/\text{KG}_0/\text{Urea}) - [ICDH_{for} + ACL](\text{Citrate}_2/\text{Citrate}) \)
3. \( \frac{d\text{Citrate}_3}{dt} = \text{CS}(\text{AcCoAm}_0/\text{AcCoAm})(\text{OAAmMal}_2/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_3/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_3/\text{Citrate}) \)

4. \( \frac{d\text{Citrate}_4}{dt} = \text{CS}(\text{AcCoAm}_2/\text{AcCoAm})(\text{OAAmMal}_0/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_4/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_4/\text{Citrate}) \)

5. \( \frac{d\text{Citrate}_5}{dt} = \text{CS}(\text{AcCoAm}_1/\text{AcCoAm})(\text{OAAmMal}_0/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_5/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_5/\text{Citrate}) \)

6. \( \frac{d\text{Citrate}_6}{dt} = \text{CS}(\text{AcCoAm}_0/\text{AcCoAm})(\text{OAAmMal}_1/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_0/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_6/\text{Citrate}) \)

7. \( \frac{d\text{Citrate}_{1,2}}{dt} = \text{CS}(\text{AcCoAm}_0/\text{AcCoAm})(\text{OAAmMal}_{3,4}/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_{1,2}/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_{1,2}/\text{Citrate}) \)

8. \( \frac{d\text{Citrate}_{1,3}}{dt} = \text{CS}(\text{AcCoAm}_0/\text{AcCoAm})(\text{OAAmMal}_{2,4}/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_{1,3}/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_{1,3}/\text{Citrate}) \)

9. \( \frac{d\text{Citrate}_{1,4}}{dt} = \text{CS}(\text{AcCoAm}_2/\text{AcCoAm})(\text{OAAmMal}_4/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_{1,4}/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_{1,4}/\text{Citrate}) \)
10. \( \text{dCitrate}_{1,5}/dt \) = \( \text{CS} \left( \text{AcCoAm}_1/\text{AcCoAm} \right) (\text{OAAmMal}_{1,4}/\text{OAAmMal}) + \)
\( \text{ICDH}_{\text{rev}}(\text{KG}_{1,5}/\text{KG})0/\text{Urea} \) - \( [\text{ICDH}_{\text{for}} + \text{ACL}] (\text{Citrate}_{1,5}/\text{Citrate}) \)

11. \( \text{dCitrate}_{1,6}/dt \) = \( \text{CS} \left( \text{AcCoAm}_0/\text{AcCoAm} \right) (\text{OAAmMal}_{1,4}/\text{OAAmMal}) + \)
\( \text{ICDH}_{\text{rev}}(\text{KG}_1/\text{KG})1/\text{Urea} \) - \( [\text{ICDH}_{\text{for}} + \text{ACL}] (\text{Citrate}_{1,6}/\text{Citrate}) \)

12. \( \text{dCitrate}_{2,3}/dt \) = \( \text{CS} \left( \text{AcCoAm}_0/\text{AcCoAm} \right) (\text{OAAmMal}_{2,3}/\text{OAAmMal}) + \)
\( \text{ICDH}_{\text{rev}}(\text{KG}_{2,3}/\text{KG})0/\text{Urea} \) - \( [\text{ICDH}_{\text{for}} + \text{ACL}] (\text{Citrate}_{2,3}/\text{Citrate}) \)

13. \( \text{dCitrate}_{2,4}/dt \) = \( \text{CS} \left( \text{AcCoAm}_0/\text{AcCoAm} \right) (\text{OAAmMal}_{2,3}/\text{OAAmMal}) + \)
\( \text{ICDH}_{\text{rev}}(\text{KG}_{2,4}/\text{KG})0/\text{Urea} \) - \( [\text{ICDH}_{\text{for}} + \text{ACL}] (\text{Citrate}_{2,4}/\text{Citrate}) \)

14. \( \text{dCitrate}_{2,5}/dt \) = \( \text{CS} \left( \text{AcCoAm}_1/\text{AcCoAm} \right) (\text{OAAmMal}_{2,3}/\text{OAAmMal}) + \)
\( \text{ICDH}_{\text{rev}}(\text{KG}_{2,5}/\text{KG})0/\text{Urea} \) - \( [\text{ICDH}_{\text{for}} + \text{ACL}] (\text{Citrate}_{2,5}/\text{Citrate}) \)

15. \( \text{dCitrate}_{2,6}/dt \) = \( \text{CS} \left( \text{AcCoAm}_0/\text{AcCoAm} \right) (\text{OAAmMal}_{1,3}/\text{OAAmMal}) + \)
\( \text{ICDH}_{\text{rev}}(\text{KG}_2/\text{KG})1/\text{Urea} \) - \( [\text{ICDH}_{\text{for}} + \text{ACL}] (\text{Citrate}_{2,6}/\text{Citrate}) \)

16. \( \text{dCitrate}_{3,4}/dt \) = \( \text{CS} \left( \text{AcCoAm}_0/\text{AcCoAm} \right) (\text{OAAmMal}_{2,3}/\text{OAAmMal}) + \)
\( \text{ICDH}_{\text{rev}}(\text{KG}_{3,4}/\text{KG})0/\text{Urea} \) - \( [\text{ICDH}_{\text{for}} + \text{ACL}] (\text{Citrate}_{3,4}/\text{Citrate}) \)

17. \( \text{dCitrate}_{3,5}/dt \) = \( \text{CS} \left( \text{AcCoAm}_1/\text{AcCoAm} \right) (\text{OAAmMal}_{2,3}/\text{OAAmMal}) + \)
\( \text{ICDH}_{\text{rev}}(\text{KG}_{3,5}/\text{KG})0/\text{Urea} \) - \( [\text{ICDH}_{\text{for}} + \text{ACL}] (\text{Citrate}_{3,5}/\text{Citrate}) \)
18. \[ \frac{d\text{Citrate}_{3,6}}{dt} = \text{CS}(\text{AcCoAm}_0/\text{AcCoAm})(\text{OAAmMal}_{1,2}/\text{OAAmMal}) + \] 
\[ \text{ICDHrev}(\text{KG}_3/\text{KG})_1/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{3,6}/\text{Citrate}) \]

19. \[ \frac{d\text{Citrate}_{4,5}}{dt} = \text{CS}(\text{AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAmMal}_0/\text{OAAmMal}) + \] 
\[ \text{ICDHrev}(\text{KG}_{4,5}/\text{KG})_0/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{4,5}/\text{Citrate}) \]

20. \[ \frac{d\text{Citrate}_{4,6}}{dt} = \text{CS}(\text{AcCoAm}_2/\text{AcCoAm})(\text{OAAmMal}_{1}/\text{OAAmMal}) + \] 
\[ \text{ICDHrev}(\text{KG}_4/\text{KG})_1/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{4,6}/\text{Citrate}) \]

21. \[ \frac{d\text{Citrate}_{5,6}}{dt} = \text{CS}(\text{AcCoAm}_1/\text{AcCoAm})(\text{OAAmMal}_1/\text{OAAmMal}) + \] 
\[ \text{ICDHrev}(\text{KG}_5/\text{KG})_1/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{5,6}/\text{Citrate}) \]

22. \[ \frac{d\text{Citrate}_{1,2,3}}{dt} = \text{CS}(\text{AcCoAm}_0/\text{AcCoAm})(\text{OAAmMal}_{2,3,4}/\text{OAAmMal}) + \] 
\[ \text{ICDHrev}(\text{KG}_{1,2,3}/\text{KG})_0/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{1,2,3}/\text{Citrate}) \]

23. \[ \frac{d\text{Citrate}_{1,2,4}}{dt} = \text{CS}(\text{AcCoAm}_2/\text{AcCoAm})(\text{OAAmMal}_{3,4}/\text{OAAmMal}) + \] 
\[ \text{ICDHrev}(\text{KG}_{1,2,4}/\text{KG})_0/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{1,2,4}/\text{Citrate}) \]

24. \[ \frac{d\text{Citrate}_{1,2,5}}{dt} = \text{CS}(\text{AcCoAm}_1/\text{AcCoAm})(\text{OAAmMal}_{3,4}/\text{OAAmMal}) + \] 
\[ \text{ICDHrev}(\text{KG}_{1,2,5}/\text{KG})_0/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{1,2,5}/\text{Citrate}) \]
25. \[ \text{dCitrate}_{1,2,6}/dt \] = \[ \text{CS}(\text{AcCoAm}_0/\text{AcCoAm})(\text{OAmMal}_{1,3,4}/\text{OAmMal}) \] + \\
[\text{ICDHrev}(\text{Kg}_{1,2}/\text{Kg})_1/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{1,2,6}/\text{Citrate})]

26. \[ \text{dCitrate}_{1,3,4}/dt \] = \[ \text{CS}(\text{AcCoAm}_2/\text{AcCoAm})(\text{OAmMal}_{2,4}/\text{OAmMal}) \] + \\
[\text{ICDHrev}(\text{Kg}_{1,3,4}/\text{Kg})_0/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{1,3,4}/\text{Citrate})]

27. \[ \text{dCitrate}_{1,3,5}/dt \] = \[ \text{CS}(\text{AcCoAm}_1/\text{AcCoAm})(\text{OAmMal}_{2,4}/\text{OAmMal}) \] + \\
[\text{ICDHrev}(\text{Kg}_{1,3,5}/\text{Kg})_0/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{1,3,5}/\text{Citrate})]

28. \[ \text{dCitrate}_{1,3,6}/dt \] = \[ \text{CS}(\text{AcCoAm}_0/\text{AcCoAm})(\text{OAmMal}_{1,2,4}/\text{OAmMal}) \] + \\
[\text{ICDHrev}(\text{Kg}_{1,3}/\text{Kg})_1/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{1,3,6}/\text{Citrate})]

29. \[ \text{dCitrate}_{1,4,5}/dt \] = \[ \text{CS}(\text{AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAmMal}_{4}/\text{OAmMal}) \] + \\
[\text{ICDHrev}(\text{Kg}_{1,4,5}/\text{Kg})_0/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{1,4,5}/\text{Citrate})]

30. \[ \text{dCitrate}_{1,4,6}/dt \] = \[ \text{CS}(\text{AcCoAm}_0/\text{AcCoAm})(\text{OAmMal}_{1,2,4}/\text{OAmMal}) \] + \\
[\text{ICDHrev}(\text{Kg}_{1,4}/\text{Kg})_1/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{1,4,6}/\text{Citrate})]

31. \[ \text{dCitrate}_{1,5,6}/dt \] = \[ \text{CS}(\text{AcCoAm}_{1}/\text{AcCoAm})(\text{OAmMal}_{1,4}/\text{OAmMal}) \] + \\
[\text{ICDHrev}(\text{Kg}_{1,5}/\text{Kg})_1/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{1,5,6}/\text{Citrate})]

32. \[ \text{dCitrate}_{2,4,6}/dt \] = \[ \text{CS}(\text{AcCoAm}_2/\text{AcCoAm})(\text{OAmMal}_{1,3}/\text{OAmMal}) \] + \\
[\text{ICDHrev}(\text{Kg}_{2,4}/\text{Kg})_1/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{2,4,6}/\text{Citrate})]
33. \( \frac{d\text{Citrate}_{2,3,4}}{dt} = \text{CS}(\text{AcCoAm}_2/\text{AcCoAm})(\text{OAAmMal}_{2,3}/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_{2,3,4}/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_{2,3,4}/\text{Citrate}) \)

34. \( \frac{d\text{Citrate}_{2,3,5}}{dt} = \text{CS}(\text{AcCoAm}_1/\text{AcCoAm})(\text{OAAmMal}_{2,3}/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_{2,3,5}/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_{2,3,5}/\text{Citrate}) \)

35. \( \frac{d\text{Citrate}_{2,3,6}}{dt} = \text{CS}(\text{AcCoAm}_0/\text{AcCoAm})(\text{OAAmMal}_{1,2,3}/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_{2,3}/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_{2,3,6}/\text{Citrate}) \)

36. \( \frac{d\text{Citrate}_{2,4,5}}{dt} = \text{CS}(\text{AcCoAm}_1,2/\text{AcCoAm})(\text{OAAmMal}_{3}/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_{2,4,5}/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_{2,4,5}/\text{Citrate}) \)

37. \( \frac{d\text{Citrate}_{2,5,6}}{dt} = \text{CS}(\text{AcCoAm}_1/\text{AcCoAm})(\text{OAAmMal}_{1,3}/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_{2,5}/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_{2,5,6}/\text{Citrate}) \)

38. \( \frac{d\text{Citrate}_{4,5,6}}{dt} = \text{CS}(\text{AcCoAm}_1,2/\text{AcCoAm})(\text{OAAmMal}_{1}/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_{4,5}/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_{4,5,6}/\text{Citrate}) \)

39. \( \frac{d\text{Citrate}_{3,4,6}}{dt} = \text{CS}(\text{AcCoAm}_2/\text{AcCoAm})(\text{OAAmMal}_{1,2}/\text{OAAmMal}) + \) 
\( \text{ICDHrev}(\text{KG}_{3,4}/\text{KG})/\text{Urea}) - [\text{ICDHfor + ACL}](\text{Citrate}_{3,4,6}/\text{Citrate}) \)
40. \( \frac{d\text{Citrate}_{3,5,6}}{dt} = \text{CS}(\text{AcCoAm}_1/\text{AcCoAm})(\text{OAmMal}_{1,2}/\text{OAmMal}) + \text{ICDHrev}(\text{KG}_{3,5}/\text{KG})/\text{Urea} - [\text{ICDHfor + ACL}](\text{Citrate}_{3,5,6}/\text{Citrate}) \)

41. \( \frac{d\text{Citrate}_{1,2,3,4}}{dt} = \text{CS}(\text{AcCoAm}_2/\text{AcCoAm})(\text{OAmMal}_{2,3,4}/\text{OAmMal}) + \text{ICDHrev}(\text{KG}_{1,2,3,4}/\text{KG})/\text{Urea} - [\text{ICDHfor + ACL}](\text{Citrate}_{1,2,3,4}/\text{Citrate}) \)

