DIFFERENTIAL BCAA METABOLISM IN TISSUES OF TUMOR-BEARING MICE: A PATH TO UNDERSTANDING CANCER CACHEXIA

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENTS OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

MASTER OF SCIENCE

GRADUATE PROGRAM IN KINESIOLOGY AND HEALTH SCIENCE

YORK UNIVERSITY, TORONTO, ONTARIO, CANADA

AUGUST 2025

Abstract

Cancer cachexia is a complex syndrome marked by muscle and fat loss, often resistant to nutritional support. While branched-chain amino acids (BCAAs) stimulate muscle protein synthesis, BCAA-targeted therapies have shown inconsistent results. In this study, a C26 colon cancer mouse model was used to examine how tumor burden alters BCAA metabolism across skeletal muscle, liver, kidney, and adipose tissue. Tumors accumulated BCAAs and showed increased enzymatic activity, whereas peripheral sites displayed widespread BCAA depletion, reduced expression of the amino acid transporter LAT1, and suppression of mechanistic target of rapamycin complex 1 (mTORC1) signaling. Notably, the soleus muscle maintained mTORC1 activity despite reduced BCAA availability, suggesting fiber-type–specific adaptations. These findings indicate that tumors act as metabolic sinks, diverting systemic amino acids away from host tissues. Such reprogramming may underlie the limited success of BCAA-based interventions in cachexia and highlight the need for therapies that address both tumor and host metabolism.

Acknowledgements

Thank you, Dr. Adegoke, for believing in me from the start, for your support and leadership over the past two years. Thank you to my committee member, Dr. Perry, for the opportunities and discussions that have helped shape this journey.

Next, thank you to my lab mates, Gagan, Stephen, Lilia, Armita and Jason. Your support and the many laughs (and coffees) along the way made this experience forever memorable.

Finally, I extend my sincere thanks to my family, my husband, and my cats, who have always inspired and encouraged me to do my best. I could not have done this without you all by my side.

Lastly, Alhamdulilah.

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List of Abbreviations

4E-BP1	4E-Binding Protein 1
AA	Amino Acids
AMBRA1	Beclin-1-associated autophagy regulator
AMPK	AMP-activated protein kinase
ATG1	Atrogin-1
ASCT2	Alanine-Serine-Cysteine Transporter 2
ATG13	Autophagy-related protein 13
ATGL	Adipose triglyceride lipase
B^{0}AT1	Sodium-dependent neutral amino acid transporter 1
BCAA	Branched Chain Amino Acids
BCAT	Branched Chain Amino Transferase
BCKA	Branched Chain Keto Acids
BCKD	Branched Chain α-keto Acid Dehydrogenase
BCKDK	Branched Chain α-Keto Acid Dehydrogenase Kinase
CASTOR1	Cellular Arginine Sensor for mTORC1
C26	Colon-26 Tumor Model
CMP	Counts per Minute
EAA	Essential Amino Acids
EIF4B	Eukaryotic initiation factor 4B
FBS	Fetal Bovine Serum
HMB	β-Hydroxy β-methylbutyrate
HSL	Hormone-sensitive lipase
IL-1β	Interleukin-1 Beta
IL-6	Interleukin-6
JAK/STAT3	Janus Kinase/Signal Transducer and Activator of Transcription 3 Pathway
KIC	α-Ketoisocaproate
KIV	α-Ketoisovalerate
KMV	α-Keto-β-Methylvalerate
LAT1	L-Type Amino Acid Transporter 1
LAT2	L-type amino acid transporter 2
LMF	Lipid-mobilizing factor
MPS	Muscle Protein Synthesis
MPB	Muscle Protein Breakdown
mTORC1	Mechanistic Target of Rapamycin Complex 1
MuRF1	Muscle-specific RING finger protein 1
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
NO	Nitric oxide
OPA	o-Phthalaldehyde

P-BCKD	Phosphorylated Branched Chain Keto Acid Dehydrogenase
PBS	Phosphate-Buffered Saline
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1alpha
PP2CM	Protein Phosphatase 2C
P-S6	Phosphorylated Ribosomal Protein S6 Kinase
P-S6K1	Phosphorylated Ribosomal Protein S6K1 Kinase
RHEB	Ras homolog enriched in brain
S6	Ribosomal Protein S6 Kinase
S6K1	Ribosomal Protein S6 Kinase
SEM	Standard Error of the Mean
SF	Supplementary Figure
SNAT1	Sodium-Coupled Neutral Amino Acid Transporter 1
SNAT2	Sodium-coupled neutral amino acid transporter 2
TBST	Tris-Buffered Saline with Tween
TCA	Tricarboxylic Acid Cycle
TFEB	Transcription Factor EB
TNF-α	Tumor Necrosis Factor Alpha
TOP	Terminal oligopyrimidine
ULK-1	Unc-51-like Autophagy Activating Kinase
ZAG	Zinc-α2-glycoprotein

1.0 Introduction

Cancer is the second leading cause of death worldwide [205], affecting millions every year [35]. Depending on the cancer type, more than half of patients experience a debilitating condition known as cachexia [36]. Cancer cachexia is a multifactorial metabolic syndrome, characterized by involuntary weight loss, skeletal muscle atrophy, and adipose tissue depletion [37,41] all of which reduce treatment tolerance [82], diminish quality of life, and increase mortality [80]. Unlike malnutrition, cachexia is not solely the result of reduced caloric intake, rather, it involves profound metabolic disturbances that impair the ability of tissues to retain nutrients and support anabolic processes [43]. These changes often occur despite nutritional support, underscoring the role of systemic metabolic reprogramming in cachexia development [43,44].

Central to the progression of muscle wasting in cachexia is the dysregulation of amino acid (AA) metabolism [48,59], particularly branched-chain amino acids (BCAAs): leucine, isoleucine, and valine [10]. BCAAs serve as both signaling molecules and metabolic substrates [11]. Leucine, in particular, activates the mechanistic target of rapamycin complex 1 (mTORC1) pathway, which promotes muscle protein synthesis and suppresses proteolysis [14,15]. In contrast, under catabolic conditions, BCAAs are oxidized via the branched-chain α-keto acid dehydrogenase (BCKD) complex [20] to supply energy and nitrogen, especially in peripheral tissues such as skeletal muscle, liver, and kidney [74]. Thus, the balance between BCAA signaling and oxidation is critical for maintaining muscle mass and whole-body metabolic health [20].

In cancer, this balance is disrupted. Tumors are known to increase their uptake and utilization of BCAAs to support both rapid proliferation and protein synthesis [29,45,46]. This

increased demand can lead to systemic depletion of BCAAs and impaired anabolic signaling in host tissues [47,48]. While previous studies have documented BCAA accumulation within tumor tissue, little is known about how this accumulation affects the distribution, metabolism, and signaling of BCAAs in peripheral organs and tissues. Further, the role of key regulatory enzymes and transporters - such as BCKD, BDK, PP2CM, LAT1, SNAT1 and P-S6 - remains poorly understood in the context of tumor-bearing cachexia models.

Additionally, disruptions in these systems may explain why BCAA-targeted therapeutic strategies have produced inconsistent results in cachexia [189]. While some studies suggest that BCAA supplementation may preserve muscle mass, others have shown that it may inadvertently fuel tumor growth or fail to activate anabolic pathways in nutrient-depleted tissues [189]. These inconsistencies highlight the need to understand how cancer alters tissue-specific BCAA metabolism and inter-organ AA redistribution.

This study investigated how colon cancer affects BCAA metabolism across multiple tissues in a C26 mouse model of cachexia. Specifically, BCAA and AA concentrations, BCKD enzymatic activity, protein expression of key enzymes in the BCAA metabolic pathway, AA transporters, and protein synthesis signaling molecules were measured in the tumor, liver, kidney, adipose tissue, and skeletal muscle. Through this work, the study aimed to identify the mechanisms by which tumor burden induces systemic metabolic shifts and contributes to tissue wasting. Findings from this study may help explain the limitations of current BCAA based interventions and inform the development of targeted therapies that restore metabolic balance in cancer cachexia.

2.0 Literature Review

2.1 Skeletal Muscle

Skeletal muscle is one of the largest organs in the human body, contributing to approximately 50% of total body mass [1]. It serves as a driver of movement, stability, and posture, while also acting as a critical reservoir for amino acids (AAs) [2,3], the building blocks of protein [4]. Skeletal muscle maintenance is governed by a delicate balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB), processes which are regulated by metabolic signals, mechanical stimuli, and nutritional status [5,6]. In healthy states, this balance ensures skeletal muscle maintenance, while fulfilling local demands for movement and systemic requirements for AA and energy.

The maintenance of skeletal muscle is essential for overall health, contributing to locomotion, thermoregulation and metabolic regulation [7]. Loss of muscle mass, commonly referred to as muscle wasting, is a hallmark of various chronic and acute diseases, including cancer [55] and chronic heart failure [56]. Muscle loss is a key feature of frailty, particularly in older adults and chronically ill populations [86,88,89]. Among others, reduced muscle mass is associated with increased risk of falls, fractures [88], hospitalization [89], all-cause mortality [86], as well as declines in strength, mobility, and independence [8,9]. These effects significantly impair quality of life and long-term health outcomes, reinforcing the urgency of addressing muscle loss of all kinds.

There are many factors involved in maintaining MPS, such as exercise and nutrition [63]. Nutritional factors, particularly the availability of essential amino acids (EAAs), play a complementary role in supporting MPS. Among these, are the branched-chain amino acids

(BCAAs) [10]. To stay within the scope of this thesis, the following section will focus on the role of BCAAs in muscle metabolism.

2.2 Branched Chain Amino Acids

A key factor in skeletal muscle maintenance is the role of BCAAs, comprising leucine, isoleucine, and valine [10]. The BCAAs are EAAs with critical roles in protein synthesis, energy production, and cellular signaling [11]. There are two types of AAs, EAA, are those that our body can not make and must be acquired through food for health and function [210], whereas non-essential amino acids (NEAA) are those that our bodies can make on their own through various process [209]. In contrast to most AAs, which are primarily metabolized in the liver [89], the initial step of BCAA catabolism occurs predominantly in extrahepatic tissues, including skeletal muscle and kidneys [90], due to the limited hepatic expression of branched-chain aminotransferase (BCAT) [12], a pyridoxal phosphate-dependent enzyme [91] that catalyzes the reversible transamination of BCAAs into their corresponding branched-chain α-keto acids (BCKAs) [13], representing the first step of BCAA catabolism. However, subsequent oxidative steps of BCAA metabolism take place primarily in the liver.