42. \( \frac{d\text{Citrate}_{1,2,3,5}}{dt} = \text{CS}(\text{AcCoAm}_1/\text{AcCoAm})(\text{OAmMal}_{2,3,4}/\text{OAmMal}) + \text{ICDHrev}(\text{KG}_{1,2,3,5}/\text{KG})/\text{Urea} - [\text{ICDHfor + ACL}](\text{Citrate}_{1,2,3,5}/\text{Citrate}) \)

43. \( \frac{d\text{Citrate}_{1,2,4,6}}{dt} = \text{CS}(\text{AcCoAm}_2/\text{AcCoAm})(\text{OAmMal}_{1,2,4}/\text{OAmMal}) + \text{ICDHrev}(\text{KG}_{1,2,4}/\text{KG})/\text{Urea} - [\text{ICDHfor + ACL}](\text{Citrate}_{1,2,4,6}/\text{Citrate}) \)

44. \( \frac{d\text{Citrate}_{1,2,5,6}}{dt} = \text{CS}(\text{AcCoAm}_1/\text{AcCoAm})(\text{OAmMal}_{1,2,4}/\text{OAmMal}) + \text{ICDHrev}(\text{KG}_{1,2,5}/\text{KG})/\text{Urea} - [\text{ICDHfor + ACL}](\text{Citrate}_{1,2,5,6}/\text{Citrate}) \)

45. \( \frac{d\text{Citrate}_{1,3,4,6}}{dt} = \text{CS}(\text{AcCoAm}_2/\text{AcCoAm})(\text{OAmMal}_{1,2,4}/\text{OAmMal}) + \text{ICDHrev}(\text{KG}_{1,3,4}/\text{KG})/\text{Urea} - [\text{ICDHfor + ACL}](\text{Citrate}_{1,3,4,6}/\text{Citrate}) \)

46. \( \frac{d\text{Citrate}_{1,3,5,6}}{dt} = \text{CS}(\text{AcCoAm}_1/\text{AcCoAm})(\text{OAmMal}_{1,2,4}/\text{OAmMal}) + \text{ICDHrev}(\text{KG}_{1,3,5}/\text{KG})/\text{Urea} - [\text{ICDHfor + ACL}](\text{Citrate}_{1,3,5,6}/\text{Citrate}) \)

47. \( \frac{d\text{Citrate}_{2,3,4,6}}{dt} = \text{CS}(\text{AcCoAm}_2/\text{AcCoAm})(\text{OAmMal}_{1,2,3}/\text{OAmMal}) + \text{ICDHrev}(\text{KG}_{2,3,4}/\text{KG})/\text{Urea} - [\text{ICDHfor + ACL}](\text{Citrate}_{2,3,4,6}/\text{Citrate}) \)
\[ \text{dCitrate}_{2,3,5,6}/dt = \text{CS}(\text{AcCoAm}_1/\text{AcCoAm})(\text{OAAnMal}_{1,2,3}/\text{OAAnMal}) + \]
\[ \text{ICDHrev}(\text{KG}_{2,3,5}/\text{KG})_{1/\text{Urea}} - [\text{ICDHfor + ACL}](\text{Citrate}_{2,3,5,6}/\text{Citrate}) \]

\[ \text{dCitrate}_{1,2,4,5}/dt = \text{CS}(\text{AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAnMal}_{3,4}/\text{OAAnMal}) + \]
\[ \text{ICDHrev}(\text{KG}_{1,2,4,5}/\text{KG})_{0/\text{Urea}} - [\text{ICDHfor + ACL}](\text{Citrate}_{1,2,4,5}/\text{Citrate}) \]

\[ \text{dCitrate}_{1,2,4,5,6}/dt = \text{CS}(\text{AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAnMal}_{1,3,4}/\text{OAAnMal}) + \]
\[ \text{ICDHrev}(\text{KG}_{1,2,4,5}/\text{KG})_{1/\text{Urea}} - [\text{ICDHfor + ACL}](\text{Citrate}_{1,2,4,5,6}/\text{Citrate}) \]

\[ \text{dCitrate}_{3,4,5}/dt = \text{CS}(\text{AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAnMal}_{2}/\text{OAAnMal}) + \]
\[ \text{ICDHrev}(\text{KG}_{3,4,5}/\text{KG})_{0/\text{Urea}} - [\text{ICDHfor + ACL}](\text{Citrate}_{3,4,5}/\text{Citrate}) \]

\[ \text{dCitrate}_{2,4,5,6}/dt = \text{CS}(\text{AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAnMal}_{1,3}/\text{OAAnMal}) + \]
\[ \text{ICDHrev}(\text{KG}_{2,4,5}/\text{KG})_{1/\text{Urea}} - [\text{ICDHfor + ACL}](\text{Citrate}_{2,4,5,6}/\text{Citrate}) \]

\[ \text{dCitrate}_{1,3,4,5}/dt = \text{CS}(\text{AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAnMal}_{2,4}/\text{OAAnMal}) + \]
\[ \text{ICDHrev}(\text{KG}_{1,3,4,5}/\text{KG})_{0/\text{Urea}} - [\text{ICDHfor + ACL}](\text{Citrate}_{1,3,4,5}/\text{Citrate}) \]

\[ \text{dCitrate}_{1,4,5,6}/dt = \text{CS}(\text{AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAnMal}_{1,4}/\text{OAAnMal}) + \]
\[ \text{ICDHrev}(\text{KG}_{1,4,5}/\text{KG})_{1/\text{Urea}} - [\text{ICDHfor + ACL}](\text{Citrate}_{1,4,5,6}/\text{Citrate}) \]
55. \( \frac{d\text{Citrate}_{2,3,4,5}}{dt} = \text{CS(AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAmMal}_{2,3}/\text{OAAmMal}) + \text{ICDHrev(KG}_{2,3,4,5}/\text{KG})0/\text{Urea}) - \left[ \text{ICDHfor + ACL}(\text{Citrate}_{2,3,4,5}/\text{Citrate}) \right] \)

56. \( \frac{d\text{Citrate}_{3,4,5,6}}{dt} = \text{CS(AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAmMal}_{1,2}/\text{OAAmMal}) + \text{ICDHrev(KG}_{3,4,5}/\text{KG})1/\text{Urea}) - \left[ \text{ICDHfor + ACL}(\text{Citrate}_{3,4,5,6}/\text{Citrate}) \right] \)

57. \( \frac{d\text{Citrate}_{1,3,4,5,6}}{dt} = \text{CS(AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAmMal}_{1,2,4}/\text{OAAmMal}) + \text{ICDHrev(KG}_{1,3,4,5}/\text{KG})1/\text{Urea}) - \left[ \text{ICDHfor + ACL}(\text{Citrate}_{1,3,4,5,6}/\text{Citrate}) \right] \)

58. \( \frac{d\text{Citrate}_{1,2,3,4,5}}{dt} = \text{CS(AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAmMal}_{2,3,4}/\text{OAAmMal}) + \text{ICDHrev(KG}_{1,2,3,4,5}/\text{KG})0/\text{Urea}) - \left[ \text{ICDHfor + ACL}(\text{Citrate}_{1,2,3,4,5}/\text{Citrate}) \right] \)

59. \( \frac{d\text{Citrate}_{2,3,4,5,6}}{dt} = \text{CS(AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAmMal}_{1,2,3,4}/\text{OAAmMal}) + \text{ICDHrev(KG}_{2,3,4,5}/\text{KG})1/\text{Urea}) - \left[ \text{ICDHfor + ACL}(\text{Citrate}_{2,3,4,5,6}/\text{Citrate}) \right] \)

60. \( \frac{d\text{Citrate}_{1,2,3,6}}{dt} = \text{CS(AcCoAm}_{0}/\text{AcCoAm})(\text{OAAmMal}_{1,2,3,4}/\text{OAAmMal}) + \text{ICDHrev(KG}_{1,2,3}/\text{KG})1/\text{Urea}) - \left[ \text{ICDHfor + ACL}(\text{Citrate}_{1,2,3,6}/\text{Citrate}) \right] \)

61. \( \frac{d\text{Citrate}_{1,2,3,4,6}}{dt} = \text{CS(AcCoAm}_{2}/\text{AcCoAm})(\text{OAAmMal}_{1,2,3,4}/\text{OAAmMal}) + \text{ICDHrev(KG}_{1,2,3,4}/\text{KG})1/\text{Urea}) - \left[ \text{ICDHfor + ACL}(\text{Citrate}_{1,2,3,4,6}/\text{Citrate}) \right] \)

62. \( \frac{d\text{Citrate}_{1,2,3,5,6}}{dt} = \text{CS(AcCoAm}_{1}/\text{AcCoAm})(\text{OAAmMal}_{1,2,3,4}/\text{OAAmMal}) + \text{ICDHrev(KG}_{1,2,3,5}/\text{KG})1/\text{Urea}) - \left[ \text{ICDHfor + ACL}(\text{Citrate}_{1,2,3,5,6}/\text{Citrate}) \right] \)
63. \( \frac{d\text{Citrate}_{1,2,3,4,5,6}}{dt} = \text{CS}(\text{AcCoAm}_{1,2}/\text{AcCoAm})(\text{OAAmMal}_{1,2,3,4}/\text{OAAmMal}) + \text{ICDHrev}(\text{KG}_{1,2,3,4,5}/\text{KG})/\text{Urea}) - [\text{ICDHfor} + \text{ACL}](\text{Citrate}_{1,2,3,4,5,6}/\text{Citrate}) \)

**Glutamate**

1. \( \frac{d\text{Glu}_1}{dt} = V_{x\_KG\text{Glu}}(\text{KG}_1/\text{KG}) + V_{\text{Gln}}(\text{IsotNA0}/\text{IsotNA}) - [\text{GluOut} + V_{x\_\text{GluKG}}](\text{Glu}_1/\text{Glu}) \)

2. \( \frac{d\text{Glu}_2}{dt} = V_{x\_KG\text{Glu}}(\text{KG}_2/\text{KG}) + V_{\text{Gln}}(\text{IsotNA0}/\text{IsotNA}) - [\text{GluOut} + V_{x\_\text{GluKG}}](\text{Glu}_2/\text{Glu}) \)

3. \( \frac{d\text{Glu}_3}{dt} = V_{x\_KG\text{Glu}}(\text{KG}_3/\text{KG}) + V_{\text{Gln}}(\text{IsotNA0}/\text{IsotNA}) - [\text{GluOut} + V_{x\_\text{GluKG}}](\text{Glu}_3/\text{Glu}) \)

4. \( \frac{d\text{Glu}_4}{dt} = V_{x\_KG\text{Glu}}(\text{KG}_4/\text{KG}) + V_{\text{Gln}}(\text{IsotNA0}/\text{IsotNA}) - [\text{GluOut} + V_{x\_\text{GluKG}}](\text{Glu}_4/\text{Glu}) \)

5. \( \frac{d\text{Glu}_5}{dt} = V_{x\_KG\text{Glu}}(\text{KG}_5/\text{KG}) + V_{\text{Gln}}(\text{IsotNA0}/\text{IsotNA}) - [\text{GluOut} + V_{x\_\text{GluKG}}](\text{Glu}_5/\text{Glu}) \)
6. \( \frac{d\text{Glu}_{1,2}}{dt} = V_x \cdot \text{KG}_{\text{Glu}}(\text{KG}_{1,2}/\text{KG}) + V_{\text{Gln}}(\text{Gln}_{\text{Liver}_{1,2}}/\text{Gln}_{\text{Liver}}) - [\text{Glu}_{\text{Out}} + V_x \cdot \text{Glu}_{\text{KG}}](\text{Glu}_{1,2}/\text{Glu}) \)

7. \( \frac{d\text{Glu}_{1,3}}{dt} = V_x \cdot \text{KG}_{\text{Glu}}(\text{KG}_{1,3}/\text{KG}) + V_{\text{Gln}}(\text{Isot}_{\text{NA0}}/\text{Isot}_{\text{NA}}) - [\text{Glu}_{\text{Out}} + V_x \cdot \text{Glu}_{\text{KG}}](\text{Glu}_{1,3}/\text{Glu}) \)

8. \( \frac{d\text{Glu}_{1,4}}{dt} = V_x \cdot \text{KG}_{\text{Glu}}(\text{KG}_{1,4}/\text{KG}) + V_{\text{Gln}}(\text{Isot}_{\text{NA0}}/\text{Isot}_{\text{NA}}) - [\text{Glu}_{\text{Out}} + V_x \cdot \text{Glu}_{\text{KG}}](\text{Glu}_{1,4}/\text{Glu}) \)

9. \( \frac{d\text{Glu}_{1,5}}{dt} = V_x \cdot \text{KG}_{\text{Glu}}(\text{KG}_{1,5}/\text{KG}) + V_{\text{Gln}}(\text{Isot}_{\text{NA0}}/\text{Isot}_{\text{NA}}) - [\text{Glu}_{\text{Out}} + V_x \cdot \text{Glu}_{\text{KG}}](\text{Glu}_{1,5}/\text{Glu}) \)

10. \( \frac{d\text{Glu}_{2,4}}{dt} = V_x \cdot \text{KG}_{\text{Glu}}(\text{KG}_{2,4}/\text{KG}) + V_{\text{Gln}}(\text{Isot}_{\text{NA0}}/\text{Isot}_{\text{NA}}) - [\text{Glu}_{\text{Out}} + V_x \cdot \text{Glu}_{\text{KG}}](\text{Glu}_{2,4}/\text{Glu}) \)
1. \[ d\text{Glu}_{2,5}/dt = V_x \text{KG}_{\text{Glu}}(\text{KG}_{2,5}/\text{KG}) + V_{\text{Gln}}(\text{IsotNA}_0/\text{IsotNA}) - [\text{GluOut} + V_x \text{GluKG}](\text{Glu}_{2,5}/\text{Glu}) \]

2. \[ d\text{Glu}_{3,4}/dt = V_x \text{KG}_{\text{Glu}}(\text{KG}_{3,4}/\text{KG}) + V_{\text{Gln}}(\text{IsotNA}_0/\text{IsotNA}) - [\text{GluOut} + V_x \text{GluKG}](\text{Glu}_{3,4}/\text{Glu}) \]

3. \[ d\text{Glu}_{3,5}/dt = V_x \text{KG}_{\text{Glu}}(\text{KG}_{3,5}/\text{KG}) + V_{\text{Gln}}(\text{IsotNA}_0/\text{IsotNA}) - [\text{GluOut} + V_x \text{GluKG}](\text{Glu}_{3,5}/\text{Glu}) \]

4. \[ d\text{Glu}_{4,5}/dt = V_x \text{KG}_{\text{Glu}}(\text{KG}_{4,5}/\text{KG}) + V_{\text{Gln}}(\text{IsotNA}_0/\text{IsotNA}) - [\text{GluOut} + V_x \text{GluKG}](\text{Glu}_{4,5}/\text{Glu}) \]

5. \[ d\text{Glu}_{1,2,3}/dt = V_x \text{KG}_{\text{Glu}}(\text{KG}_{1,2,3}/\text{KG}) + V_{\text{Gln}}(\text{IsotNA}_0/\text{IsotNA}) - [\text{GluOut} + V_x \text{GluKG}](\text{Glu}_{1,2,3}/\text{Glu}) \]

6. \[ d\text{Glu}_{1,2,4}/dt = V_x \text{KG}_{\text{Glu}}(\text{KG}_{1,2,4}/\text{KG}) + V_{\text{Gln}}(\text{IsotNA}_0/\text{IsotNA}) - [\text{GluOut} + V_x \text{GluKG}](\text{Glu}_{1,2,4}/\text{Glu}) \]

7. \[ d\text{Glu}_{1,3,4}/dt = V_x \text{KG}_{\text{Glu}}(\text{KG}_{1,3,4}/\text{KG}) + V_{\text{Gln}}(\text{IsotNA}_0/\text{IsotNA}) - [\text{GluOut} + V_x \text{GluKG}](\text{Glu}_{1,3,4}/\text{Glu}) \]

8. \[ d\text{Glu}_{1,2,5}/dt = V_x \text{KG}_{\text{Glu}}(\text{KG}_{1,2,5}/\text{KG}) + V_{\text{Gln}}(\text{IsotNA}_0/\text{IsotNA}) - [\text{GluOut} + V_x \text{GluKG}](\text{Glu}_{1,2,5}/\text{Glu}) \]
\[ \begin{align*}
9. \quad dGlu_{1,3,5}/dt &= Vx_{KG}Gl(KG_{1,3,5}/KG) + Vgln(IsotNA0/IsotNA) - [GluOut + \\
&\quad Vx_{GluKG}](Glu_{1,3,5}/Glu) \\
10. dGlu_{1,4,5}/dt &= Vx_{KG}Gl(KG_{1,4,5}/KG) + Vgln(IsotNA0/IsotNA) - [GluOut + \\
&\quad Vx_{GluKG}](Glu_{1,4,5}/Glu) \\
11. dGlu_{2,3,4}/dt &= Vx_{KG}Gl(KG_{2,3,4}/KG) + Vgln(IsotNA0/IsotNA) - [GluOut + \\
&\quad Vx_{GluKG}](Glu_{2,3,4}/Glu) \\
12. dGlu_{2,3,5}/dt &= Vx_{KG}Gl(KG_{2,3,5}/KG) + Vgln(IsotNA0/IsotNA) - [GluOut + \\
&\quad Vx_{GluKG}](Glu_{2,3,5}/Glu) \\
13. dGlu_{2,4,5}/dt &= Vx_{KG}Gl(KG_{2,4,5}/KG) + Vgln(IsotNA0/IsotNA) - [GluOut + \\
&\quad Vx_{GluKG}](Glu_{2,4,5}/Glu) \\
14. dGlu_{3,4,5}/dt &= Vx_{KG}Gl(KG_{3,4,5}/KG) + Vgln(IsotNA0/IsotNA) - [GluOut + \\
&\quad Vx_{GluKG}](Glu_{3,4,5}/Glu) \\
15. dGlu_{1,2,3,4}/dt &= Vx_{KG}Gl(KG_{1,2,3,4}/KG) + Vgln(IsotNA0/IsotNA) - [GluOut + \\
&\quad Vx_{GluKG}](Glu_{1,2,3,4}/Glu) \\
\end{align*} \]
16. $\frac{d\text{Glu}_{1,2,3,5}}{dt} = V_{x\_KG\text{Glu}}(\text{KG}_{1,2,3,5}/\text{KG}) + V_{\text{gln}}(\text{IsotNA0}/\text{IsotNA}) - [\text{GluOut} + V_{x\_\text{GluKG}}(\text{Glu}_{1,2,3,5}/\text{Glu})]$

17. $\frac{d\text{Glu}_{1,2,4,5}}{dt} = V_{x\_KG\text{Glu}}(\text{KG}_{1,2,4,5}/\text{KG}) + V_{\text{gln}}(\text{IsotNA0}/\text{IsotNA}) - [\text{GluOut} + V_{x\_\text{GluKG}}(\text{Glu}_{1,2,4,5}/\text{Glu})]$

18. $\frac{d\text{Glu}_{1,3,4,5}}{dt} = V_{x\_KG\text{Glu}}(\text{KG}_{1,3,4,5}/\text{KG}) + V_{\text{gln}}(\text{IsotNA0}/\text{IsotNA}) - [\text{GluOut} + V_{x\_\text{GluKG}}(\text{Glu}_{1,3,4,5}/\text{Glu})]$

19. $\frac{d\text{Glu}_{2,3,4,5}}{dt} = V_{x\_KG\text{Glu}}(\text{KG}_{2,3,4,5}/\text{KG}) + V_{\text{gln}}(\text{IsotNA0}/\text{IsotNA}) - [\text{GluOut} + V_{x\_\text{GluKG}}(\text{Glu}_{2,3,4,5}/\text{Glu})]$

20. $\frac{d\text{Glu}_{1,2,3,4,5}}{dt} = V_{x\_KG\text{Glu}}(\text{KG}_{1,2,3,4,5}/\text{KG}) + V_{\text{gln}}(\text{IsotNA0}/\text{IsotNA}) - [\text{GluOut} + V_{x\_\text{GluKG}}(\text{Glu}_{1,2,3,4,5}/\text{Glu})]$

**Glycerol 3 Phosphate**

1. $\frac{d\text{Glyc3p1}}{dt} = \text{GlcTriNeo}(\text{PEP}/\text{PEP}) + V_{\text{glycerol}}(\text{IsotNA0}/\text{IsotNA}) - [\text{GlyTri} + \text{GlcNeo}(\text{Glyc3p1}/\text{Glyc3p})]$
2. $\frac{d\text{Glyc}_{3p2}}{dt} = \text{GlcTriNeo}(\text{PEP}_2/\text{PEP}) + \text{Vglycerol}(\text{IsotNA}0/\text{IsotNA}) - \left[\text{GlyTri} + \text{GlcNeo}\right](\text{Glyc}_{3p2}/\text{Glyc}_{3p})$

3. $\frac{d\text{Glyc}_{3p3}}{dt} = \text{GlcTriNeo}(\text{PEP}_3/\text{PEP}) + \text{Vglycerol}(\text{Glycerol}_3/\text{Glycerol}) - \left[\text{GlyTri} + \text{GlcNeo}\right](\text{Glyc}_{3p3}/\text{Glyc}_{3p})$

4. $\frac{d\text{Glyc}_{3p1,2}}{dt} = \text{GlcTriNeo}(\text{PEP}_1,2/\text{PEP}) + \text{Vglycerol}(\text{IsotNA}0/\text{IsotNA}) - \left[\text{GlyTri} + \text{GlcNeo}\right](\text{Glyc}_{3p1,2}/\text{Glyc}_{3p})$

5. $\frac{d\text{Glyc}_{3p1,3}}{dt} = \text{GlcTriNeo}(\text{PEP}_1,3/\text{PEP}) + \text{Vglycerol}(\text{IsotNA}0/\text{IsotNA}) - \left[\text{GlyTri} + \text{GlcNeo}\right](\text{Glyc}_{3p1,3}/\text{Glyc}_{3p})$

6. $\frac{d\text{Glyc}_{3p2,3}}{dt} = \text{GlcTriNeo}(\text{PEP}_2,3/\text{PEP}) + \text{Vglycerol}(\text{IsotNA}0/\text{IsotNA}) - \left[\text{GlyTri} + \text{GlcNeo}\right](\text{Glyc}_{3p2,3}/\text{Glyc}_{3p})$

7. $\frac{d\text{Glyc}_{3p1,2,3}}{dt} = \text{GlcTriNeo}(\text{PEP}_1,2,3/\text{PEP}) + \text{Vglycerol}(\text{IsotNA}0/\text{IsotNA}) - \left[\text{GlyTri} + \text{GlcNeo}\right](\text{Glyc}_{3p1,2,3}/\text{Glyc}_{3p})$
\[d\text{KG}_0/dt = -\left[V_{x\_KG\text{Glu}} + \text{ICDH}_{\text{rev}} + \text{KGDH}\right](\text{KG}_0/\text{KG})\]

2. \[d\text{KG}_1/dt = \text{ICDH}_{\text{for}}(\text{Citrate}_1/\text{Citrate}) + V_{x\_\text{GluKG}}(\text{Glu}_1/\text{Glu}) + \text{ICDH}_{\text{for}}(\text{Citrate}_1,6/\text{Citrate}) - \left[V_{x\_\text{KG}\text{Glu}} + \text{ICDH}_{\text{rev}} + \text{KGDH}\right](\text{KG}_1/\text{KG})\]

3. \[d\text{KG}_2/dt = \text{ICDH}_{\text{for}}(\text{Citrate}_2/\text{Citrate}) + V_{x\_\text{GluKG}}(\text{Glu}_2/\text{Glu}) + \text{ICDH}_{\text{for}}(\text{Citrate}_{2,6}/\text{Citrate}) - \left[V_{x\_\text{KG}\text{Glu}} + \text{ICDH}_{\text{rev}} + \text{KGDH}\right](\text{KG}_2/\text{KG})\]

4. \[d\text{KG}_3/dt = \text{ICDH}_{\text{for}}(\text{Citrate}_3/\text{Citrate}) + V_{x\_\text{GluKG}}(\text{Glu}_3/\text{Glu}) + \text{ICDH}_{\text{for}}(\text{Citrate}_{3,6}/\text{Citrate}) - \left[V_{x\_\text{KG}\text{Glu}} + \text{ICDH}_{\text{rev}} + \text{KGDH}\right](\text{KG}_3/\text{KG})\]

5. \[d\text{KG}_4/dt = \text{ICDH}_{\text{for}}(\text{Citrate}_4/\text{Citrate}) + V_{x\_\text{GluKG}}(\text{Glu}_4/\text{Glu}) + \text{ICDH}_{\text{for}}(\text{Citrate}_{4,6}/\text{Citrate}) - \left[V_{x\_\text{KG}\text{Glu}} + \text{ICDH}_{\text{rev}} + \text{KGDH}\right](\text{KG}_4/\text{KG})\]

6. \[d\text{KG}_5/dt = \text{ICDH}_{\text{for}}(\text{Citrate}_5/\text{Citrate}) + V_{x\_\text{GluKG}}(\text{Glu}_5/\text{Glu}) + \text{ICDH}_{\text{for}}(\text{Citrate}_{5,6}/\text{Citrate}) - \left[V_{x\_\text{KG}\text{Glu}} + \text{ICDH}_{\text{rev}} + \text{KGDH}\right](\text{KG}_5/\text{KG})\]

7. \[d\text{KG}_{1,2}/dt = \text{ICDH}_{\text{for}}(\text{Citrate}_{1,2}/\text{Citrate}) + V_{x\_\text{GluKG}}(\text{Glu}_{1,2}/\text{Glu}) + \text{ICDH}_{\text{for}}(\text{Citrate}_{1,2,6}/\text{Citrate}) - \left[V_{x\_\text{KG}\text{Glu}} + \text{ICDH}_{\text{rev}} + \text{KGDH}\right](\text{KG}_{1,2}/\text{KG})\]

8. \[d\text{KG}_{1,3}/dt = \text{ICDH}_{\text{for}}(\text{Citrate}_{1,3}/\text{Citrate}) + V_{x\_\text{GluKG}}(\text{Glu}_{1,3}/\text{Glu}) + \text{ICDH}_{\text{for}}(\text{Citrate}_{1,3,6}/\text{Citrate}) - \left[V_{x\_\text{KG}\text{Glu}} + \text{ICDH}_{\text{rev}} + \text{KGDH}\right](\text{KG}_{1,3}/\text{KG})\]
9. \[ \frac{dKG_{1,4}}{dt} = \text{ICDHfor(Citrate}_{1,4}/\text{Citrate}) + Vx_{GluKG}(\text{Glu}_{1,4}/\text{Glu}) + \]
   \[\text{ICDHfor(Citrate}_{1,4,6}/\text{Citrate}) - \left[Vx_{KG\text{Glu}} + \text{ICDHrev} + \text{KGDH}\right](\text{KG}_{1,4}/\text{KG}) \]

10. \[ \frac{dKG_{1,5}}{dt} = \text{ICDHfor(Citrate}_{1,5}/\text{Citrate}) + Vx_{GluKG}(\text{Glu}_{1,5}/\text{Glu}) + \]
    \[\text{ICDHfor(Citrate}_{1,5,6}/\text{Citrate}) - \left[Vx_{KG\text{Glu}} + \text{ICDHrev} + \text{KGDH}\right](\text{KG}_{1,5}/\text{KG}) \]

11. \[ \frac{dKG_{2,3}}{dt} = \text{ICDHfor(Citrate}_{2,3}/\text{Citrate}) + Vx_{GluKG}(\text{Glu}_{2,3}/\text{Glu}) + \]
    \[\text{ICDHfor(Citrate}_{2,3,6}/\text{Citrate}) - \left[Vx_{KG\text{Glu}} + \text{ICDHrev} + \text{KGDH}\right](\text{KG}_{2,3}/\text{KG}) \]