There are two isoforms of BCAT:

1. **BCAT1**: which is found in cytosolic compartments of extrahepatic tissues such as the brain and immune cells [65], and
2. **BCAT2:** which is localized to mitochondria and highly expressed in skeletal muscle, kidneys, and other extrahepatic tissues [64].

The key distinction between the two is their subcellular localization and tissue distribution, with BCAT2 playing a predominant role in peripheral tissue BCAA metabolism [66]. Beyond localization, BCAT1 is linked to nitrogen shuttling and proliferative processes (ex:

in brain and cancer cells) [257], whereas BCAT2 supports energy metabolism by coupling BCAA catabolism to the mitochondrial TCA cycle [258].

Leucine is a potent activator of the mechanistic target of rapamycin complex 1 (mTORC1), a central regulator of protein synthesis and muscle growth [14]. mTORC1 activation depends on its localization to the lysosomal surface, a process that is dynamically regulated by the presence of AAs [15]. When BCAAs, particularly leucine, are available, they stimulate the recruitment of mTORC1 to lysosomes through the Rag GTPase complex, enabling mTORC1 activation [70]. Upon activation, mTORC1 phosphorylates downstream targets, including ribosomal protein S6 kinase (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), initiating mRNA translation and promoting protein synthesis and muscle fiber growth [16,17,18,]. Phosphorylation of S6K1(P-S6K1) subsequently leads to phosphorylation of ribosomal protein S6 (P-S6), which enhances the translation of mRNAs involved in ribosome biogenesis and protein synthesis [92], this is discussed in more detail in section 2.2.1.

While leucine is the most potent activator of mTORC1 and the most extensively researched [71], all three BCAAs, leucine, isoleucine, and valine, contribute to this process. They collectively provide critical nutrient signals and metabolic substrates that support anabolic signaling and energy production, further promoting muscle maintenance and growth [72,73]. Through these mechanisms, BCAAs play an essential role in anabolic signaling and preserving muscle mass under normal physiological conditions.

Beyond their anabolic roles, BCAAs also act as metabolic substrates, contributing carbon skeletons for gluconeogenesis and the tricarboxylic acid (TCA) cycle particularly in tissues such as the liver and skeletal muscle [74]. The liver primarily utilizes BCAAs for gluconeogenesis, especially during fasting, while skeletal muscle catabolizes BCAAs for energy production

through the TCA cycle during periods of increased metabolic demand such as exercise or illness [2].

Additionally, BCAAs modulate protein degradation pathways, including the ubiquitin-proteasome system and autophagy-lysosomal pathways [75,76], which are central to muscle protein breakdown. By activating mTORC1, BCAAs suppress these proteolytic processes through several mechanisms. For instance, mTORC1 directly inhibits autophagy by phosphorylating and inactivating key regulators such as Unc-51-like autophagy activating kinase (ULK-1), transcription factor EB (TFEB), autophagy-related protein 13 (ATG13), and Beclin-1-associated autophagy regulator (AMBRA1) [18, 67, 68, 69]. This suppression of autophagic activity reduces the degradation of intracellular proteins and organelles, thereby preserving muscle protein content and muscle mass [18]. Albeit these effects, sustained elevations of the BCAAs in plasma and skeletal muscle have been associated with insulin resistance and type 2 diabetes mellitus, emphasizing the need for balanced regulation of their metabolism [18]. In all, these metabolic and signaling properties position BCAAs as integral to muscle health and a key focus of research in conditions characterized by muscle wasting, such as cancer cachexia [19].

2.2.1 mTORC1 Signalling: Specific Roles of P-S6 and P-S6K1

As discussed in section 2.2, mTORC1 is a central regulator of cell growth and MPS, particularly responsive to AA availability [15,16,17,18], especially leucine. Among its downstream effectors, P-S6 and P-S6K1 play critical and distinct roles in promoting translational activity in skeletal muscle [92].

P-S6K1, phosphorylation at Thr389 [93], is a direct substrate of mTORC1 and serves as a key intermediary in initiating mRNA translation to MPS [93]. Upon activation, P-S6K1

phosphorylates multiple targets involved in ribosomal biogenesis and translation initiation, including eukaryotic initiation factor 4B (eIF4B) and P-S6 [92, 94].

P-S6, phosphorylated at Ser235/236 [95], and central to this study design, is a downstream readout of S6K1 activity [94]. Elevated levels of P-S6 are commonly used as a proxy for mTORC1 activity in skeletal muscle and tumor models [95], serving as an indirect indicator that MPS is active or inactive. Increased P-S6 is typically associated with anabolic states that promote muscle growth and hypertrophy [96]. Conversely, reduced or absent P-S6 signaling reflects suppressed mTORC1 activity, a hallmark of muscle wasting observed in conditions such as disuse, cancer cachexia, and other chronic illnesses [98].

These phosphorylation events are tightly regulated by nutrients, such as AAs [98] and hormonal signals, such as insulin [99]. For example, and as discussed in section 2.2, leucine directly stimulates mTORC1 localization to the lysosome via the Rag-GTPase complex [70], enabling the phosphorylation events of S6K1 and S6 [92]. Building on this, tumor-driven BCAA depletion or inflammation-induced suppression can impair mTORC1 signaling upstream [95], thereby reducing P-S6 levels and contributing to muscle wasting observed in cachexia [100]. Measuring P-S6 and P-S6K1 thus provides mechanistic insight into translational control in various tissues and reflects the balance between anabolic and catabolic states in cancer-related muscle wasting.

2.2.2 AA Transporters and Tissue-Specific Regulation

As discussed in section 2.2, the availability of intracellular AAs is essential for MPS and anabolic signaling, particularly through activation of mTORC1 [14]. This process is tightly regulated by AA transporters, which mediate the uptake and exchange of AAs across cell membranes [101,102,103,104].

In skeletal muscle, large amino acid transporter 1 (LAT1) and small neutral amino acid transporter 1 (SNAT1) function together to regulate AA uptake and signaling [101]. LAT1 can mediate leucine uptake in exchange for intracellular glutamine, directly promoting mTORC1 activation and protein synthesis [102]. SNAT1 maintains intracellular glutamine levels [103], supplying the substrate required for LAT1 exchange activity. Although SNAT1 is well-studied in neurons for its role in neurotransmitter cycling [207], in skeletal muscle it supports AA exchange and mTORC1 signaling by sustaining intracellular glutamine pools [104]. Together, these transporters form a functional axis that facilitates AA and promotes muscle anabolism [101,102,103,104].

Beyond skeletal muscle, AA transporters also play important roles in the liver, kidney, adipose tissue, and tumor, where they regulate local AA availability to support metabolic demands [105,106]. For example, tumors often upregulate LAT1 and Alanine-Serine-Cysteine Transporter 2 (ASCT2) to enhance leucine and glutamine uptake for rapid growth [112]. This increased demand may reduce circulating AA [107,108] and disrupt nutrient balance in peripheral tissues [109], contributing to systemic effects like muscle wasting and metabolic alterations [110].

Although transporter activity is context-dependent, alterations in their expression across tissues may influence anabolic signaling, AA redistribution, and the progression of cancer cachexia [111]. As such, they represent important regulatory nodes in the maintenance of muscle mass and systemic metabolic homeostasis.

2.3 BCAA Metabolism

As shown in *Figure 1*, the initial steps of BCAA metabolism involve a shared, two-step enzymatic process for all three BCAAs. The first step is a reversible transamination reaction

catalyzed by BCAT. In skeletal muscle, which highly expresses BCAT2, this reaction converts BCAAs into branched-chain keto acids (BCKAs): α-ketoisocaproate (KIC) from leucine, α-keto-β-methylvalerate (KMV) from isoleucine, and α-ketoisovalerate (KIV) from valine [20,21]. These BCKAs then follow one of two primary pathways:

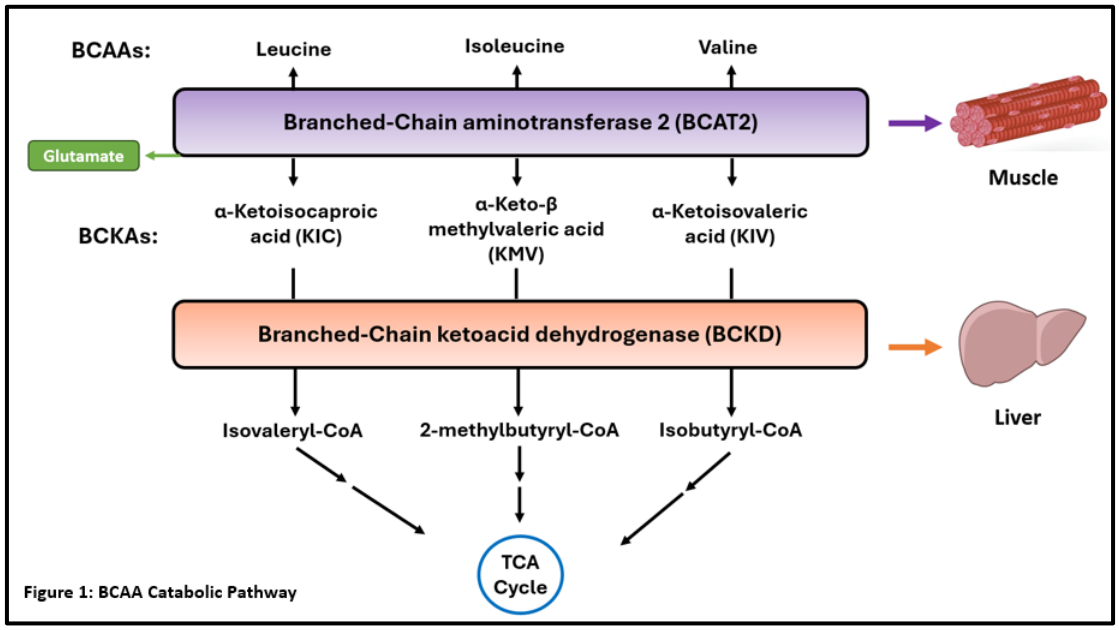

Figure 1: BCAA Catabolic Pathway

1. **Oxidative Decarboxylation:** In the liver, BCKD complex catalyzes the second, irreversible step, converting BCKAs into acyl-CoA derivatives. These derivatives enter the TCA cycle for ATP production or serve as substrates for gluconeogenesis [22,23]. This is the pathway shown in Figure 1**.** Notably, in the case of leucine, its ketoacid KIC can also be converted to β-Hydroxy β-methylbutyrate (HMB) via KIC dioxygenase [216], a less prominent yet biologically relevant pathway, especially in relation to MPS as HMB downregulate ubiquitin-proteasome proteolysis pathways and upregulate mTORC1 signalling. [216,217].
2. **Local Utilization in Skeletal Muscle:** While the BCKD complex is generally considered to be expressed at lower levels in skeletal muscle compared to the liver [218], it can still contribute to local BCKA oxidation under certain conditions. During periods of metabolic

stress, such as fasting or exercise, skeletal muscle may increase its reliance on BCAA catabolism to support energy demands [20].