12. \[ \frac{dKG_{2,4}}{dt} = \text{ICDHfor(Citrate}_{2,4}/\text{Citrate}) + Vx_{GluKG}(\text{Glu}_{2,4}/\text{Glu}) + \]
    \[\text{ICDHfor(Citrate}_{2,4,6}/\text{Citrate}) - \left[Vx_{KG\text{Glu}} + \text{ICDHrev} + \text{KGDH}\right](\text{KG}_{2,4}/\text{KG}) \]

13. \[ \frac{dKG_{2,5}}{dt} = \text{ICDHfor(Citrate}_{2,5}/\text{Citrate}) + Vx_{GluKG}(\text{Glu}_{2,5}/\text{Glu}) + \]
    \[\text{ICDHfor(Citrate}_{2,5,6}/\text{Citrate}) - \left[Vx_{KG\text{Glu}} + \text{ICDHrev} + \text{KGDH}\right](\text{KG}_{2,5}/\text{KG}) \]

14. \[ \frac{dKG_{3,4}}{dt} = \text{ICDHfor(Citrate}_{3,4}/\text{Citrate}) + Vx_{GluKG}(\text{Glu}_{3,4}/\text{Glu}) + \]
    \[\text{ICDHfor(Citrate}_{3,4,6}/\text{Citrate}) - \left[Vx_{KG\text{Glu}} + \text{ICDHrev} + \text{KGDH}\right](\text{KG}_{3,4}/\text{KG}) \]

15. \[ \frac{dKG_{3,5}}{dt} = \text{ICDHfor(Citrate}_{3,5}/\text{Citrate}) + Vx_{GluKG}(\text{Glu}_{3,5}/\text{Glu}) + \]
    \[\text{ICDHfor(Citrate}_{3,5,6}/\text{Citrate}) - \left[Vx_{KG\text{Glu}} + \text{ICDHrev} + \text{KGDH}\right](\text{KG}_{3,5}/\text{KG}) \]
16. \( \frac{dKG_{4,5}}{dt} = ICDH_{for}(Citrate_{4,5}/Citrate) + Vx\_GluKG(Glu_{4,5}/Glu) + ICDH_{for}(Citrate_{4,5,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{4,5}/KG) \)

17. \( \frac{dKG_{1,2,3}}{dt} = ICDH_{for}(Citrate_{1,2,3}/Citrate) + Vx\_GluKG(Glu_{1,2,3}/Glu) + ICDH_{for}(Citrate_{1,2,3,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{1,2,3}/KG) \)

18. \( \frac{dKG_{1,2,4}}{dt} = ICDH_{for}(Citrate_{1,2,4}/Citrate) + Vx\_GluKG(Glu_{1,2,4}/Glu) + ICDH_{for}(Citrate_{1,2,4,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{1,2,4}/KG) \)

19. \( \frac{dKG_{1,2,5}}{dt} = ICDH_{for}(Citrate_{1,2,5}/Citrate) + Vx\_GluKG(Glu_{1,2,5}/Glu) + ICDH_{for}(Citrate_{1,2,5,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{1,2,5}/KG) \)

20. \( \frac{dKG_{1,4,5}}{dt} = ICDH_{for}(Citrate_{1,4,5}/Citrate) + Vx\_GluKG(Glu_{1,4,5}/Glu) + ICDH_{for}(Citrate_{1,4,5,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{1,4,5}/KG) \)

21. \( \frac{dKG_{1,3,4}}{dt} = ICDH_{for}(Citrate_{1,3,4}/Citrate) + Vx\_GluKG(Glu_{1,3,4}/Glu) + ICDH_{for}(Citrate_{3,4,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{1,3,4}/KG) \)

22. \( \frac{dKG_{1,3,5}}{dt} = ICDH_{for}(Citrate_{1,3,5}/Citrate) + Vx\_GluKG(Glu_{1,3,5}/Glu) + ICDH_{for}(Citrate_{1,3,5,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{1,3,5}/KG) \)
23. \( \frac{dK_{G2,3,4}}{dt} = ICDH_{for}(Citrate_{2,3,4}/Citrate) + Vx\_GluKG(Glu_{2,3,4}/Glu) + ICDH_{for}(Citrate_{2,3,4,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{2,3,4}/KG) \)

24. \( \frac{dK_{G2,3,5}}{dt} = ICDH_{for}(Citrate_{2,3,5}/Citrate) + Vx\_GluKG(Glu_{2,3,5}/Glu) + ICDH_{for}(Citrate_{2,3,5,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{2,3,5}/KG) \)

25. \( \frac{dK_{G2,4,5}}{dt} = ICDH_{for}(Citrate_{2,4,5}/Citrate) + Vx\_GluKG(Glu_{2,4,5}/Glu) + ICDH_{for}(Citrate_{2,4,5,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{2,4,5}/KG) \)

26. \( \frac{dK_{G3,4,5}}{dt} = ICDH_{for}(Citrate_{3,4,5}/Citrate) + Vx\_GluKG(Glu_{3,4,5}/Glu) + ICDH_{for}(Citrate_{3,4,5,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{3,4,5}/KG) \)

27. \( \frac{dK_{G1,2,3,4}}{dt} = ICDH_{for}(Citrate_{1,2,3,4}/Citrate) + Vx\_GluKG(Glu_{1,2,3,4}/Glu) + ICDH_{for}(Citrate_{1,2,3,4,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{1,2,3,4}/KG) \)

28. \( \frac{dK_{G1,2,3,5}}{dt} = ICDH_{for}(Citrate_{1,2,3,5}/Citrate) + Vx\_GluKG(Glu_{1,2,3,5}/Glu) + ICDH_{for}(Citrate_{1,2,3,5,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{1,2,3,5}/KG) \)

29. \( \frac{dK_{G1,2,4,5}}{dt} = ICDH_{for}(Citrate_{1,2,4,5}/Citrate) + Vx\_GluKG(Glu_{1,2,4,5}/Glu) + ICDH_{for}(Citrate_{1,2,4,5,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{1,2,4,5}/KG) \)

30. \( \frac{dK_{G1,3,4,5}}{dt} = ICDH_{for}(Citrate_{1,3,4,5}/Citrate) + Vx\_GluKG(Glu_{1,3,4,5}/Glu_{28}) + ICDH_{for}(Citrate_{1,3,4,5,6}/Citrate) - [Vx\_KG\_Glu + ICDH_{rev} + KGDH](KG_{1,3,4,5}/KG) \)
31. \[ dK_{G2,3,4,5}/dt = \text{ICDHfor}(\text{Citrate}_{2,3,4,5}/\text{Citrate}) + V_x\_\text{GluKG}(\text{Glu}_{2,3,4,5}/\text{Glu}) + \]
\[ \text{ICDHfor}(\text{Citrate}_{2,3,4,5,6}/\text{Citrate}) - [V_x\_\text{KGGlu} + \text{ICDHex} + \text{KGDH}](\text{KG}_{2,3,4,5}/\text{KG}) \]

32. \[ dK_{G1,2,3,4,5}/dt = \text{ICDHfor}(\text{Citrate}_{1,2,3,4,5}/\text{Citrate}) + V_x\_\text{GluKG}(\text{Glu}_{1,2,3,4,5}/\text{Glu}) + \]
\[ \text{ICDHfor}(\text{Citrate}_{1,2,3,4,5,6}/\text{Citrate}) - [V_x\_\text{KGGlu} + \text{ICDHex} + \text{KGDH}](\text{KG}_{1,2,3,4,5}/\text{KG}) \]

**Lactate**

1. \[ dLacPy/\text{pyr0}/dt = - [PDH + PC](LacPy/\text{pyr0}/LacPy/\text{pyr}) \]

2. \[ dLacPy/\text{yr1}/dt = \text{PK}(\text{PEP1}/\text{PEP}) + V\text{lac}(\text{IsotNA0}/\text{IsotNA}) - [PDH + PC](LacPy/\text{yr1}/LacPy/\text{pyr}) \]

3. \[ dLacPy/\text{yr2}/dt = \text{PK}(\text{PEP2}/\text{PEP}) + V\text{lac}(\text{IsotNA0}/\text{IsotNA}) - [PDH + PC](LacPy/\text{yr2}/LacPy/\text{pyr}) \]

4. \[ dLacPy/\text{yr3}/dt = \text{PK}(\text{PEP3}/\text{PEP}) + V\text{lac}(\text{LacBlood3}/\text{LacBlood}) - [PDH + PC](LacPy/\text{yr3}/LacPy/\text{pyr}) \]

5. \[ dLacPy/\text{yr1,2}/dt = \text{PK}(\text{PEP1,2}/\text{PEP}) + V\text{lac}(\text{IsotNA0}/\text{IsotNA}) - [PDH + PC](LacPy/\text{yr1,2}/LacPy/\text{pyr}) \]
6. \( \frac{d\text{LacPyr}_{1,3}}{dt} = PK(\text{PEP}_{1,3}/\text{PEP}) + \text{Vlac}(\text{IsotNA}_0/\text{IsotNA}) - [\text{PDH} + \text{PC}](\text{LacPyr}_{1,3}/\text{LacPyr}) \)

7. \( \frac{d\text{LacPyr}_{2,3}}{dt} = PK(\text{PEP}_{2,3}/\text{PEP}) + \text{Vlac}(\text{IsotNA}_0/\text{IsotNA}) - [\text{PDH} + \text{PC}](\text{LacPyr}_{2,3}/\text{LacPyr}) \)

8. \( \frac{d\text{LacPyr}_{1,2,3}}{dt} = PK(\text{PEP}_{1,2,3}/\text{PEP}) + \text{Vlac}(\text{IsotNA}_0/\text{IsotNA}) - [\text{PDH} + \text{PC}](\text{LacPyr}_{1,2,3}/\text{LacPyr}) \)

**Malate**

1. \( \frac{d\text{OAAmMal}_0}{dt} = - [\text{Vx}_\text{OAAAspm} + \text{PEPCK} + \text{CS}](\text{OAAmMal}_0/\text{OAAmMal}) \)

2. \( \frac{d\text{OAAmMal}_1}{dt} = \text{PC}(\text{LacPyr}_1/\text{LacPyr})_0/\text{Urea}) + \text{Vx}_\text{AspOAAm}(\text{Asp}_1/\text{Asp}) + \text{ACL}(\text{OAAc}_1/\text{OAAc}) + 0.5\text{SDH}(\text{Suc}_1/\text{Suc}) + 0.5\text{SDH}(\text{Suc}_4/\text{Suc}) - [\text{Vx}_\text{OAAAspm} + \text{PEPCK} + \text{CS}](\text{OAAmMal}_1/\text{OAAmMal}) \)

3. \( \frac{d\text{OAAmMal}_2}{dt} = \text{PC}(\text{LacPyr}_2/\text{LacPyr})_0/\text{Urea}) + \text{Vx}_\text{AspOAAm}(\text{Asp}_2/\text{Asp}) + \text{ACL}(\text{OAAc}_2/\text{OAAc}) + 0.5\text{SDH}(\text{Suc}_3/\text{Suc}) + 0.5\text{SDH}(\text{Suc}_2/\text{Suc}) - [\text{Vx}_\text{OAAAspm} + \text{PEPCK} + \text{CS}](\text{OAAmMal}_2/\text{OAAmMal}) \)
4. \[ \frac{d(OAA_{mMal_3})}{dt} = PC(LacPyr_3/LacPyr)_0/IsotNA) + Vx_{AspOAAm}(Asp_3/Asp) + \\
ACL(OAAc_3/OAAc) + 0.5SDH(Suc_3/Suc) + 0.5SDH(Suc_2/Suc) - [Vx_{OAAAspm} + \\
PEPCK + CS](OAA_{mMal_3}/OAA_{mMal}) \]

5. \[ \frac{d(OAA_{mMal_4})}{dt} = PC(LacPyr_0/LacPyr)_0/IsotNA) + Vx_{AspOAAm}(Asp_4/Asp) + \\
ACL(OAAc_4/OAAc) + 0.5SDH(Suc_1/Suc) + 0.5SDH(Suc_4/Suc_{5}) - [Vx_{OAAAspm} + \\
PEPCK + CS](OAA_{mMal_4}/OAA_{mMal}) \]

6. \[ \frac{d(OAA_{mMal_{1,2}})}{dt} = PC(LacPyr_{1,2}/LacPyr)_0/Urea) + Vx_{AspOAAm}(Asp_{1,2}/Asp) + \\
ACL(OAAc_{1,2}/OAAc) + 0.5SDH(Suc_{1,2}/Suc) + 0.5SDH(Suc_{3,4}/Suc) - \\
[Vx_{OAAAspm} + PEPCK + CS](OAA_{mMal_{1,2}}/OAA_{mMal}) \]

7. \[ \frac{d(OAA_{mMal_{1,3}})}{dt} = PC(LacPyr_{1,3}/LacPyr)_0/Urea) + Vx_{AspOAAm}(Asp_{1,3}/Asp) + \\
ACL(OAAc_{1,3}/OAAc) + 0.5SDH(Suc_{1,3}/Suc) + 0.5SDH(Suc_{2,4}/Suc) - \\
[Vx_{OAAAspm} + PEPCK + CS](OAA_{mMal_{1,3}}/OAA_{mMal}) \]

8. \[ \frac{d(OAA_{mMal_{1,4}})}{dt} = PC(LacPyr_{1}/LacPyr)_0/IsotNA) + Vx_{AspOAAm}(Asp_{1,4}/Asp) + \\
ACL(OAAc_{1,4}/OAAc) + SDH(Suc_{1,4}/Suc) - [Vx_{OAAAspm} + PEPCK + \\
CS](OAA_{mMal_{1,4}}/OAA_{mMal}) \]

9. \[ \frac{d(OAA_{mMal_{2,3}})}{dt} = PC(LacPyr_{2,3}/LacPyr)_0/Urea) + Vx_{AspOAAm}(Asp_{2,3}/Asp) + \\
ACL(OAAc_{2,3}/OAAc) + SDH(Suc_{2,3}/Suc) - [Vx_{OAAAspm} + PEPCK + \\
CS](OAA_{mMal_{2,3}}/OAA_{mMal}) \]
10. $\frac{dOAAmMal_{2,4}}{dt} = PC(LacPyr_2/LacPyr)1/Urea) + Vx_{AspOAAm}(Asp_{2,4}/Asp) + ACL(OAAc_{2,4}/OAAc) + 0.5SDH(Suc_{1,3}/Suc) + 0.5SDH(Suc_{2,4}/Suc) - [Vx_{OAAAspm} + PEPCK + CS](OAAmMal_{2,4}/OAAmMal)$

11. $\frac{dOAAmMal_{3,4}}{dt} = PC(LacPyr_3/LacPyr)0/IsotNA) + Vx_{AspOAAm}(Asp_{3,4}/Asp) + ACL(OAAc_{3,4}/OAAc) + 0.5SDH(Suc_{1,2}/Suc) + 0.5SDH(Suc_{3,4}/Suc) - [Vx_{OAAAspm} + PEPCK + CS](OAAmMal_{3,4}/OAAmMal)$

12. $\frac{dOAAmMal_{1,2,4}}{dt} = PC(LacPyr_{1,2}/LacPyr)1/Urea) + Vx_{AspOAAm}(Asp_{1,2,4}/Asp) + ACL(OAAc_{1,2,4}/OAAc) + 0.5SDH(Suc_{1,2,4}/Suc) + 0.5SDH(Suc_{1,3,4}/Suc) - [Vx_{OAAAspm} + PEPCK + CS](OAAmMal_{1,2,4}/OAAmMal)$

13. $\frac{dOAAmMal_{1,3,4}}{dt} = PC(LacPyr_{1,3}/LacPyr)1/Urea) + Vx_{AspOAAm}(Asp_{1,3,4}/Asp) + ACL(OAAc_{1,3,4}/OAAc) + 0.5SDH(Suc_{1,2,4}/Suc) + 0.5SDH(Suc_{1,3,4}/Suc) - [Vx_{OAAAspm} + PEPCK + CS](OAAmMal_{1,3,4}/OAAmMal)$

14. $\frac{dOAAmMal_{2,3,4}}{dt} = PC(LacPyr_{2,3}/LacPyr)0/IsotNA) + Vx_{AspOAAm}(Asp_{2,3,4}/Asp) + ACL(OAAc_{2,3,4}/OAAc) + 0.5SDH(Suc_{1,2,3}/Suc) + 0.5SDH(Suc_{2,3,4}/Suc) - [Vx_{OAAAspm} + PEPCK + CS](OAAmMal_{2,3,4}/OAAmMal)$

15. $\frac{dOAAmMal_{1,2,3}}{dt} = PC(LacPyr_{1,2,3}/LacPyr)0/Urea) + Vx_{AspOAAm}(Asp_{1,2,3}/Asp) + ACL(OAAc_{1,2,3}/OAAc) + 0.5SDH(Suc_{1,2,3}/Suc) + 0.5SDH(Suc_{2,3,4}/Suc) - [Vx_{OAAAspm} + PEPCK + CS](OAAmMal_{1,2,3}/OAAmMal)$
Oxaloacetate

1. $\frac{d\text{OAAc}_1}{dt} = \text{ACL(Citrate}_6/\text{Citrate)} + \text{ACL(Citrate}_{4,6}/\text{Citrate)} +$
   $\text{ACL(Citrate}_{5,6}/\text{Citrate)} + \text{ACL(Citrate}_{4,5,6}/\text{Citrate)} - \text{ACL(OAAc}_1/\text{OAAc)}$

2. $\frac{d\text{OAAc}_2}{dt} = \text{ACL(Citrate}_3/\text{Citrate)} + \text{ACL(Citrate}_{3,4}/\text{Citrate)} +$
   $\text{ACL(Citrate}_{3,5}/\text{Citrate)} + \text{ACL(Citrate}_{3,4,5}/\text{Citrate)} - \text{ACL(OAAc}_2/\text{OAAc)}$

3. $\frac{d\text{OAAc}_3}{dt} = \text{ACL(Citrate}_2/\text{Citrate)} + \text{ACL(Citrate}_{2,4}/\text{Citrate)} +$
   $\text{ACL(Citrate}_{2,5}/\text{Citrate)} + \text{ACL(Citrate}_{2,4,5}/\text{Citrate)} - \text{ACL(OAAc}_3/\text{OAAc)}$

4. $\frac{d\text{OAAc}_4}{dt} = \text{ACL(Citrate}_1/\text{Citrate)} + \text{ACL(Citrate}_{1,4}/\text{Citrate)} +$
   $\text{ACL(Citrate}_{1,5}/\text{Citrate)} + \text{ACL(Citrate}_{1,4,5}/\text{Citrate)} - \text{ACL(OAAc}_4/\text{OAAc)}$

5. $\frac{d\text{OAAc}_{1,2}}{dt} = \text{ACL(Citrate}_3,6/\text{Citrate)} + \text{ACL(Citrate}_{3,4,6}/\text{Citrate)} +$
   $\text{ACL(Citrate}_{3,5,6}/\text{Citrate)} + \text{ACL(Citrate}_{3,4,5,6}/\text{Citrate)} - \text{ACL(OAAc}_{1,2}/\text{OAAc)}$

6. $\frac{d\text{OAAc}_{1,3}}{dt} = \text{ACL(Citrate}_2,6/\text{Citrate}_{11}) + \text{ACL(Citrate}_{2,4,6}/\text{Citrate)} +$
   $\text{ACL(Citrate}_{2,5,6}/\text{Citrate)} + \text{ACL(Citrate}_{2,4,5,6}/\text{Citrate)} - \text{ACL(OAAc}_{1,3}/\text{OAAc)}$

7. $\frac{d\text{OAAc}_{1,4}}{dt} = \text{ACL(Citrate}_1,6/\text{Citrate)} + \text{ACL(Citrate}_{1,4,6}/\text{Citrate)} +$
   $\text{ACL(Citrate}_{1,5,6}/\text{Citratec}) + \text{ACL(Citrate}_{1,4,5,6}/\text{Citrate)} - \text{ACL(OAAc}_{1,4}/\text{OAAc)}$
8. \[ \frac{d\text{OAAc}_{2,3}}{dt} = \text{ACL(Citrate}_{2,3}/\text{Citrate}) + \text{ACL(Citrate}_{2,3,4}/\text{Citrate}) + \text{ACL(Citrate}_{2,3,5}/\text{Citrate}) + \text{ACL(Citrate}_{2,3,4,5}/\text{Citrate}) - \text{ACL(OAAc}_{2,3}/\text{OAAc}) \]

9. \[ \frac{d\text{OAAc}_{2,4}}{dt} = \text{ACL(Citrate}_{1,3}/\text{Citrate}) + \text{ACL(Citrate}_{1,3,4}/\text{Citrate}) + \text{ACL(Citrate}_{1,3,5}/\text{Citrate}) + \text{ACL(Citrate}_{1,3,4,5}/\text{Citrate}) - \text{ACL(OAAc}_{2,4}/\text{OAAc}) \]

10. \[ \frac{d\text{OAAc}_{3,4}}{dt} = \text{ACL(Citrate}_{1,2}/\text{Citrate}) + \text{ACL(Citrate}_{1,2,4}/\text{Citrate}) + \text{ACL(Citrate}_{1,2,5}/\text{Citrate}) + \text{ACL(Citrate}_{1,2,4,5}/\text{Citrate}) - \text{ACL(OAAc}_{3,4}/\text{OAAc}) \]

11. \[ \frac{d\text{OAAc}_{1,3,4}}{dt} = \text{ACL(Citrate}_{1,2,6}/\text{Citrate}) + \text{ACL(Citrate}_{1,2,4,6}/\text{Citrate}) + \text{ACL(Citrate}_{1,2,5,6}/\text{Citrate}) + \text{ACL(Citrate}_{1,2,4,5,6}/\text{Citrate}) - \text{ACL(OAAc}_{1,3,4}/\text{OAAc}) \]

12. \[ \frac{d\text{OAAc}_{1,2,4}}{dt} = \text{ACL(Citrate}_{1,3,6}/\text{Citrate}) + \text{ACL(Citrate}_{1,3,4,6}/\text{Citrate}) + \text{ACL(Citrate}_{1,3,5,6}/\text{Citrate}) + \text{ACL(Citrate}_{1,3,4,5,6}/\text{Citrate}) - \text{ACL(OAAc}_{1,2,4}/\text{OAAc}) \]

13. \[ \frac{d\text{OAAc}_{1,2,3}}{dt} = \text{ACL(Citrate}_{2,3,6}/\text{Citrate}) + \text{ACL(Citrate}_{2,3,4,6}/\text{Citrate}) + \text{ACL(Citrate}_{2,3,5,6}/\text{Citrate}) + \text{ACL(Citrate}_{2,3,4,5,6}/\text{Citrate}) - \text{ACL(OAAc}_{1,2,3}/\text{OAAc}) \]

14. \[ \frac{d\text{OAAc}_{2,3,4}}{dt} = \text{ACL(Citrate}_{1,2,3}/\text{Citrate}) + \text{ACL(Citrate}_{1,2,3,4}/\text{Citrate}) + \text{ACL(Citrate}_{1,2,3,5}/\text{Citrate}) + \text{ACL(Citrate}_{1,2,3,4,5}/\text{Citrate}) - \text{ACL(OAAc}_{2,3,4}/\text{OAAc}) \]
15. \( \frac{d\text{OAAc}1,2,3,4}{dt} = \text{ACL(Citrate}_{1,2,3,6}/\text{Citrate}) + \text{ACL(Citrate}_{1,2,3,4,6}/\text{Citrate}) + \text{ACL(Citrate}_{1,2,3,5,6}/\text{Citrate}) + \text{ACL(Citrate}_{1,2,3,4,5,6}/\text{Citrate}) - \text{ACL(OAAC}_{1,2,3,4}/\text{OAAc}) \)

16. \( \frac{d\text{OAAmMal}_{1,2,3,4}}{dt} = \text{PC(LacPyr}_{1,2,3}/\text{LacPyr})0/\text{IsotNA}) + \text{Vx}_\text{AspOAAm(Asp}_{1,2,3,4}/\text{Asp}) + \text{ACL(OAAC}_{1,2,3,4}/\text{OAAc}) + \text{SDH(Suc}_{1,2,3,4}/\text{Suc}) - [\text{Vx}_\text{OAAAspm} + \text{PEPCK} + \text{CS}]\text{(OAAmMal)}_{1,2,3,4}/\text{OAAmMal}) \)

**PEP**

1. \( \frac{d\text{PEP}_1}{dt} = \text{PEPCK(OAAmMal}_{1}/\text{OAAmMal}) + \text{GlyTri(Glyc3p}_{1}/\text{Glyc3p}) + \text{PEPCK(OAAmMal}_{1,4}/\text{OAAmMal}) - [\text{PK} + \text{GlcTriNeo}]\text{(PEP}_1/\text{PEP}) \)

2. \( \frac{d\text{PEP}_2}{dt} = \text{PEPCK(OAAmMal}_{2}/\text{OAAmMal}) + \text{GlyTri(Glyc3p}_{2}/\text{Glyc3p}) + \text{PEPCK(OAAmMal}_{2,4}/\text{OAAmMal}) - [\text{PK} + \text{GlcTriNeo}]\text{(PEP}_2/\text{PEP}) \)

3. \( \frac{d\text{PEP}_3}{dt} = \text{PEPCK(OAAmMal}_{3,4}/\text{OAAmMal}) + \text{GlyTri(Glyc3p}_{3}/\text{Glyc3p}) + \text{PEPCK(OAAmMal}_{3}/\text{OAAmMal}) - [\text{PK} + \text{GlcTriNeo}]\text{(PEP}_3/\text{PEP}) \)

4. \( \frac{d\text{PEP}_{1,2}}{dt} = \text{PEPCK(OAAmMal}_{1,2}/\text{OAAmMal}) + \text{GlyTri(Glyc3p}_{1,2}/\text{Glyc3p}) + \text{PEPCK(OAAmMal}_{1,2,4}/\text{OAAmMal}) - [\text{PK} + \text{GlcTriNeo}]\text{(PEP}_{1,2}/\text{PEP}) \)
5. \( \frac{d\text{PEP}_{1,3}}{dt} = \text{PEPCK(OAAmMal}_{1,3,4}/\text{OAAmMal}) + \text{GlyTri(Glyc3p}_{1,3}/\text{Glyc3p}) + \text{PEPCK(OAAmMal}_{1,3}/\text{OAAmMal}) - [\text{PK + GlcTriNeo}(\text{PEP}_{1,3}/\text{PEP})] \)

6. \( \frac{d\text{PEP}_{2,3}}{dt} = \text{PEPCK(OAAmMal}_{2,3}/\text{OAAmMal}) + \text{GlyTri(Glyc3p}_{2,3}/\text{Glyc3p}) + \text{PEPCK(OAAmMal}_{2,3,4}/\text{OAAmMal}) - [\text{PK + GlcTriNeo}(\text{PEP}_{2,3}/\text{PEP})] \)

7. \( \frac{d\text{PEP}_{1,2,3}}{dt} = \text{PEPCK(OAAmMal}_{1,2,3}/\text{OAAmMal}) + \text{GlyTri(Glyc3p}_{1,2,3}/\text{Glyc3p}) + \text{PEPCK(OAAmMal}_{1,2,3,4}/\text{OAAmMal}) - [\text{PK + GlcTriNeo}(\text{PEP}_{1,2,3}/\text{PEP})] \)

**Succinate**

1. \( \frac{d\text{Suc}_{1}}{dt} = \text{KGDH(KG}_{1,2}/\text{KG}) + \text{Vprop(IsotNA0/IsotNA}) + \text{KGDH(KG}_{2}/\text{KG}) - \text{SDH(Suc}_{1}/\text{Suc}) \)

2. \( \frac{d\text{Suc}_{2}}{dt} = \text{KGDH(KG}_{3}/\text{KG}) + \text{Vprop(IsotNA0/IsotNA}) + \text{KGDH(KG}_{1,3}/\text{KG}) - \text{SDH(Suc}_{2}/\text{Suc}) \)