The BCKD complex in the liver, represents the rate-limiting step in BCAA catabolism and is therefore, tightly regulated by phosphorylation. BCKD kinase (BCKDK) inhibits BCKD activity by phosphorylating its E1α subunit, while protein phosphatase 2C (PP2CM) reverses this inhibition, allowing for dynamic metabolic adaptation based on nutrient availability and energy demands [20]. This is represented in *Figure 2*.

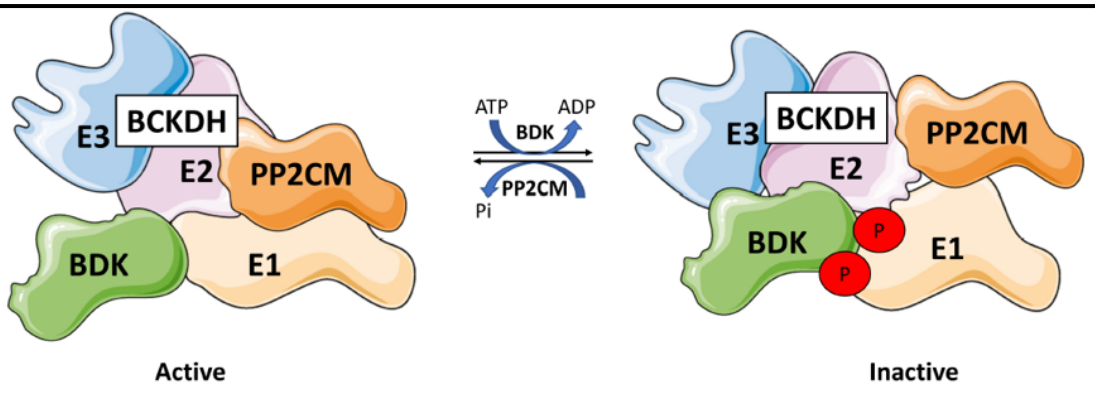

Figure 2 Regulation of BCKD Activity: The activity of the branched-chain α-ketoacid dehydrogenase (BCKD) complex is controlled by reversible phosphorylation of its E1 subunit. On the right, in its inactive state, BCKD is phosphorylated at Ser293 and Ser303 by branched-chain ketoacid dehydrogenase kinase (BDK), which suppresses its enzymatic function. On the left, BCKD is shown in its active state, where protein phosphatase 2Cm (PP2CM) removes these phosphate groups, restoring its activity and allowing for continued BCAA catabolism. Adapted from Mann et al [18].

2.4 Tissue-Specific Contributions to BCAA Metabolism

BCAA metabolism is tightly regulated across multiple organs and tissues, including skeletal muscle, liver, adipose tissue, and kidneys [131], which will be the primary focus of my thesis. Each tissue plays a distinct yet interconnected role in maintaining systemic BCAA balance, highlighting their significance in metabolic homeostasis and disease progression.

As discussed previously in section 2.3, skeletal muscle serves a central role in the initial step of BCAA catabolism due to its high expression of BCAT2. BCAT2 initiates the transamination of

BCAAs into their respective BCKAs, a critical step in BCAA metabolism [20]. The resulting BCKAs can be exported from skeletal muscle into circulation via monocarboxylate transporters, including MCT1 (SLC16A1), which has been shown to mediate BCKA efflux in cellular models [259]. Additionally, sodium-coupled monocarboxylate transporters such as SMCT1 are capable of transporting BCKAs, supporting their systemic movement [260]. This mechanism allows skeletal muscle to offload excess nitrogen-free carbon skeletons, where these circulating BCKAs are subsequently taken up by the liver through corresponding transporter systems. Additionally, leucine's activation of mTORC1 promotes MPS and maintenance, particularly in response to nutritional and mechanical stimuli [14]. This dynamic role positions skeletal muscle as central to both anabolic signaling and energy homeostasis.

The liver complements skeletal muscle by completing the oxidative decarboxylation of BCKAs through the activity of the BCKD complex. This irreversible step converts BCKAs into acyl-CoA derivatives, which are either utilized in the TCA cycle for ATP production or directed toward gluconeogenesis during fasting, ensuring glucose availability for systemic energy needs [23]. Importantly, the liver expresses very little BCAT2, making it reliant on skeletal muscle for the initial step of BCAA metabolism [15]. This inter-organ collaboration highlights the liver's pivotal role in systemic BCAA regulation and metabolic adaptation under stress conditions. Adipose tissue, though less metabolically active than skeletal muscle or the liver, contributes to systemic BCAA homeostasis by acting as a metabolic buffer [133]. It exhibits moderate BCAT2 activity, enabling it to regulate circulating BCAA concentrations during periods of nutritional excess or deprivation [7].

Lastly, the kidneys play a significant role in BCAA metabolism, particularly in the transamination of BCAAs, a function that has been less emphasized in literature. Similar to

skeletal muscle, the kidneys express high levels of BCAT2, enabling the initial conversion of BCAAs into BCKAs [7, 20]. Although kidneys do not participate in transamination as much as skeletal muscle, this process supports gluconeogenesis, particularly in the renal cortex, which helps meet both local energy demands and systemic glucose needs during fasting or metabolic stress [134]. It also supports acid-base balance, and facilitates the synthesis of AAs such as arginine and tyrosine from BCAA-derived intermediates [24].

Taken together, BCAA metabolism is a dynamic process that integrates the functions of various organs in the body to maintain balance and overall health. However, this balance is easily disrupted in pathological conditions and can influence disease progression. Understanding these disruptions is essential for exploring how altered BCAA metabolism contributes to muscle-wasting conditions like cancer cachexia.

2.5 AAs Beyond BCAAs

While BCAAs play a central role in skeletal muscle maintenance and metabolic regulation, other AAs are equally important in supporting whole-body homeostasis, anabolic processes, and inter-organ communication [113]. Among these, and to stay within the scope of this thesis, are NEAAs glutamate, alanine, and arginine, which stand out for their versatile metabolic roles, particularly in skeletal muscle, liver, and their involvement in immune function [114].

Glutamate, the most abundant AA in the body [211], is a central product of transamination, serving as a key nitrogen donor and participates in various metabolic pathways [115]. It contributes to energy production via conversion to α-ketoglutarate in the TCA cycle [116] and serves as a precursor for glutamine, which plays a major role in acid-base balance and immune cell function [117].

Alanine is synthesized in skeletal muscle through the transamination of pyruvate and serves as a carrier of carbon and nitrogen to the liver via the glucose-alanine cycle [118]. There, it is converted to glucose through gluconeogenesis, supporting energy homeostasis during fasting and exercise [119].

Arginine contributes to protein synthesis, nitric oxide (NO) production, and detoxification through the urea cycle [120]. It also indirectly activates the mTORC1 pathway by engaging nutrient-sensing mechanisms [118]. Specifically, arginine is detected by cytosolic sensors such as Cellular Arginine Sensor for mTORC1 (CASTOR1) and by lysosomal transporters like SLC38A9, both of which promote the activation of Rag GTPases. This leads to the recruitment of mTORC1 to the lysosomal surface, where it can be activated by Ras homolog enriched in brain (Rheb), ultimately enhancing protein synthesis and cell growth [255].

Together, these NEAAs contribute to the regulation of metabolic balance, tissue function, and nutrient signaling [122], emphasizing the importance of studying a broad range of AAs when evaluating muscle health and whole-body metabolism.

2.6 Overview of AA Metabolism

While BCAAs represent a distinct subset of EAAs with unique metabolic handling in extrahepatic tissues, they function within a broader framework of AA metabolism that spans multiple organ systems. General AA metabolism involves multiple tightly coordinated pathways that regulate protein turnover, energy balance, and nitrogen homeostasis across organ systems [120]. Unlike BCAAs, which are predominantly catabolized in extrahepatic tissues [123], most AAs undergo initial transamination and subsequent degradation within the liver [124], where they feed into the urea cycle or serve as precursors for other metabolic processes [125].
AA metabolism generally follows a two-step process:

1. **Transamination:** Most AAs undergo reversible transamination catalyzed by aminotransferases, transferring their amino group to α-ketoglutarate to form glutamate. This reaction occurs in multiple tissues, including skeletal muscle and liver, and serves to funnel nitrogen into central intermediates [126].
2. **Deamination and Ureagenesis:** In the liver, glutamate is deaminated by glutamate dehydrogenase to release free ammonia, which enters the urea cycle. Through this process, known as ureagenesis, the liver safely disposes of nitrogen by converting ammonia into urea for excretion by the kidneys [127].

In parallel, several AAs serve as key metabolic intermediates. For example, alanine and glutamine synthesized in skeletal muscle are transported to the liver for gluconeogenesis and nitrogen disposal [128]. This inter-organ coordination allows skeletal muscle to function as both a source and sink of AAs depending on physiological demands.

The kidney and adipose tissue also contribute to systemic AA metabolism [129,130]. Kidneys participate in transamination and assist in maintaining acid-base balance by modulating AA-derived ammonium excretion [129], while adipose tissue can buffer AA levels during periods of nutrient deprivation or stress [130].

Furthermore, these processes highlight the importance of viewing AA metabolism as a distributed, multi-organ network, capable of dynamic adaptation in response to energy demand, hormonal regulation, and nutrient availability [114].

2.7 Tissue-Specific Contributions to AA Metabolism

As lightly discussed in section 2.6, in addition to BCAAs, other AAs are also regulated across multiple tissues, each contributing uniquely to, among others, the coordination of nitrogen balance, energy metabolism, and systemic flexibility [122]. The regulation of these AAs is

tightly controlled and adapts to a variety of physiological demands, including fasting, physical activity, and stress [124].

Skeletal muscle plays a central role in inter-organ AA handling [134]. It synthesizes glucogenic AAs such as alanine and glutamine [118], which are released into circulation and transported to the liver [133]. Further, alanine, formed through the transamination of pyruvate [125], participates in the glucose-alanine cycle [118], shuttling nitrogen and carbon from muscle to liver for gluconeogenesis [118]. Glutamine, derived from glutamate, also serves as a nitrogen carrier and plays important roles in immune support and acid-base regulation [117]. Together, these functions position skeletal muscle as both a reservoir and regulator of AAs during metabolic stress [138].

The liver serves as the primary site for AA catabolism, where it carries out transamination and oxidative deamination of most AAs [114]. Glutamate, produced from transamination, is further deaminated to release ammonia, which enters the urea cycle [138]. Through ureagenesis, the liver converts this potentially toxic ammonia into urea, a water-soluble compound excreted by the kidneys [125,139]. This pathway is essential for nitrogen disposal and is particularly active during catabolic conditions, such as fasting, injury, or inflammation, when AA turnover increases [139].

The kidneys contribute to AA metabolism by facilitating transamination reactions and playing a key role in acid-base balance [140]. Ammonium ions, derived from AA metabolism, are excreted to regulate systemic pH [141], while bicarbonate is reabsorbed to maintain acid-buffering capacity [136]. Additionally, the renal cortex participates in gluconeogenesis using AA derived substrates, particularly during periods of prolonged fasting or increased metabolic demand [133].

Although less frequently discussed, adipose tissue also participates in systemic AA regulation. It exhibits moderate transamination activity and may act as a metabolic buffer [132], by taking up excess AAs during nutrient abundance and releasing them during deprivation. In doing so, adipose tissue helps stabilize circulating AA levels and preserve muscle and organ function under stress [130]. Altogether, these tissue-specific roles highlight the integrated nature of AA metabolism and its importance in supporting systemic metabolic flexibility and homeostasis.

2.8 AA Metabolism in Health

Collectively, these tissue-specific functions emphasize that AA metabolism is not confined to isolated organs, rather it is distributed across a dynamic, multi-organ network. This coordination allows the body to adapt to changing physiological states and maintain systemic homeostasis. However, when this balance is disrupted, particularly in pathological conditions such as cancer, tumor growth may alter host metabolism, disturb inter-organ AA handling [45,46], and contribute to metabolic dysfunction and muscle-wasting conditions such as cancer cachexia [47]. As a result, understanding how cancer affects this network, especially in the context of systemic dysfunction and muscle loss, is essential. The next section will introduce the biology of cancer, with a focus on colon cancer and its relevance to the metabolic disruptions explored in this thesis.

2.9 Cancer

Closely behind cardiovascular disease, cancer is the second leading cause of death globally [28]. Cancer is characterized by uncontrolled cell proliferation within a given tissue [25]. Cancer cells have the unique ability to invade surrounding tissues and metastasize to distant parts of the body, while evading stop signals [25,26,27]. These aberrant growth processes are

driven by genetic mutations, epigenetic changes, and alterations in metabolic pathways, which disrupt normal cellular functions [28,29]. Among the many types of cancer, colon cancer is particularly relevant to this study due to its profound impact on systemic metabolism and its association with muscle wasting [77] which will be further discussed.

2.10 Colon Cancer

Colon cancer represents a major global health challenge [30]. Each year, approximately 1 million new cases are diagnosed worldwide [31], with a prominent increase in incidence, particularly among individuals under 50 years old [32]. This disease often originates from adenomatous polyps and is influenced by a complex interplay of genetic predisposition, environmental factors, and lifestyle choices [33]. Among others, risk factors such as poor diet, tobacco use, obesity, and chronic inflammatory conditions (i.e. inflammatory bowel disease) further contribute to its development [31,34]. Advancing our understanding of the causes and focusing on effective prevention and treatment strategies remain critical to mitigating its global impact. Importantly, colon cancer is also one of the cancer types most frequently associated with the development of cachexia [6] – a metabolic syndrome characterized my involuntary weight loss and muscle wasting [37]. This connection is particularly relevant to the present thesis, as cachexia contributes significantly to the poor outcomes observed in colon cancer patients [219] and is tied to disruptions in AA metabolism.

2.11 Cachexia

With cancer affecting millions worldwide [35], more than half of patients, depending on the cancer type, experience a debilitating condition known as cachexia [36]. As lightly discussed in section 2.10, cachexia is a multifactorial syndrome characterized by the involuntary weight loss and muscle wasting, significantly affecting patients' quality of life and treatment outcomes

[37]. The syndrome often involves a loss of over 5% of body weight within six months, which cannot be reversed by traditional nutritional support alone [38,39,40]. This is accompanied by a decline in lean body mass, including both muscle and fat tissue, and symptoms such as fatigue, inflammation and anorexia, complicating treatment [41]. The abnormalities associated with cachexia stem from a combination of altered metabolism and reduced food intake in cancer patients, leading to a negative protein and energy balance [42].

Cachexia is particularly prevalent in advanced cancers, such as colon cancer, where metabolic imbalances driven by tumor growth and systemic inflammation disrupt normal muscle and tissue homeostasis [43]. Pro-inflammatory cytokines, including Interleukin 6 (IL-6), Tumor Necrosis Factor Alpha (TNF-α), and Interleukin-1 Beta (IL-1β), play a central role by activating pathways such as JAK/STAT3 and NF-κB [57, 58]. These pathways promote muscle proteolysis, adipose lipolysis, and the production of reactive oxidative species, further accelerating tissue loss [43,44]. Various management strategies, including nutritional support, pharmacological treatments, and exercise, aim to mitigate muscle loss and improve overall outcomes [38]. However, despite its profound impact, cancer cachexia remains an unresolved challenge in oncology, highlighting the need for continued research to better understand its underlying mechanisms [41].

2.12 Implications of Cachexia

The effects of cachexia extend beyond muscle wasting and weight loss, significantly impairing quality of life and reducing survival rates in cancer patients [80]. Additionally, cachexia complicates cancer treatment by adversely impacting muscle mass, which is critical for both functional capacity and chemotherapy tolerance [81]. Reduced muscle mass increases the risk of chemotherapy toxicity, as lower lean body mass alters drug metabolism and clearance

[82]. Consequently, patients may experience greater treatment-related side effects, necessitating dose reductions or even the early termination of therapy [83].

In addition to its metabolic disruptions, cachexia presents a range of systemic and functional challenges that impact nearly every aspect of patient well-being. Symptoms such as fatigue, reduced appetite, and early satiety contribute to decreased energy intake and physical inactivity [141], accelerating muscle loss and weakening the body overall [45]. This decline in physical function often leads to frailty, diminished mobility, and increased risk of falls, especially in older or vulnerable populations [142]. Beyond the physical consequences, cachexia is associated with emotional and psychological burdens, including changes in body image, social withdrawal, and depression [143], factors further diminishing quality of life. These compounding effects emphasize that cachexia is both a physiological condition as well as a multidimensional syndrome that affects patients physically, emotionally, and socially throughout the course of their illness.

These challenges compromise treatment efficacy and also exacerbate the physical and emotional toll on patients, emphasizing the need for effective interventions to mitigate the devastating impacts of cachexia. One developing area of attention is the role of BCAA metabolism, which is intricately linked to both cancer progression and the development of cachexia. This will be discussed in later section 2.14 and 2.15.

2.13 Inflammatory Regulation of Cachexia

As lightly discussed in section 2.9, chronic systemic inflammation is a defining feature of cancer cachexia and a key driver of the metabolic alterations that underpin this syndrome [141]. Elevated levels of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β, initiate and sustain catabolic processes that contribute to ongoing muscle wasting, adipose tissue breakdown,

and metabolic inefficiency [47]. These cytokines exert their effects through the activation of several signaling cascades, most notably the NF-κB and JAK/STAT3 pathways [57,58].

TNF-α is among the most extensively studied cytokines in cachexia [144] and has been shown to promote proteolysis by enhancing ubiquitin-proteasome activity [41] and suppressing protein synthesis pathways, including mTORC1 [145]. IL-6, meanwhile, activates STAT3 signaling [146] and is the most effective inducer of hepatic acute-phase protein synthesis [147], leading to increased AA demand and a shift in protein metabolism [147]. IL-1β contributes to both muscle wasting and appetite suppression [148,149], in part through its effects on hypothalamic signaling [148] and serotonergic tone [149]. These cytokines, particularly TNF- α, may also stimulate the production of reactive oxygen species (ROS), further exacerbate tissue degradation and impair mitochondrial function [150].

Importantly, these inflammatory mediators are elevated systemically [151] and also act locally within tissues [152], amplifying metabolic disruption. Their persistent elevation is associated with resistance to anabolic stimuli, including nutrition and exercise [153]. As a result, inflammation is increasingly recognized as both a trigger and perpetuator of cachexia [6], making anti-inflammatory targets an area of growing therapeutic interest.

2.14 Cytokine Effects on Tissue Metabolism

While inflammation is a defining feature of cachexia [141], the effects of pro-inflammatory cytokines extend beyond general metabolic dysregulation to include tissue-specific responses that contribute to coordinated systemic wasting. Cytokines such as TNF-α, IL-6, and IL-1β act on multiple organs [155].