3. \( \frac{d\text{Suc}_{3}}{dt} = \text{KGDH(KG}_{1,4}/\text{KG}) + \text{Vprop(IsotNA0/IsotNA}) + \text{KGDH(KG}_{4}/\text{KG}) - \text{SDH(Suc}_{3}/\text{Suc}) \)
4. \( \frac{d\text{Suc}_4}{dt} = \text{KGDH}(\text{KG}_5/\text{KG}) + \text{Vprop}(\text{IsotNA}_0/\text{IsotNA}) + \text{KGDH}(\text{KG}_{1,5}/\text{KG}) - \text{SDH}(\text{Suc}_4/\text{Suc}) \)

5. \( \frac{d\text{Suc}_{1,2}}{dt} = \text{KGDH}(\text{KG}_{2,3}/\text{KG}) + \text{Vprop}(\text{IsotNA}_0/\text{IsotNA}) + \text{KGDH}(\text{KG}_{1,2,3}/\text{KG}) - \text{SDH}(\text{Suc}_{1,2}/\text{Suc}) \)

6. \( \frac{d\text{Suc}_{1,3}}{dt} = \text{KGDH}(\text{KG}_{1,2,4}/\text{KG}) + \text{Vprop}(\text{IsotNA}_0/\text{IsotNA}) + \text{KGDH}(\text{KG}_{2,4}/\text{KG}) - \text{SDH}(\text{Suc}_{1,3}/\text{Suc}) \)

7. \( \frac{d\text{Suc}_{1,4}}{dt} = \text{KGDH}(\text{KG}_{1,2,5}/\text{KG}) + \text{Vprop}(\text{IsotNA}_0/\text{IsotNA}) + \text{KGDH}(\text{KG}_{2,5}/\text{KG}) - \text{SDH}(\text{Suc}_{1,4}/\text{Suc}) \)

8. \( \frac{d\text{Suc}_{2,3}}{dt} = \text{KGDH}(\text{KG}_{3,4}/\text{KG}) + \text{Vprop}(\text{IsotNA}_0/\text{IsotNA}) + \text{KGDH}(\text{KG}_{1,3,4}/\text{KG}) - \text{SDH}(\text{Suc}_{2,3}/\text{Suc}) \)

9. \( \frac{d\text{Suc}_{2,4}}{dt} = \text{KGDH}(\text{KG}_{3,5}/\text{KG}) + \text{Vprop}(\text{IsotNA}_0/\text{IsotNA}) + \text{KGDH}(\text{KG}_{1,3,5}/\text{KG}_{17}) - \text{SDH}(\text{Suc}_{2,4}/\text{Suc}) \)

10. \( \frac{d\text{Suc}_{3,4}}{dt} = \text{KGDH}(\text{KG}_{4,5}/\text{KG}) + \text{Vprop}(\text{IsotNA}_0/\text{IsotNA}) + \text{KGDH}(\text{KG}_{1,4,5}/\text{KG}) - \text{SDH}(\text{Suc}_{3,4}/\text{Suc}) \)

11. \( \frac{d\text{Suc}_{1,2,3}}{dt} = \text{KGDH}(\text{KG}_{2,3,4}/\text{KG}) + \text{Vprop}(\text{IsotNA}_0/\text{IsotNA}) + \text{KGDH}(\text{KG}_{1,2,3,4}/\text{KG}) - \text{SDH}(\text{Suc}_{1,2,3}/\text{Suc}) \)
12. \( \frac{d\text{Suc}_{1,2,4}}{dt} = \text{KGDH}(\text{KG}_{2,3,5}/\text{KG}) + \text{Vprop}(\text{IsotNA0}/\text{IsotNA}) + \text{KGDH}(\text{KG}_{1,2,3,5}/\text{KG}) \)
   - SDH(\text{Suc}_{1,2,4}/\text{Suc})

13. \( \frac{d\text{Suc}_{1,3,4}}{dt} = \text{KGDH}(\text{KG}_{1,2,4,5}/\text{KG}) + \text{Vprop}(\text{IsotNA0}/\text{IsotNA}) + \text{KGDH}(\text{KG}_{2,4,5}/\text{KG}) \)
   - SDH(\text{Suc}_{1,3,4}/\text{Suc})

14. \( \frac{d\text{Suc}_{2,3,4}}{dt} = \text{KGDH}(\text{KG}_{3,4,5}/\text{KG}) + \text{Vprop}(\text{IsotNA0}/\text{IsotNA}) + \text{KGDH}(\text{KG}_{1,3,4,5}/\text{KG}) \)
   - SDH(\text{Suc}_{2,3,4}/\text{Suc})

15. \( \frac{d\text{Suc}_{1,2,3,4}}{dt} = \text{KGDH}(\text{KG}_{2,3,4,5}/\text{KG}) + \text{Vprop}(\text{IsotNA0}/\text{IsotNA}) + \text{KGDH}(\text{KG}_{1,2,3,4,5}/\text{KG}) \)
   - SDH(\text{Suc}_{1,2,3,4}/\text{Suc})

S2. MIMOSA equations with derivations

Below we describe the stepwise derivation and solution for all metabolic fluxes, beginning with establishing key phi ratios to establish relative flux contributions that aid in the calculation of metabolic flux analysis
Establishing Preliminary Relationships:

*Mass Balance of anaplerosis and cataplerosis:*

\[
\frac{d\text{OAA}}{dt} = V_{\text{SDH}} + V_{\text{PC}} + V_{\text{ACly}} - (V_{\text{CS}} + V_{\text{PEPCK}})
\]

At steady state: \(V_{\text{SDH}} + V_{\text{PC}} + V_{\text{ACly}} = V_{\text{CS}} + V_{\text{PEPCK}}\)

Thus:

\[
\frac{V_{\text{SDH}} + V_{\text{PC}} + V_{\text{ACly}}}{V_{\text{CS}} + V_{\text{PEPCK}}} = 1
\]

We make the following definitions of fractional fluxes:

- **F\text{SDH}:** \(\frac{V_{\text{SDH}}}{V_{\text{CS}} + V_{\text{PEPCK}}} = f_s\)
- **F\text{ACly}:** \(\frac{V_{\text{ACly}}}{V_{\text{CS}} + V_{\text{PEPCK}}} = f_c\)
- **F\text{PC}:** \(\frac{V_{\text{PC}}}{V_{\text{CS}} + V_{\text{PEPCK}}} = f_p\)

The sum of the fractional fluxes then equals 1.

\[
f_s + f_p + f_c = 1
\]

Rearranging to solve for \(f_p\) \(f_p = 1 - f_s - f_c\)

**Solving for \(f_c\)**

Since malate and OAA are in isotopic equilibrium across all carbons, we can substitute malate for OAA.

Then isotope balance for malate M+2 is:
\[
\frac{d(MalM+2)}{dt} = V_{SDH}(Succ M + 2) + V_{PC}(Pyr M + 2) + V_{ACLY}(Cit M + 2) - 
(Mal M + 2)(V_{CS} + V_{PEPCK+ME})
\]

At steady state:

\[
V_{SDH}(Succ M + 2) + V_{PC}(Pyr M + 2) + V_{ACLY}(Cit M + 2) = (Mal M + 2)(V_{CS} + V_{PEPCK+ME})
\]

Since there was no detectable M+2 succinate or pyruvate, this simplifies to:

\[
V_{ACLY}(Cit M + 2) = (V_{CS} + V_{PEPCK+ME})(Mal M + 2)
\]

Rearranging:

\[
\frac{V_{ACLY}}{V_{CS} + V_{PEPCK+ME}} = \frac{Malate_{M+2}}{Citrate_{[1,2]}}
\]

Substituting previously defined variables and relationships

\[
f_c = \frac{Malate_{M+2}}{Citrate_{[1,2]}} = \frac{Malate_c}{Citrate_a}
\]

**Solving for \(f_s\)**

Isotope balance for malate M+1:
\[
\frac{d(Mal_{M+1})}{dt} = v_{SDH}(Succ_{M+1}) + v_{PC}(Pyr_{M+1}) + v_{ACLY} \left( [(1)(2)(3)(6)\text{C}_1]Cit \right) - (Mal_{M+1})(V_{CS} + V_{PEPCK+ME})
\]

At steady state:
\[
v_{SDH}(Succ_{M+1}) + v_{PC}(Pyr_{M+1}) + v_{ACLY} \left( [(1)(2)(3)(6)\text{C}_1]Cit \right) = (Mal_{M+1})(V_{CS} + V_{PEPCK+ME})
\]

Divide by \(V_{CS} + V_{PEPCK+ME}\)
\[
\frac{(Succ_{M+1})v_{SDH}}{(V_{CS} + V_{PEPCK+ME})} + \frac{(Pyr_{M+1})v_{PC}}{(V_{CS} + V_{PEPCK+ME})} + \frac{\left( [(1)(2)(3)(6)\text{C}_1]Cit \right)v_{ACLY}}{(V_{CS} + V_{PEPCK+ME})} = (Mal_{M+1})
\]

Substitute fraction relations
\[
f_s(Succ_{M+1}) + (1 - f_c - f_s)(Pyr_{M+1}) + f_c\left( [(1)(2)(3)(6)\text{C}_1]Cit \right) = Mal_{M+1}
\]

Expand
\[
f_s(Succ_{M+1}) + Pyr_{M+1} - f_c(Pyr_{M+1}) - f_s(Pyr_{M+1}) + f_c\left( [(1)(2)(3)(6)\text{C}_1]Cit \right) = Mal_{M+1}
\]

Combine terms
\[
f_s(Succ_{M+1} - Pyr_{M+1}) + f_c\left( [(1)(2)(3)(6)\text{C}_1]Cit - Pyr_{M+1} \right) = Mal_{M+1} - Pyr_{M+1}
\]

Solve for \(f_s\)
\[
f_s = ((Mal_{M+1} - Pyr_{M+1}) + f_c(Pyr_{M+1} - [(1)(2)(3)(6)\text{C}_1]Cit))/(Succ_{M+1} - yr_{M+1})
\]

[Note: New isotopomer definition assuming C5 enrichment is negligible]
\[
\left( [(1)(2)(3)\text{C}_1]Cit \right) = \frac{\left( 1-\left[ (4)\text{C}_1 \right]glu \right)}{\left( (1)(2)(3)(4)\text{C}_1 \right)glu} \left( [(1)(2)(3)(4)\text{C}_1]Cit \right)
\]
\[
\left( [(1)(2)(3)\text{C}_1]Cit + \frac{1}{2}Cit_c \right) = \left( [(1)(2)(3)(6)\text{C}_1]Cit \right)
\]
\[(1)(2)(3)(6)^{13}C_i]Cit\]
\[
= \frac{(1 - (147/42 + 148/42))}{Glu_{M+1}} \ast (192/112 - 192/111) + 2 \ast 192/111
\]

**Metabolic Flux Analysis**

**Establishing fluxes relative to OGDH**

**Solving for \(V_{iDH}\) relative to \(V_{OGDH}\)**

**Mass Balance**

\[
\frac{daKG}{dt} = V_{iDH} + V_{GDH} - V_{GDH} + V_{OGDH}
\]

**Isotope Balance**

\[
\frac{d(aKG_{M+2})}{dt} = V_{iDH}(\text{Cit}[M + 2]) + V_X(\text{Glu} M + 2) + V_{GDH}(\text{Gln} M + 2) - aKG M + 2 \ast (V_{OGDH} + V_{GDH} + V_X)
\]

Because \(V_X\) is fast:: \(aKG_{M+2} = Glu_{M+2}\)

then GDH: \(\text{Gln} M+2 \rightarrow \text{Glu} M+2\)

**Rearrange:**

\[
\frac{d(aKG \ M+2)}{dt} = V_{iDH}((\text{Cit} \ M+ 2)) + V_X(\text{Glu} \ M + 2) + V_{GDH}(\text{Gln} \ M + 2)
\]
\[ = \alpha KG \cdot M + 2 \cdot (V_{OGDH} + V_X + V_{GDH}) \]

Solve:
\[
\frac{V_{IDH}}{V_{OGDH}} = \frac{(\text{Glu} M+2 - \text{Gln} M+2)}{(\text{Cit} M+2 - \text{Gln} M+2)} = \frac{(\text{Glu} M+2_{OAA} - \text{Gln} M+2)}{(\text{Citrate}_{[1,2]} - \text{Gln} M+2)}
\]

Solving for GDH relative to OGDH

Mass Balance:
\[
\frac{d\alpha KG}{dt} = V_{\text{IDH}} + V_{\text{GDH}} - V_{\text{OGDH}}
\]

Isotope Balance:
\[
\frac{d(\alpha KG \cdot M + 1)}{dt} = V_{\text{IDH}}((\text{Cit}[M + 1])) + V_X (\text{Glu} M + 1) + V_{\text{GDH}} (\text{Gln} M + 1) - \alpha KG \cdot M + 1 \cdot (V_{\text{OGDH}} + V_{\text{GDH}} + V_X)
\]

Rearrange:
\[
V_{\text{IDH}} + V_{\text{GDH}} = V_{\text{OGDH}}
\]

Solve:
\[
\frac{V_{IDH}}{V_{OGDH}} + \frac{V_{GDH}}{V_{OGDH}} = 1
\]
\[
\frac{V_{GDH}}{V_{OGDH}} = 1 - \frac{V_{IDH}}{V_{OGDH}} = 1 - \frac{(\text{Glu} M+2_{OAA} - \text{Gln} M+2)}{(\text{Citrate}_{[1,2]} - \text{Gln} M+2)}
\]
Solving fluxes relative to SDH

Isotope Balance: \[ \frac{d\text{Succ}}{dt} = V_{\text{OGDH}} (\text{GluM}+2\text{OAA}+\text{GluM}) - V_{\text{SDH}} (\text{Succ}) \]

Rearrange: \[ V_{\text{OGDH}} (\text{GluM}+2\text{OAA}+\text{GluM}) = V_{\text{SDH}} (\text{Succ}) \]

Solve: \[ \frac{V_{\text{OGDH}}}{V_{\text{SDH}}} = \frac{\text{Succ}}{\text{GluM}+2\text{OAA}+\text{GluM}} = \frac{\text{Succ}_a}{\text{Glu}_a+\text{Glu}_b+\text{Glu}_c+\text{d}} \]