In skeletal muscle, TNF-α and IL-6 promote proteolysis by activating the ubiquitin-proteasome system and suppressing mTORC1 signaling [41]. These cytokines also upregulate

the expression of muscle-specific E3 ligases such as MuRF1 and Atrogin-1, leading to breakdown of contractile proteins and muscle atrophy [155]. TNF-α further disrupts insulin signaling, contributing to anabolic resistance in muscle tissue [156].

In the liver, IL-6 is a potent inducer of the acute-phase response [147], redirecting AAs away from peripheral tissues toward hepatic protein synthesis, promoting gluconeogenesis and the production of inflammatory proteins [157], increasing energy expenditure while limiting AA availability for muscle repair [158]. TNF-α can also impair lipid metabolism in the liver and contribute to hepatic insulin resistance [159].

Adipose tissue is highly responsive to inflammatory signals, particularly TNF-α, which stimulates lipolysis and downregulates genes involved in lipid storage [160]. The resulting increase in free fatty acids contributes to systemic inflammation and further metabolic imbalance [161]. IL-6 has been shown to influence adipokine secretion and insulin sensitivity [162], both of which play important roles in whole-body energy regulation. Specifically, IL-6 can suppress adiponectin and increase pro-inflammatory cytokines secreted by adipose tissue, contributing to insulin resistance and altered lipid metabolism [163]. In addition, IL-6 has been implicated in the browning of white adipose tissue in certain cancer models, a process where white adipocytes take on characteristics of thermogenic brown fat [164,165]. This phenotypic shift increases energy expenditure and may contribute to the accelerated fat loss observed in cancer-associated cachexia [164,165].

Within tumors, pro-inflammatory cytokines are produced both by cancer cells and surrounding stromal, such as endothelial and fibroblasts [167], or immune cells, such as T-cells and dendric cells [168], in the tumor microenvironment and contribute to tumor progression

[166]. IL-6 can promote tumor growth via STAT3 signaling [57,58], while TNF-α may support angiogenesis and matrix remodeling [169, 170].

Furthermore, the way in which inflammatory cytokines from tumors affect peripheral tissues reminds us that cancer's impact extends well beyond the tumor itself. It can reprogram metabolism across multiple organs. Just as cytokines drive tissue-specific changes in muscle and fat, BCAA and AA metabolism is also likely altered throughout the body in response to tumor growth. This idea is central to my study, which focuses on how BCAA metabolism is regulated across tumor, liver, kidney, adipose tissue and skeletal muscle in tumor-bearing mice. The next section will explore how these disruptions in BCAA metabolism may contribute to cancer-associated muscle wasting, with an emphasis on the tissue-specific dysregulation that underpins cachexia.

2.15 Dysregulated BCAA metabolism in Cancer

BCAA metabolism plays a central role in cancer biology, influencing tumor growth, immune modulation, and systemic metabolic regulation [48,59]. In colon cancer, metabolic reprogramming enables tumor cells to exploit BCAAs as critical substrates for growth and proliferation [45]. Alterations in BCAA metabolism is particularly evident in colorectal cancer, where reduced BCAT2-mediated BCAA catabolism in tumor and stromal tissues leads to chronic activation of mTORC1 signaling, promoting tumorigenesis [29, 46]. This metabolic shift underscores the dual role of BCAAs, as while they are necessary for normal cellular function, their reprogramming in tumors supports anabolic growth and may disrupt systemic homeostasis. Tumor cells use BCAAs for nucleotide synthesis and energy production in addition to sustaining their rapid proliferation [60]. As a result, enhanced BCAA uptake and utilization in tumors may

reduce their availability in peripheral tissues such as skeletal muscle, contributing to muscle wasting and the progression of cancer-associated cachexia [47,48,49].

In addition, the disruption of BCAA metabolism in cancer contributes to the onset of cachexia [61], affecting multiple tissues beyond the tumor itself. In skeletal muscle, increased BCAA oxidation and impaired mTORC1 signaling disrupts protein synthesis and enhances proteolysis, contributing to muscle wasting. In the liver, there is a metabolic shift toward gluconeogenesis at the expense of other anabolic processes. BCAA catabolism generates alanine, which serves as a gluconeogenic precursor, thereby enhancing glucose production while indirectly influencing lipid metabolism [50, 51]. Additionally, glutamine, a metabolite interconnected with BCAA metabolism, plays a crucial role in this metabolic shift [62]. As discussed in section 2.3, glutamine is primarily synthesized in skeletal muscle, where BCAAs undergo transamination catalyzed by BCAT, transferring their amino group to α-ketoglutarate to form glutamate. Subsequently, glutamate combines with ammonia through the action of glutamine synthetase to produce glutamine [78]. The glutamine generated through this process supports both gluconeogenesis and also serves as a key nitrogen carrier, facilitating nitrogen transport and disposal between tissues. This dual role highlights glutamine's importance in maintaining metabolic homeostasis, particularly during periods of increased metabolic stress [62]. The interdependence of these processes highlights the broader metabolic network influenced by BCAA dysregulation, with far-reaching impacts on energy and AA homeostasis.

Adipose tissue also plays a pivotal role in BCAA metabolism. In cachexia, reduced BCKD activity in adipose tissue results in elevated circulating BCAA levels, a phenomenon linked to insulin resistance and systemic metabolic dysregulation [12, 51]. Together, these

disruptions underscore the complex interplay between tissues in the metabolic derangements associated with cancer-induced cachexia.

Additionally, inflammatory cytokines released from adipose tissue and tumors further disrupt systemic metabolic homeostasis, compounding the progression of cachexia [19]. Systemic inflammation, driven primarily by cytokines such as IL-6 and TNF-α [43], worsens BCAA dysregulation by impairing enzymatic activity across multiple tissues. These inflammatory signals disrupt mTORC1 signaling pathways, reduce protein synthesis, and amplify tissue catabolism [52,53]. Together, these interconnected processes highlight the complex interplay between skeletal muscle, liver, adipose tissue, kidneys, and tumor metabolism in the progression of cancer cachexia.

While dysregulation of other AAs in cancer is a growing area of interest, a detailed exploration of these pathways falls outside the scope of this thesis. Although several AAs are included in the experimental analyses, the primary focus remains on BCAAs and their metabolic regulation across tissues. This targeted approach allows for a more in-depth investigation of BCAA focused alterations and their potential contribution to cancer cachexia.

2.16 BCAA Therapeutic Implications for Cachexia Treatment

Despite BCAAs being hijacked by tumor cells for tumor progression, targeting BCAA metabolism offers a promising approach to restore muscle muss and address both colon cancer progression and cachexia. While tumors often exhibit enhanced BCAA catabolism to generate TCA cycle substrates and fuel their rapid growth [45], strategies that modulate this pathway could potentially disrupt tumor energy metabolism, thereby limiting proliferation and reducing the tumor-driven metabolic demands that contribute to systemic imbalances. Moreover, restoring anabolic signaling in peripheral tissues could help suppress tumor growth while reducing muscle

wasting [54]. However, the dual role of BCAAs in cancer - where they can both support and hinder tumor growth - means a more precise, tissue-specific approach is needed. Despite its potential, research into BCAA metabolism in cancer progression and cachexia remains limited, particularly regarding its effects across multiple tissues.

2.17 Challenges in Current BCAA-Based Therapies

While the therapeutic potential of BCAAs has gained interest, outcomes in current studies remain inconsistent. Evidence suggests that BCAA supplementation may preserve muscle mass in some cases, however, these effects are highly variable [84]. Moreover, the underlying mechanism of cachexia goes beyond simple caloric or protein deficits. Systemic drivers such as inflammation and tumor-induced metabolic reprogramming often overpower the anabolic effects of nutritional interventions, rendering them as insufficient standalone treatments [85]. These limitations highlight the need for more complete research to clarify the role of BCAA metabolism in cachexia. Investigating tissue specific effects, particularly in tumor, liver, kidneys, adipose and skeletal muscle, may uncover mechanisms that explain the variability in therapeutic outcomes.

3.0 Rationale

Cancer cachexia is a debilitating condition characterized by severe muscle wasting and metabolic dysfunction. Central to this syndrome is the dysregulation of BCAA metabolism. While the role of BCAA metabolism in tumor cells is well-established, the impact on peripheral organs and tissues, such as skeletal muscle, liver, adipose tissue and kidneys, remains under studied. By investigating BCAA metabolism within these peripheral sites we can gain a greater understanding of their specific contributions to cachexia. Pairing such studies, we can potentially understand why BCAA-targeted therapeutic strategies presents itself as inconclusive.

3.1 Objectives

1. Investigate if protein expression of key regulators of BCAA metabolism (such as BCKD, P-BCKD, PP2CM, BDK, SNAT1, LAT1) is altered in tumor, liver, kidneys, adipose and skeletal muscle of tumor-bearing mice.
2. Measure the levels of BCAAs and AAs in tumor, liver, kidneys, adipose and skeletal muscle of tumor-bearing mice.
3. Assess tissue-specific capacity for BCAA oxidation by measuring BCKD activity in tumor, liver, kidneys, adipose and skeletal muscle of tumor-bearing mice.
4. Interpret findings in relation to existing BCAA-targeted therapies, highlighting potential reasons for their limited or inconsistent effectiveness.

3.2 Hypothesis

It is hypothesized that tumor-bearing mice will exhibit significant reductions in BCAA levels, metabolism and oxidation across liver, kidneys, adipose and skeletal muscle, driven by the tumor's utilization of BCAAs to support its growth and proliferation. These changes will

reflect tissue-specific metabolic disruptions associated with cachexia and may help explain why BCAA-targeted therapies have produced inconsistent or limited outcomes in cancer.

3.3 Author Contributions

This work is part of a collaboration between Dr. Adegoke's lab and Dr. Perry's lab at York University.

The original animal study and tissue collection were conducted by Dr. Luca J. Delfinis (LJD) as part of his doctoral research under the supervision of Dr. Christopher R.G. Perry (CRGP) [79]. That phase of the work is complete and formed the basis for the current study.

The present study, involving additional analysis of tissues from the original cohort, was conceptualized by Miriam Zerrouk (MZ) and Dr. Olasunkanmi A. Adegoke (OAJA). MZ performed all subsequent experiments and completed all data analysis. MZ drafted the initial version of the thesis, OAJA edited and reviewed the thesis.

3.4 Additional Contributions

The following represents additional contributions made during my MSc that are not part of my thesis:

Co-Author: In Submission

1. **Zerrouk, M**., Boateng, G.O., Adegoke, O.A. Racial and Ethnic Differences in the Incidence, Prevalence, Risk Factors and Severity of Cachexia: A Scoping Review. 2025

4.0 Materials and Methods

The animal experiments and tissue collection were conducted by-then PhD student Dr. Luca Delfinis in Dr. Perry's lab. My role in this project focused on analyzing the frozen tissues that were prepared and stored following the completion of the animal experiments. Below is an outline of the methods employed during the original animal experiments, as described by Dr. Delfinis [79]. After tissue collection, all procedures, including tissue analysis, were conducted by me with an n=10 for control mice (C) and n=9/10 for C26 tumor bearing mice (C26) for all peripheral organs and tissues except the soleus. For the soleus there was an n=5 for C and n=5 for C26.

4.1 Animal Care

Male CD2F1 mice (8 weeks old) were purchased from Envigo and acclimatized for a minimum of 72 hours upon arrival. During this period, mice were housed in standard laboratory conditions with ad libitum access to chow and water. Although food intake was not directly assessed, prior research with this model has shown that cachexia-induced muscle atrophy occurs independently of reduced food intake as pair-fed control mice maintained normal muscle weights, fiber cross-sectional areas, and muscle strength, in contrast to cachectic mice [261,262]. Mice were monitored daily for overall health, tumor size, and signs of distress [79].

4.2 C26 Cell Culture and Tumor Implantation

C26 cancer cells (purchased from NIH National Cancer Institute) were cultured in T-75 flasks using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were maintained at passages 2–3, and once confluent, they were trypsinized, counted, and suspended in sterile phosphate-buffered saline

(PBS). C26 cells (5×10^5) in 100 µL of PBS were subcutaneously injected into the flanks of experimental mice at 8 weeks of age. Control mice received identical subcutaneous injections of 100 µL of PBS and were aged for two to four weeks. Tumors were allowed to grow for 26–29 days, during which tumor dimensions were measured daily using digital calipers. All procedures adhered to the guidelines established by the York University Animal Care Committee [79]. An infographic of this procedure can be seen in *Figure 3*.

4.3 Tissue Collection

At the endpoint of the study, and when mice were aged 10 to 12 weeks, the plantaris, soleus, red gastrocnemius, adipose tissue, liver and kidney were collected at week 4 under isoflurane anesthesia. Tumors were excised at both week 2 and week 4. All tissues were rapidly excised, weighed, and snap-frozen in liquid nitrogen and stored at –80°C for subsequent analysis [79].

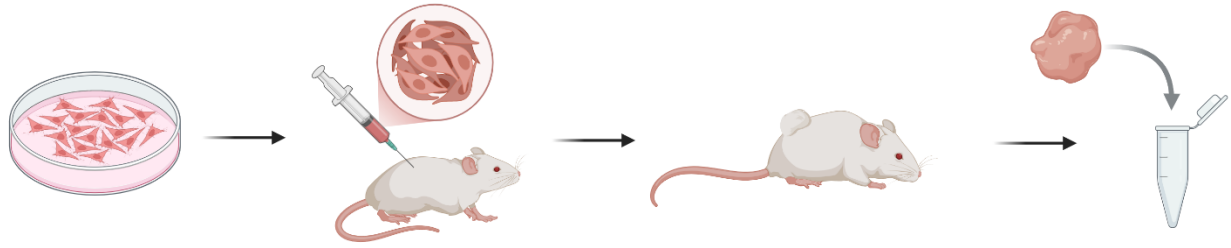

Figure 3: C26 mouse inoculation experimental design (Made with Biorender)

4.4 Tissue Homogenization

The skeletal muscle, liver and kidney tissue samples were weighed and homogenized in a 7-volume homogenization buffer. The tumor tissue samples were weighted and homogenized in a 5-volume homogenization buffer and the Adipose tissue samples were weighted and homogenized in a 1-volume homogenization buffer (composition: 20 mM HEPES, pH 7.4; 2 mM EGTA; 50 mM NaF; 100 mM KCl; 0.2 mM EDTA; 50 mM glycerophosphate),

supplemented with 1 mM DTT, 1 mM benzamidine, 0.5 mM sodium vanadate, and protease and phosphatase inhibitor cocktails (10 µl/ml of buffer). The homogenization was performed on ice to maintain protein stability. The homogenate was centrifuged at 1,000 g for 3 minutes at 4°C, and the resulting supernatant was collected. For all tissues excluding Adipose, this supernatant was further centrifuged at 10,000 g for 30 minutes at 4°C to obtain clarified samples. The processed samples were subsequently used for western blotting and ultra-high-pressure liquid chromatography analyses.

4.5 Protein Assay, Gels and Western Blotting of BCAA Metabolism

Protein concentrations were determined for each sample using the Pierce BCA Protein Assay Kit (Thermo Scientific, #23225). Absorbance readings were acquired at 550 nm using the KC4 plate reader software (Bio-Tek Instruments Inc., Winooski, Vermont, United States). A standard curve was generated using seven standards (0, 0.2, 0.4, 0.8, 1.0, 1.5, and 2.0 mg/mL) to calculate the sample volume required to load either 25 µg or 40 µg of protein per well, depending on the experimental conditions and sample amount.

Proteins were separated using 10% SDS-polyacrylamide gels with 10 or 15 wells per gel. Following electrophoresis, the separated proteins were transferred overnight onto 0.2 µm-pore polyvinylidene difluoride (PVDF) membranes (Bio-RAD, #1620177). Successful transfer was confirmed by staining membranes with Ponceau S dye for 15 minutes. Full Ponceau stained membranes were imaged using the BioRad ChemiDoc MP system. After imaging, membranes were followed by three 5-minute washes in Tris-buffered saline with Tween 20 (TBST).

To block non-specific antigen binding, membranes were incubated in a 5% milk solution (10 g skim milk powder in 190 mL TBST) at room temperature for 1 hour. After blocking,

membranes were washed three times for 5 minutes each with TBST and incubated overnight at 4°C with the primary antibody of interest, prepared at the appropriate dilution in the blocking solution, as shown in *Table 1*.

Table 1: List of Antibodies Used

Primary Antibody	Dilution	Purchased From	Secondary Antibody
BCKD	1:1000	Sigma Aldrich #90198	Anti-Rabbit (CST #7074)
P-BCKD (Ser293)	1:1000	Sigma Aldrich #40368	Anti-Rabbit (CST #7074)
BDK	1:1000	Thermo Fisher #PA5-31455	Anti-Rabbit (CST #7074)
PP2CM	1:1000	ProteinTech #14573-1-AP	Anti-Rabbit (CST #7074)
SNAT1	1:1000	Cell Signalling (CST) #36057T	Anti-Rabbit (CST #7074)
LAT1	1:500	Invitrogen #PA5-50485	Anti-rabbit (CST #7074)
P-S6K1 (Thr389)	1:1000	Cell Signaling (CST) #9234	Anti-Rabbit (CST #7074)
P-S6 (Ser235/6)	1:2000	CST #4858	Anti-rabbit (CST#7074)

The following day, membranes were washed again (3 × 5 minutes in TBST) and incubated with the appropriate secondary antibody (anti-rabbit, CST #7074) conjugated to HRP, and diluted at 1:10,000 in the blocking solution. Secondary antibody incubation was carried out for 3 hours at room temperature, after which membranes were washed again (3 × 5 minutes in TBST).

To visualize protein bands, HRP chemiluminescent substrate (Clarity Western ECL BioRad, #1705060S) was applied to each membrane. Signal detection and imaging were performed using the BioRad ChemiDoc MP system, and all images were quantified using Image Lab Software v7.

4.6 BCKD assay

Frozen samples from red gastrocnemius, liver, kidney, adipose and tumor tissues were crushed in liquid nitrogen and homogenized using a Bio-Gen PRO200 Homogenizer. Homogenization was performed in 250µL of ice-cold buffer 1 (30 mM potassium phosphate, pH 7.4; 3 mM EDTA; 5 mM DTT; 1 mM valine; 3% FBS; 5% Triton X-100; and 1µM leupeptin). The homogenate was centrifuged at 10,000 g for 10 minutes at 4°C, and the supernatant was collected.

For the reaction, 50µL of the supernatant was added to 300µL of reaction buffer 2 (50 mM HEPES, pH 7.4; 30 mM potassium phosphate; 0.4 mM CoA; 3 mM NAD+; 5% FBS; 2 mM thiamine; 2 mM magnesium chloride; and 7.8µM [14C]-valine (Perkin Elmer, #NEC291EU050UC)). The reaction was carried out in a 1.5 mL Eppendorf tube with a 2 M NaOH soaked wick attached to the inner surface of the lid to capture released 14CO2. Tubes were tightly sealed with tape and incubated at 37°C for 30 minutes.

After incubation, the NaOH wick was removed and counted using a liquid scintillation counter to quantify the 14CO2 released during the BCKD reaction. Additionally, 50µL of the reaction mix was counted in the scintillation counter to measure total radioactivity. Protein concentrations were determined for each sample, and BCKD activity (measured from the wick) was normalized to total radioactivity and protein content.

Valine was used as the substrate for this assay, as valine-derived α-ketoisovalerate (KIV) is the preferred substrate for BCKD over leucine-derived intermediates.

4.7 HPLC for BCAA and AA Quantification

4.7.1 Sample Preparation

Homogenized tissue samples containing free amino acids were diluted in a 1:2:1:8 ratio (sample: potassium phosphate buffer: 0.1 N hydrochloric acid: HPLC-grade water). The diluted samples were pre-column derivatized with a 1:1 ratio of sample to o-phthalaldehyde (OPA, 29.28 mM; Sigma Aldrich, #P1378) to enhance detection sensitivity.