\( V_{\text{GDH}} \) relative to \( V_{\text{SDH}} \) can be established from the above relationships of \( V_{\text{OGDH}}/V_{\text{SDH}} \) and \( V_{\text{GDH}}/V_{\text{OGDH}} \)

\[ \frac{V_{\text{GDH}}}{V_{\text{SDH}}} = \frac{V_{\text{OGDH}}}{V_{\text{SDH}}} \times \frac{V_{\text{GDH}}}{V_{\text{OGDH}}} \]

Substitute: \[ \frac{V_{\text{OGDH}}}{V_{\text{SDH}}} = 1 - \frac{(\text{Glu M}+2\text{OAA} - \text{Gln M}+2)}{(\text{Citrate}_{[1,2]} - \text{Gln M}+2)} \]

\[ \frac{V_{\text{GDH}}}{V_{\text{OGDH}}} = \frac{\text{Succ}_a}{\text{Glu}_a+\text{Glu}_b+\text{Glu}_c+\text{d}} \]
Solve

\[
\frac{V_{GDH}}{V_{SDH}} = \left(1 - \frac{(\text{Glu} \ M + 2 \text{OAA} - \text{Gln} \ M + 2)}{\text{Citrate}_{1,2} - \text{Gln} \ M + 2}\right) \times \left(\frac{\text{Succ}_a}{\text{Glu}_a + \text{Glu}_b + \text{Glu}_{c+d}}\right)
\]

Establishing \(V_{PROP}\) relative to \(V_{SDH}\)

Mass Balance:

\[
\frac{d\text{Succ}}{dt} = V_{\text{Prop}} + V_{\text{OGDH}} - V_{\text{SDH}}
\]

Rearrange:

\[V_{\text{Prop}} + V_{\text{OGDH}} = V_{\text{SDH}}\]

Solve:

\[
\frac{V_{\text{Prop}}}{V_{\text{SDH}}} + \frac{V_{\text{OGDH}}}{V_{\text{SDH}}} = 1
\]

\[
\frac{V_{\text{Prop}}}{V_{\text{SDH}}} = 1 - \frac{V_{\text{OGDH}}}{V_{\text{SDH}}} = 1 - \frac{\text{Succ}_{(1)(4)}}{\text{Glu}M + 2\text{OAA} + \text{Glu}M + 1}
\]
Solving for anaplerotic and cataplerotic fluxes relative to SDH

At steady state anaplerosis equals cataplerosis

Balance Equation:

\[
\frac{V_{ANA}}{V_{SDH}} = \frac{V_{PC}}{V_{SDH}} + \frac{V_{Prop}}{V_{SDH}} + \frac{V_{GDH}}{V_{SDH}}
\]

Substitute:

\[
\frac{V_{ACLy}}{V_{SDH}} = \frac{f_C}{f_S} = \frac{Malate_{M+2}}{Citrate_{[1,2]}} \div \frac{(Malate_{M+1} - PyrM+1 + fc(PyrM+1 - [(1)(2)(3)(6)\text{C}_3\text{Cit}]))}{(S_{M+1} - P_{M+1})}
\]

\[
\frac{V_{PC}}{V_{SDH}} = \frac{f_P}{f_S} = \left(1 - \frac{(M_{M+1} - f_C^{\text{Cit}([2)(3)(4)(6)]} + P_{M+1} - P_{M+1})}{(S_{M+1} - P_{M+1})} \right) \div \frac{Malate_{M+2}}{Citrate_{[1,2]}}
\]

\[
\frac{V_{GDH}}{V_{SDH}} = \left(1 - \frac{(Glu M+2\text{OAA} - Gln M+2)}{(Citrate_{[1,2]} - Gln M+2)} \right) \star \frac{Succ_a}{Glu_a + Glu_b + Glu_{c+d}}
\]

Rearrange:

\[
\frac{V_{ANA}}{V_{SDH}} = \frac{V_{GDH}}{V_{SDH}} \div \frac{f_C}{f_S} + \frac{f_P}{f_S}
\]

Solve:

\[
\left(1 - \frac{(Glu M+2\text{OAA} - Gln M+2)}{(Citrate_{[1,2]} - Gln M+2)} \right) \star \frac{Succ_a}{Glu_a + Glu_b + Glu_{c+d}}
\]
\[
\left(1 - \frac{(M_{M+1} - f_c \text{Cit}_1(2)(3)(6)) + P_{M+1}}{P_{M+1}}\right) \cdot \frac{Malate_{M+2}}{\text{Citrate}_{[1,2]}} \div \left(\frac{(MalM+1 - PyrM+1) + fc(PyrM+1 - [(1)(2)(3)(6)^{13}C_1\text{Cit}])}{P_{M+1}}\right) + \left(\frac{Malate_{M+2}}{\text{Citrate}_{[1,2]}} \div \left(\frac{(MalM+1 - PyrM+1) + fc(PyrM+1 - [(1)(2)(3)(6)^{13}C_1\text{Cit}])}{P_{M+1}}\right)\right)
\]

Mass Balance: \[\frac{d\text{Cit}}{dt} = V_{CS} + V_{IDHr} - V_{ACLY} - V_{IDHf}\]

Rearrange: \[\frac{V_{CS}}{V_{SDH}} = \frac{V_{ACLY}}{V_{SDH}} + \frac{V_{IDH}}{V_{SDH}}\]

Substitute: \[\frac{V_{IDH}}{V_{SDH}} = \frac{V_{OGDH} \cdot V_{IDH}}{V_{SDH}} \frac{V_{OGDH}}{V_{OGDH}}\]

Rearrange: \[\frac{V_{CS}}{V_{SDH}} = \frac{V_{ACLY}}{V_{SDH}} + \frac{V_{OGDH}}{V_{SDH}} \cdot \frac{V_{IDH}}{V_{OGDH}}\]

Substitute: \[\frac{f_c}{f_s} = \left(\frac{Malate_{M+2}}{\text{Citrate}_{[1,2]}} \div \left(\frac{(MalM+1 - PyrM+1) + fc(PyrM+1 - [(1)(2)(3)(6)^{13}C_1\text{Cit}])}{P_{M+1}}\right)\right)\]

\[\frac{V_{OGDH}}{V_{SDH}} = 1 - \left(\frac{\text{Glu}_{M+2}^{+} \text{QAA} - \text{Gln}_{M+2}}{\text{Citrate}_{[1,2]} - \text{Gln}_{M+2}}\right)\]
\[
\frac{V_{IDH}}{V_{OGDH}} = \frac{(\text{Glu M+2} - \text{Gln M+2})}{(\text{Cit M+2} - \text{Gln M+2})} = \frac{(\text{Glu M+2OAA} - \text{Gln M+2})}{(\text{Citrate}_{1,2} - \text{Gln M+2})}
\]

Solve:

\[
\frac{V_{CS}}{V_{SDH}} = \left(\frac{\text{Malate}_{M+2}}{\text{Citrate}_{[1,2]}}\right) \div \left(\frac{(\text{MalM+1} - \text{PyrM+1}) + \text{fc(PyrM+1} - \left[\frac{(1)(2)(3)(6)^{13}\text{C}_1]\text{Cit}\right]}{(S_{M+1} - P_{M+1})} + \left(1 - \frac{(\text{Glu M+2OAA} - \text{Gln M+2})}{(\text{Citrate}_{[1,2]} - \text{Gln M+2})}\right)\right) \cdot \frac{(\text{Glu M+2OAA} - \text{Gln M+2})}{(\text{Citrate}_{[1,2]} - \text{Gln M+2})}
\]

**In terms of CS:**

Establishing PDH relative to CS

\[
\frac{V_{PDH}}{V_{CS}} = \frac{\text{Glu M+1PDH}}{\phi_{\text{Cit} - \text{Glu}}} \div \text{PyrM+1}
\]

Establishing IDHf relative to CS

Mass Balance: \( \frac{d\text{Cit}}{dt} = V_{CS} + V_{IDHr} - V_{ACLy} - V_{IDHf} \)

Rearrange: \( V_{CS} + V_{IDHr} = V_{ACLy} + V_{IDHf} \)
\[
\begin{align*}
\frac{V_{CS}}{V_{CS}} + \frac{V_{IDHr}}{V_{CS}} &= \frac{V_{ACLY}}{V_{CS}} + \frac{V_{IDHF}}{V_{CS}} \\
1 + \frac{V_{IDHr}}{V_{CS}} &= \frac{V_{ACLY}}{V_{CS}} + \frac{V_{IDHF}}{V_{CS}} \\
\frac{V_{IDHF}}{V_{CS}} &= 1 - \frac{V_{ACLY}}{V_{CS}} + \frac{V_{IDHr}}{V_{CS}} = 1 - \frac{(\text{Mal}_M + 2 - \text{Cit}_M + 2)}{(\text{Cit}_M + 2 - \text{Glu}_M + 2)} \\
\end{align*}
\]

\[
\frac{V_{IDHF}}{V_{CS}} = \frac{(\text{Mal}_M + 2 - \text{Cit}_M + 2)}{(\text{Cit}_M + 2 - \text{Glu}_M + 2)}
\]

Mass Balance: \[V_{IDHNet} = V_{IDHF} - V_{IDHr}\]

Isotope Balance: \[\frac{d\text{Cit}_M + 2}{dt} = a\text{KG}_M + 2 \cdot V_{IDHr} - \text{Cit}_M + 2 \cdot V_{IDHF}\]

Because \(V_x\) is fast: \[a\text{KG}_M + 2 = \text{Glu}_M + 2\]

Substitute: \[\text{Glu}_M + 2 \cdot V_{IDHr} = \text{Cit}_M + 2 \cdot V_{IDHF}\]

Rearrange: \[V_{IDHF} = V_{IDHr} (\frac{\text{Glu}_M + 2}{\text{Cit}_M + 2})\]

Substitute for \(V_{IDHr}\): \[V_{IDHF} = (V_{IDHF} - V_{IDHNet}) (\frac{\text{Glu}_M + 2}{\text{Cit}_M + 2})\]

Solve: \[\frac{V_{IDHF}}{V_{IDHNet}} = \frac{\text{Glu}_M + 2}{\text{Cit}_M + 2} (\frac{\text{Glu}_M + 2}{\text{Cit}_M + 2} - 1)\]
Net IDH flux is the difference between forward and reverse IDH flux:

\[
\frac{V_{\text{IDH}\text{net}}}{V_{CS}} = \frac{V_{\text{IDH}f}}{V_{CS}} - \frac{V_{\text{IDH}r}}{V_{CS}}
\]

From the above established relationships the following relative fluxes can be established:

\[
\frac{V_{\text{OGDH}}}{V_{CS}} = \frac{V_{\text{OGDH}}}{V_{SDH}} - \frac{V_{CS}}{V_{SDH}}
\]

Establishing SDH relative CS

From the above fluxes relative to \(V_{SDH}\) fluxes relative to CS can be established as

\[
\frac{V_{\text{ACL}Y}}{V_{SDH}} = \frac{f_c}{f_s} = \left(\frac{\text{Malate}_{M+2}}{\text{Citrate}_{[1,2]}^+}\right) \div \left(\frac{(\text{Malate}_{M+1} - \text{Pyr}_{M+1}) + fc(\text{Pyr}_{M+1} - \left[(1)(2)(3)(6)^{13}C_1\text{Cit}\right])}{(S_{M+1} - P_{M+1})}\right)
\]

\[
\frac{V_{PC}}{V_{SDH}} = \frac{f_p}{f_s} = \left(1 - \frac{(\text{Malate}_{M+1} - \text{Pyr}_{M+1})}{(S_{M+1} - P_{M+1})}\right) \div \left(\frac{\text{Malate}_{M+2}}{\text{Citrate}_{[1,2]}^+}\right)
\]
\[
\frac{V_{GDH}}{V_{SDH}} = (1 - \frac{(Glu M+2 OAA - Gln M+2)}{(Citrate_{[1,2]} - Gln M+2)}) \times \frac{Succ_a}{Glu_a + Glu_b + Glu_{c+d}}
\]

\[
\frac{V_{CS}}{V_{SDH}} = \frac{(Malate_{M+2})}{(Citrate_{[1,2]})} \div \left(\frac{(MalM+1 - PyrM+1) + fc(PyrM+1 - [(1)(2)(3)(6)\text{^{13}C}_1]Clit))}{(S_M+1 - P_{M+1})}\right)
+ (1 - \frac{(Glu M+2 OAA - Gln M+2)}{(Citrate_{[1,2]} - Gln M+2)}) \times \frac{Glu M+2 OAA - Gln M+2}{(Citrate_{[1,2]} - Gln M+2)}
\]

\[
\frac{V_{DGDH}}{V_{SDH}} = 1 - \frac{(Glu M+2 OAA - Gln M+2)}{(Citrate_{[1,2]} - Gln M+2)}
\]

\[
\frac{V_{Prop}}{V_{SDH}} = 1 - \frac{V_{DGDH}}{V_{SDH}} = 1 - \frac{Succ_{(1)(4)}}{GluM+2 OAA + GluM+1}
\]
Establishing SDH relative to CS

Equation:
\[
\frac{V_{SDH}}{V_{CS}} = \frac{1}{\frac{V_{CS}}{V_{SDH}}}
\]

Solve:
\[
\frac{V_{SDH}}{V_{CS}} = 1 + \left( \frac{Malate_{M+2}}{Citrate_{[1,2]}} + \frac{(Malate_{M+1} - PyrM+1) + fc(PyrM+1 - [(1)(2)(3)(6)C_{13}]Cit))}{(S_{M+1} - P_{M+1})} \right) + \\
\left( 1 - \frac{(Glu \ M+2 \text{OAA} - Gln \ M+2)}{(Citrate_{[1,2]} - Gln \ M+2)} \right) \times \left( \frac{(Glu \ M+2 \text{OAA} - Gln \ M+2)}{(Citrate_{[1,2]} - Gln \ M+2)} \right) - \frac{Malate_{M+2}}{Citrate_{[1,2]}}
\]

Establishing PC relative to CS

Equation:
\[
\frac{V_{PC}}{V_{CS}} = \frac{V_{PC}}{V_{SDH}} \div \frac{V_{CS}}{V_{SDH}}
\]

Solve:
\[
\frac{V_{PC}}{V_{CS}} = (1 - \frac{(Malate_{M+1} - PyrM+1) + fc(PyrM+1 - [(1)(2)(3)(6)C_{13}]Cit))}{(S_{M+1} - P_{M+1})} - \frac{Malate_{M+2}}{Citrate_{[1,2]}})
\]
Establishing Prop relative to CS

**Equation:**

\[
\frac{V_{Prop}}{V_{CS}} = \frac{V_{Prop}}{V_{SDH}} + \frac{V_{CS}}{V_{SDH}}
\]

**Solve:**

\[
\frac{V_{Prop}}{V_{CS}} = \left(1 - \frac{\text{Succ}_{(1)(4)}}{\text{GluM+2}_{AA}+\text{GluM+1}} \div \left(\frac{\text{Malate}_{M+2}}{\text{Citrate}_{[1,2]}}\right)\right) + \\
\left(\frac{(\text{MalM+1} - \text{PyrM+1}) + \text{fc}(\text{PyrM+1} - [(1)(2)(3)(6)^{13}\text{C}_{1}]\text{Cit})}{{(S_{M+1} - P_{M+1})}}\right) + \\
\left(1 - \frac{(\text{GluM+2}_{AA} - \text{Gln M+2})}{(\text{Citrate}_{[1,2]} - \text{Gln M+2})}\right) \times \left(\frac{(\text{GluM+2}_{AA} - \text{Gln M+2})}{(\text{Citrate}_{[1,2]} - \text{Gln M+2})}\right)
\]
Establishing GDH relative to CS

Equation:
\[
\frac{V_{GDH}}{V_{CS}} = \frac{V_{GDH}}{V_{SDH}} + \frac{V_{CS}}{V_{SDH}}
\]

Solve:
\[
\frac{V_{GDH}}{V_{CS}} = \left(1 - \frac{(\text{Glu} M+2_{OAA} - \text{Gln} M+2)}{\text{Citrate}_{[1,2]} - \text{Gln} M+2}\right) \times \left(\frac{\text{Succ} a}{\text{Glu}_a + \text{Glu}_b + \text{Glu}_{c+d}}\right) \div
\]
\[
\left(\frac{\text{Mal}M+1 - \text{Pyr} M+1 + \text{fc}(\text{Pyr} M+1 - \left[\frac{1(2)(3)(6)^{13}C_1}{S M+1 - P M+1}\right])}{\text{Succ} a}\right) +
\]
\[
\left(1 - \frac{(\text{Glu} M+2_{OAA} - \text{Gln} M+2)}{\text{Citrate}_{[1,2]} - \text{Gln} M+2}\right) \times \frac{\text{Glu} M+2_{OAA} - \text{Gln} M+2}{\text{Citrate}_{[1,2]} - \text{Gln} M+2}
\]

Since at steady state anaplerosis is equal to cataplerosis

\[
\frac{V_{PEPCK+ME}}{V_{CS}} = \frac{V_{ANA}}{V_{CS}} = \frac{V_{ANA}}{V_{SDH}} + \frac{V_{CS}}{V_{SDH}}
\]

Establishing ACLy relative to CS

Equation:
\[
\frac{V_{ACLy}}{V_{CS}} = \frac{V_{ACLy}}{V_{SDH}} + \frac{V_{CS}}{V_{SDH}}
\]
Solve:

\[
\frac{\text{V}_{\text{ACL}_{\text{cy}}}}{\text{V}_{\text{CS}}} = \frac{\left(\frac{\text{Malate}_{M+2}}{\text{Citrate}_{[1,2]}}\right)}{\frac{\left(\frac{\left(\text{Malate}_{M+1} - \text{PyrM+1}\right) + \text{fc}\left(\text{PyrM+1} - \left[\left(1\right)\left(2\right)\left(3\right)\left(6\right)^{13}\text{C}_1\right]\text{Cit}\right)}{\left(S_{M+1} - P_{M+1}\right)}\right)}{\left(\left(S_{M+1} - P_{M+1}\right) + \left(\frac{\left(\text{Glu M}+2\text{DAA} - \text{Gln M}+2\right)}{\left(\text{Citrate}_{[1,2]} - \text{Gln M}+2\right)}\right)\right)}}{\frac{\left(\left(\text{Malate}_{M+1} - \text{PyrM+1}\right) + \text{fc}\left(\text{PyrM+1} - \left[\left(1\right)\left(2\right)\left(3\right)\left(6\right)^{13}\text{C}_1\right]\text{Cit}\right)}{\left(S_{M+1} - P_{M+1}\right)}\right)}{\left(\left(S_{M+1} - P_{M+1}\right) + \left(\frac{\left(\text{Glu M}+2\text{DAA} - \text{Gln M}+2\right)}{\left(\text{Citrate}_{[1,2]} - \text{Gln M}+2\right)}\right)\right)}} + \left(1 - \frac{\left(\text{Glu M}+2\text{DAA} - \text{Gln M}+2\right)}{\left(\text{Citrate}_{[1,2]} - \text{Gln M}+2\right)}\right)
\]

S3. Mass Spectrometry (MS) (additional description)

Since Mass spectrometry is primarily an ex vivo and in vitro technique MS based assays oftentimes incorporate additional technical steps to obtain higher resolution, including chromatographic separation, ionization of molecules, separation of molecular ions mass analyzer, counting of the ions by a detector and lastly processing of the spectral data.

S3.1 Chromatography

Tissue and plasma samples from tracer experiments usually consist of a highly complex mixture of various analytes along with additional matrix molecules, such as proteins and salts. If not purified and separated properly, co-eluting analytes can cause overlapping signals on the mass spectrum, while interfering matrix molecules can lead to ion suppression, adduct formation and poor signal to noise ratios. This will result in poor spectral resolution, difficulties discerning molecules of interest and spurious flux analysis. Therefore, chromatography is commonly set up as an initial step of separation before the
analysis by Mass Spectrometry. Chromatography was first described by Russian Botanist Mikhail S. Tswett in 1900 as a technique to separate plant pigments. He took ground-up plant extract, poured it into a glass column and saw different colored “bands” develop as the plant pigments separated. He named the procedure chromatography based on the Greek words ‘chroma’ for “color” and ‘graphein’ for “to write” [147]. Since its discovery the basic principles of chromatography have remained the same: A mobile phase containing the compounds of interest moves through a chromatography column in which the sample interacts with the immobilized “stationary” phase. The time it takes for an analyte in the mobile phase to elute from a chromatographic column is called retention time (RT). The velocity of the RT is governed by the dynamic equilibrium of adsorption to and desorption from the stationary phase. Some molecules will interact more strongly with the stationary phase than molecules with only weakly interaction. The latter compounds move faster through the column, meaning that they elude at a shorter retention time than molecules that have a high affinity to the stationary phase. Retention time is characteristic for a specific molecule in a given chromatographic set up. However, molecules with similar chemical properties, such as the same atomic weight but different chemical structures (isobars), can be difficult to separate chromatographically and thus can show significant overlap in RT. The two types of chromatography coupled with Mass Spectrometry are Gas Chromatography (GC) and Liquid Chromatography (LC).

GC is a well-established method for analyzing and quantifying small molecules (<1kDa) and their isotopic composition. Before analysis, GC requires 1) extraction of metabolites from the sample matrix, 2) addition of an internal standard to the sample and 3) derivatization of the molecules of interest [55, 69, 70]. The derivatization step is crucial
for chromatographic separation in GC because it renders the compounds of interest volatile and allows them to be taken up by an inert carrier gas, i.e. He, N, H, that functions as the mobile phase. Derivatization reagent can add a large number of additional atoms, i.e., carbons, to the compound of interest and can thereby artificially dilute its isotopic enrichment. This as well as the fragmentation pattern of the derivatized molecule need to be taken into consideration in the analysis of GC data [69]. Different derivatization protocols are used depending of the type of stable isotope investigated. After the initial preparation steps, the derivatized sample is injected into the GC column and evaporated in the heated injector. The volatile compounds are taken up by the mobile phase and moved through a very long high-resolution column. Based on their partitioning coefficient K, different compounds interact longer with the stationary phase of the column than others and thereby have different retention times [55].

In LC, the mobile phase is a liquid solvent and the stationary phase a surface of solid particles. The polarity of the mobile phase, stationary phase, and sample impact the adsorption and desorption equilibrium, that is, how long different compounds are retained on the column. In Normal Phase Chromatography the stationary phase is polar, and the mobile phase is a nonpolar or less polar solvent. Therefore, polar compounds have a higher affinity for the stationary phase, causing them to be retained longer on the column (i.e., have a longer retention time), whereas non-polar molecules are attracted to the mobile phase of equal polarity and therefore elute faster from the column (shorter retention time). In Reverse Phase Chromatography the polarity is opposite to Normal Phase Chromatography. Hence, the stationary phase is nonpolar, whereas the mobile phase is polar. Nonpolar compounds are retained longer on the column (longer retention
time) than polar compounds which elute faster (shorter retention time). Of note, by mixing two solvents of different polarity, i.e., a polar and a non-polar one, at different ratios, the overall polarity of the mobile phase can be adjusted during or between runs. The process of gradually increasing concentrations of organic solvent and decreasing the concentration of the polar solvent in the mobile phase to mobilize analytes of decreasing polarity off the stationary phase is called a ‘gradient – solvent run”. During an isocratic solvent run on the other hand the solvent composition of the mobile phase is not change.

The stationary phase of chromatography columns can consist of tightly packed silica beads whose surface is coated with an organic layer, such as siloxanes with various alkyl or aryl end groups. The end groups determine the interaction of the surface with the analyte. Long alkyl chains (C18) bind stronger to organic molecule of low polarity and let high polar/ionic analytes pass through whereas shorter alkyl chains (C8) are less retentiveness for low polarity analytes. Non-polar analytes typically require a higher fraction of organic solvent to move along the stationary phase.

S3.2 Ionization

Since mass analyzers take advantage of the movement of charged molecules through an electromagnetic field, neutral molecules cannot be studied by mass spectrometry (see Mass Analyzer below). Therefore, an ion source as part of a mass spectrometer ensures the generation of charged molecules (ions) before entering the mass analyzer. Once a molecule is charged it can then be accelerated in the electromagnetic field of the mass analyzer and directed towards a detector. Many different types of ionization have been developed, but the ones discussed here are electron ionization, chemical ionization, and
Electrospray ionization. In electron Ionization EI gas molecules enter a high-energy beam of electrons (70eV) that is generated by a heated filament and then directed towards a positively charged plate. The bombarded and subsequent collision of electrons with the neutral sample molecules results in the formation of cations (M+) and anions (M¬). Due to the high energy impact of EI this type of ionization leads to multiple highly reproducible and unique fragmentation patterns of each/individual molecule(s). In the process of chemical Ionization (CI) a reagent gas, such as methane or ammonia, reacts with molecules under high pressure leading to their ionization. The ionized bulk of reagent gas is usually present in excess to the sample molecule (1000:1). Both negative and positive ions are produced by CI. Since CI employs very little energy in the process of ionization it leads to little fragmentation. Therefore, EI is often used to identify positional labeling information whereas CI is more suitable for non-positional, total mass enrichment information. However, especially when ammonia is used as a reagent gas adduct formation can lead to higher masses of the sample molecules than seen with EI [55, 69, 70].

Electrospray Ionization (ESI) is a soft ionization technique which results in little to no fragmentation of labile analytes. ESI begins at atmospheric pressure and then proceeds into a high vacuum in the mass analyzer. The overall principal underlying ESI is the transfer of ions from a volatile solution to a gas phase, while only very low concentrations of the ionic analyte are required. The process of ESI starts with the production of a charged droplet, followed by droplet size reduction and fission, and finally gas phase ion formation. ESI is highly efficient in ion production and both positively and negatively charged ions can be formed. The type of ion produced depends on the functional group
of the sample molecule, that is, whether it readily accepts or donates a proton. Anions are usually observed in the process of protonization of amine groups, while cations are seen with the deprotonization of carboxylic groups. ESI serves a wide analytical range, especially of small polar molecules; large, non-volatile, chargeable molecules, ionic metal complexes, soluble inorganic analytes and thermally labile molecules. The ionization processed predominantly used in conjunction with LC in studies of polar molecules is electrospray ionization, while EI and CI are traditionally interfaced with GC-MS [55, 69, 70]
Glossary

\[ \beta\text{Ox/FAO} = \text{Fatty acid beta oxidation} \]

ACC = Acetyl-CoA carboxylase

ACS = Acetyl COA Synthetase

ACLy = ATP citrate Lyase

APE: Atom Percent Excess (%)

ATP= adenosine triphosphate

C = carbon atom

CAC= Citric Acid Cycle (Krebs Cycle)

CS =Citrate synthase

D= aminotransferase

DD= di-deuterated

DMS= Differential Mobility Spectrometry

GC= Gas Chromatography

GDH= Glutamate dehydrogenase

IDHf,r,net= Isocitrate dehydrogenase, (f) forward, (r) reverse, (net)

Isotopologue: position non specific label information

Isotopomer: position specific label information
LC= Liquid Chromatography
LDH= Lactate dehydrogenase
ME= Malic enzyme
MFA= Metabolic Flux Analysis
MS= Mass Spectrometry
MS/MS= Tandem Mass Spectrometry
NA= Natural Abundance
NAFLD= non alcoholic fatty liver disease
NMR= Nuclear Magnetic Resonance Spectroscopy
OGDH= α Ketoglutarate dehydrogenase
PC= Pyruvate carboxylase
PCC= Propionate CoA carboxylase
PDH= Pyruvate dehydrogenase
PEPCK = phosphoenolpyruvate carboxykinase
SDH= Succinate dehydrogenase
T2DM= Type 2 diabetes mellitus
TCA= Tricarboxylic Acid Cycle (Krebs Cycle)
V=Flux (metabolic flow)
X = aminotransferase
REFERENCES