4.7.2 HPLC Setup and Conditions

Samples were injected into a YMC-Triart C18 column (C18, 1.9µm, 75 × 3.0 mm; YMC America, Allentown, PA, USA) fitted onto a Nexera X2 ultra-high-pressure liquid chromatography system (Shimadzu, Kyoto, Japan) equipped with a fluorescence detector. Detection was performed at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

4.7.3 Mobile Phases and Gradient

Amino acids were separated using a gradient elution with two mobile phases:

- **Mobile Phase A**: 20 mM potassium phosphate buffer, pH 6.5.
- **Mobile Phase B**: A solution consisting of 45% HPLC-grade acetonitrile, 40% HPLC-grade methanol, and 15% HPLC-grade water.

The gradient was programmed to increase from 5% to 100% Mobile Phase B over 21 minutes at a flow rate of 0.8 mL/min.

4.7.4 Quantification

Amino acid concentrations were calculated by integrating chromatographic peaks and comparing them to standard curves generated using BCAA and AA standards (Sigma Aldrich, #AAS18). For all samples, concentrations were normalized to total protein content.

4.7.5 Buffers and Reagents

Buffers were prepared using potassium phosphate dibasic and monobasic salts (BioShop Canada Inc., #PPD555.1 and #PPM302.1). Mobile phases were freshly prepared in the lab to ensure optimal running conditions. The OPA derivatization kit (Sigma Aldrich, #P1378) was used for pre-column sample preparation. A more detailed protocol is outlined in Appendix B.

4.8 Statistical Analysis

BCKD activity data were calculated as counts per minute (CPM) of CO_2 released and bound to the wick, corrected for total radioactivity and normalized to the protein content (μg) of each sample. For western blot analyses, protein loading was normalized using Ponceau values.

Statistical analyses were performed using GraphPad Prism software (versions 7 or 8; GraphPad, Massachusetts, United States). Data are expressed as mean ± standard error of the mean (SEM). Unpaired t-tests were used to compare group means. Statistical significance was set at $p < 0.05$.

5.0 Results

To confirm that mice had entered a cachectic, muscle-wasting state, parameters such as body weight, tumor-free body weight, and skeletal muscle mass were evaluated during the original animal experiments [79]. These assessments are presented in *Supplementary Figure 1*, which demonstrate significant reductions in total body weight, tumor-free body weight, and percent change in tumor-free body mass. Additionally, in *Supplementary Figure 2*, marked decreases in muscle mass compared to control counterparts is observed [79].

5.1 Tumor

Previous studies have reported that tumors may exhibit increased uptake and accumulation of BCAAs to support their rapid growth and proliferation [45,46]. Given these findings, we sought to determine whether similar changes occur in the C26 tumor model. To do this, we assessed BCAA and total AA content using HPLC. We also examined BCKD enzymatic activity capacity and used western blotting to evaluate protein expression of key components of BCAA metabolism, including BCKD regulatory enzymes, AA transporters (LAT1 and SNAT1), and the mTORC1 signaling marker P-S6. This multi-level analysis provides insight into how the tumor alters AA handling to support its metabolic demands.

Tumor tissue shows increased BCAA levels and BCKD activity at Week 4

HPLC analysis revealed significantly elevated concentrations of valine ($p < 0.001$), isoleucine ($p < 0.05$), and total BCAAs ($p < 0.05$) in Week 4 tumors compared to Week 2 (**Figure 4A**), while leucine levels showed a trend toward an increase ($p = 0.09$). Glutamate was also increased by 65% at Week 4, though this was not statistically significant (Fig 4**B**).

Additionally, while some AAs, such as histidine, arginine, phenylalanine, and proline appeared modestly elevated at Week 4, none reached statistical significance (Fig 4**C**).

Further, to assess whether increased BCAA levels were accompanied by altered catabolic activity, we measured the activity of the BCKD complex. BCKD activity was significantly elevated ($p < 0.05$) in Week 4 tumors (Fig 4**D**), suggesting that in addition to accumulating BCAAs, the tumor may also upregulate their oxidation to support its metabolic demands.

Upregulation of PP2CM, SNAT1 and P-S6 in tumor-tissue at week 4

To explore the mechanism underlying increased AA concentration and BCKD activity in tumor tissue, we assessed the expression of key BCAA catabolic enzymes, transporters, and signaling proteins by western blotting (**Figure 5A**). While there was no statistically significant difference in BCKD abundance between tumors collected at Week 2 and Week 4 (Fig 5**B**), PP2CM, the phosphatase responsible for BCKD activation, was significantly upregulated ($p < 0.05$) at Week 4 (Fig 5**C**). In contrast, P-BCKD (Fig 5**D**) and BDK (Fig 5**E**) protein levels were not significantly different between groups. These findings suggest that the observed increase in BCKD activity is likely due to the increase in PP2CM. LAT1 expression was not significantly different between timepoints (Fig 5**F**), whereas SNAT1 was significantly upregulated ($p < 0.01$) at Week 4 (Fig 5**G**), indicating increased transporter expression specific to certain AA pathways. Expression of P-S6, a downstream effector of mTORC1 signaling, was also significantly elevated ($p < 0.05$) at Week 4 (Fig 5**H**), suggesting enhanced nutrient-driven signaling and protein synthesis during tumor progression. Collectively, these results indicate that tumor growth is accompanied by selective upregulation of BCAA catabolic regulation, AA transport, and mTORC1 activation.

Tumor Figure Results

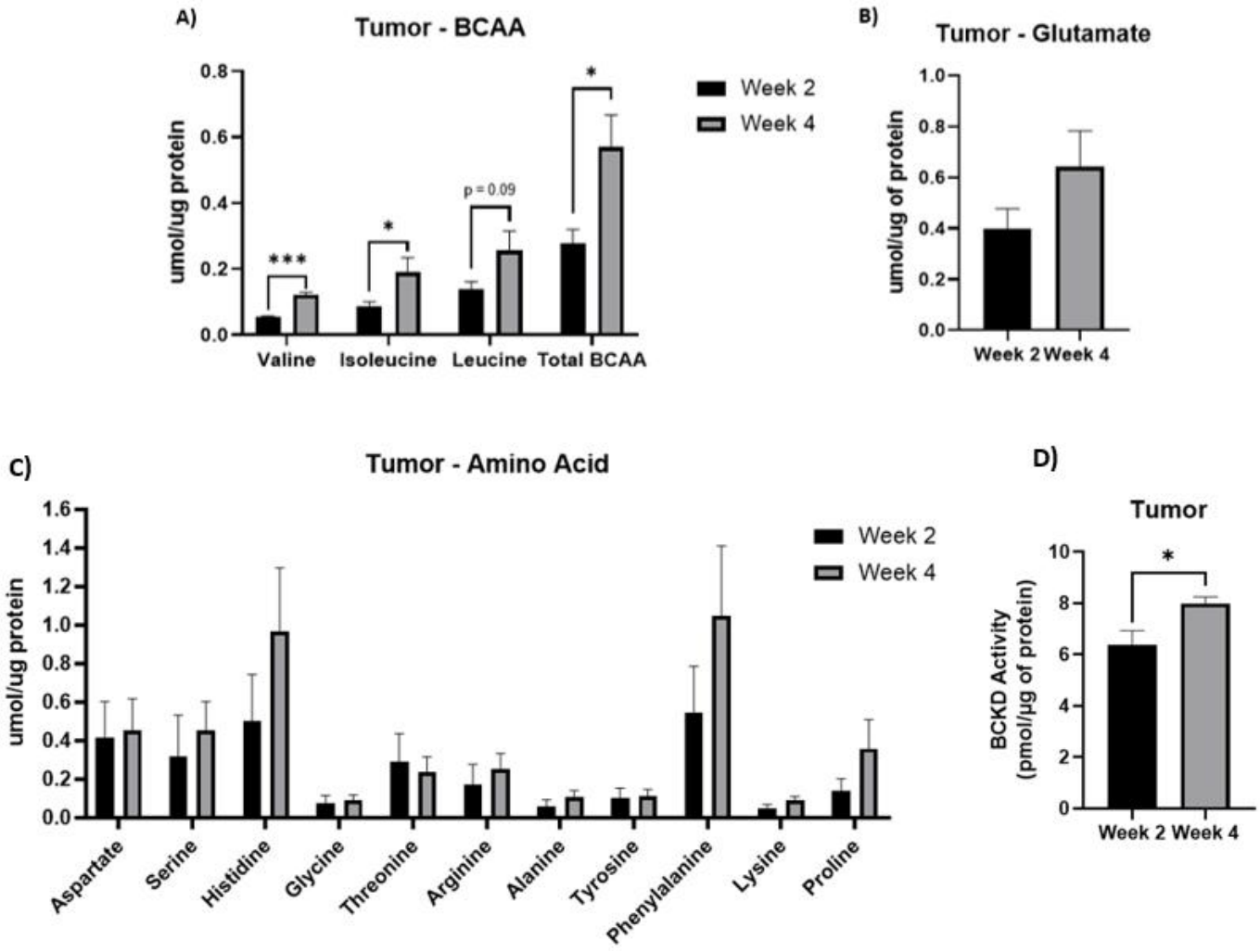

Figure 4: Increased BCAA concentrations and BCKD activity in C26 tumor tissue at week 4
Valine, isoleucine, leucine, total BCAAs (**A**), glutamate (**B**) other AAs (**C**) and BCKD activity assay (**D**) were measured in tumor tissue samples collected at Week 2 (n = 5) and Week 4 (n = 10) following C26 cell inoculation. AA concentrations (**A-C**) are expressed in µmol per µg of protein. BCKD activity (**D**), measured using [^{14}C]-CO_2 released per µg of protein per minute. Statistical significance was determined using unpaired t-tests. Data are presented as mean ± SEM. ***p < 0.001, *p < 0.05.

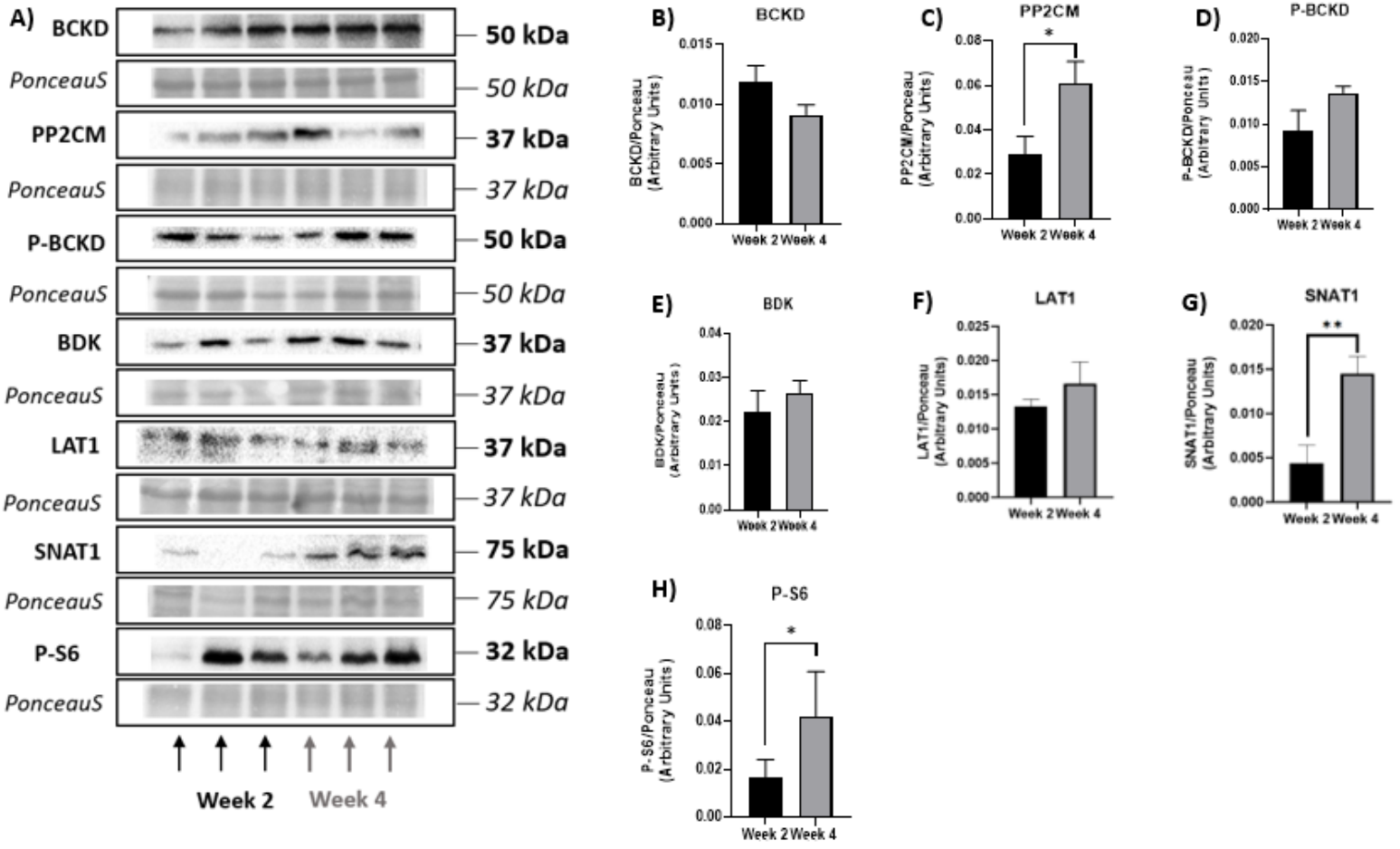

Figure 5: Upregulation of PP2CM, SNAT1, and P-S6 expression in tumor tissue at week 4
Representative blots (**A**) and quantified protein expression of BCKD (**B**), PP2CM (**C**), P-BCKD (**D**), BDK (**E**), LAT1 (**F**), SNAT1 (**G**), and P-S6 (**H**) were measured by western blotting in tumor tissue collected at Week 2 (n = 5) and Week 4 (n = 10). Protein levels were normalized to Ponceau staining. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. **p < 0.01, *p < 0.05.

5.2 Liver

Given the significant increase in BCAA content, enzyme activity, SNAT1 and P-S6 expression, observed in the week 4 tumors, we next examined the liver, a central organ in AA metabolism and homeostasis [171]. The liver plays a key role in BCAA catabolism via the

BCKD complex [22,23] and has been implicated in systemic metabolic adaptations during cancer progression [212].

Altered AA concentrations in liver of C26 tumor-bearing mice

Liver BCAA and glutamate concentrations were not significantly different between groups (**Figure 6A–B**), suggesting preserved hepatic BCAA content during tumor progression. Several other AAs were altered as aspartate ($p < 0.001$), arginine ($p < 0.001$), and alanine ($p < 0.01$) were significantly decreased, while lysine was significantly increased ($p < 0.01$) (Fig 6**C**). These changes suggest that tumor burden may contribute to selective hepatic AA depletion, particularly affecting NEAAs. BCKD activity was decreased by 20% in the C26 group, however, was not statistically significant (Fig 6**D**), indicating a modest suppression of hepatic BCAA catabolism in response to tumor burden.

Upregulated PP2CM, P-BCKD, LAT1 and P-S6 expression in the liver of C26 tumor-bearing mice.

BCKD expression was not significantly different between groups (**Figure 7A-B**), while PP2CM was significantly elevated ($p < 0.01$) in the C26 group (Fig 7A, **C**). P-BCKD showed a trend toward increased expression ($p = 0.06$) (Fig 7A, **D**), and BDK levels were unchanged (Fig 7A, **E**), suggesting a shift in hepatic BCAA regulatory balance during tumor progression. LAT1 expression was significantly decreased ($p < 0.05$) in tumor-bearing mice (Fig 7A, **F**), while SNAT1 was unchanged (Fig 7A, **G**). P-S6 expression trended higher ($p = 0.06$) in the C26 group (Fig 7A, **H**), indicating potential upregulation of hepatic mTORC1 signaling.

Liver Figure Results

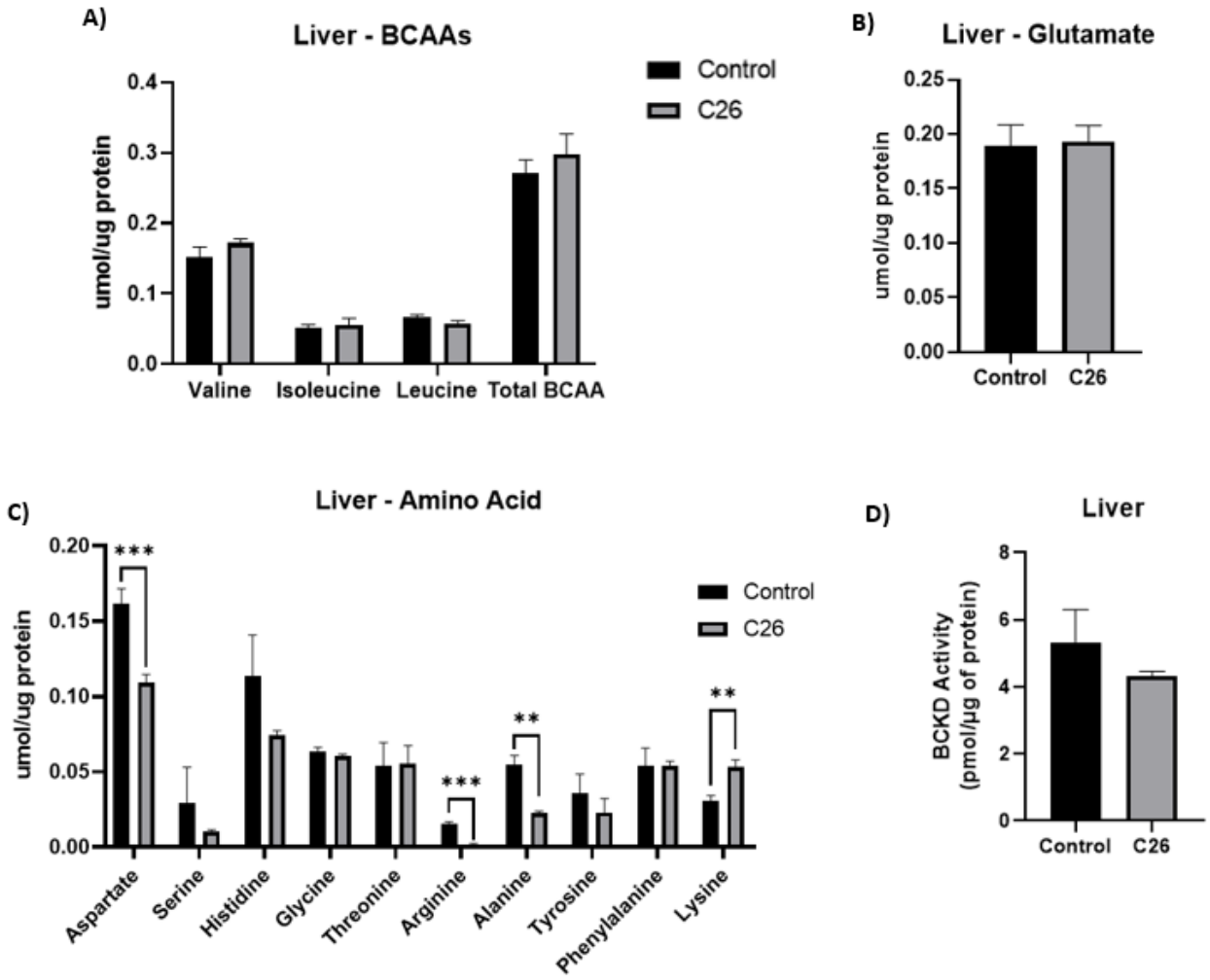

Figure 6: Altered AA concentrations in liver tissue of C26 tumor-bearing mice
BCAA concentrations (**A**), glutamate (**B**), other AAs (**C**), and BCKD activity (**D**) were measured in liver tissue collected from control (n = 10) and C26 tumor-bearing (n = 10) mice. AA (A–C) are expressed in µmol per µg of protein. BCKD activity (D), measured using [^{14}C]-valine, is expressed as pmol of [^{14}C]-CO_2 released per µg of protein per minute. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. ****$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

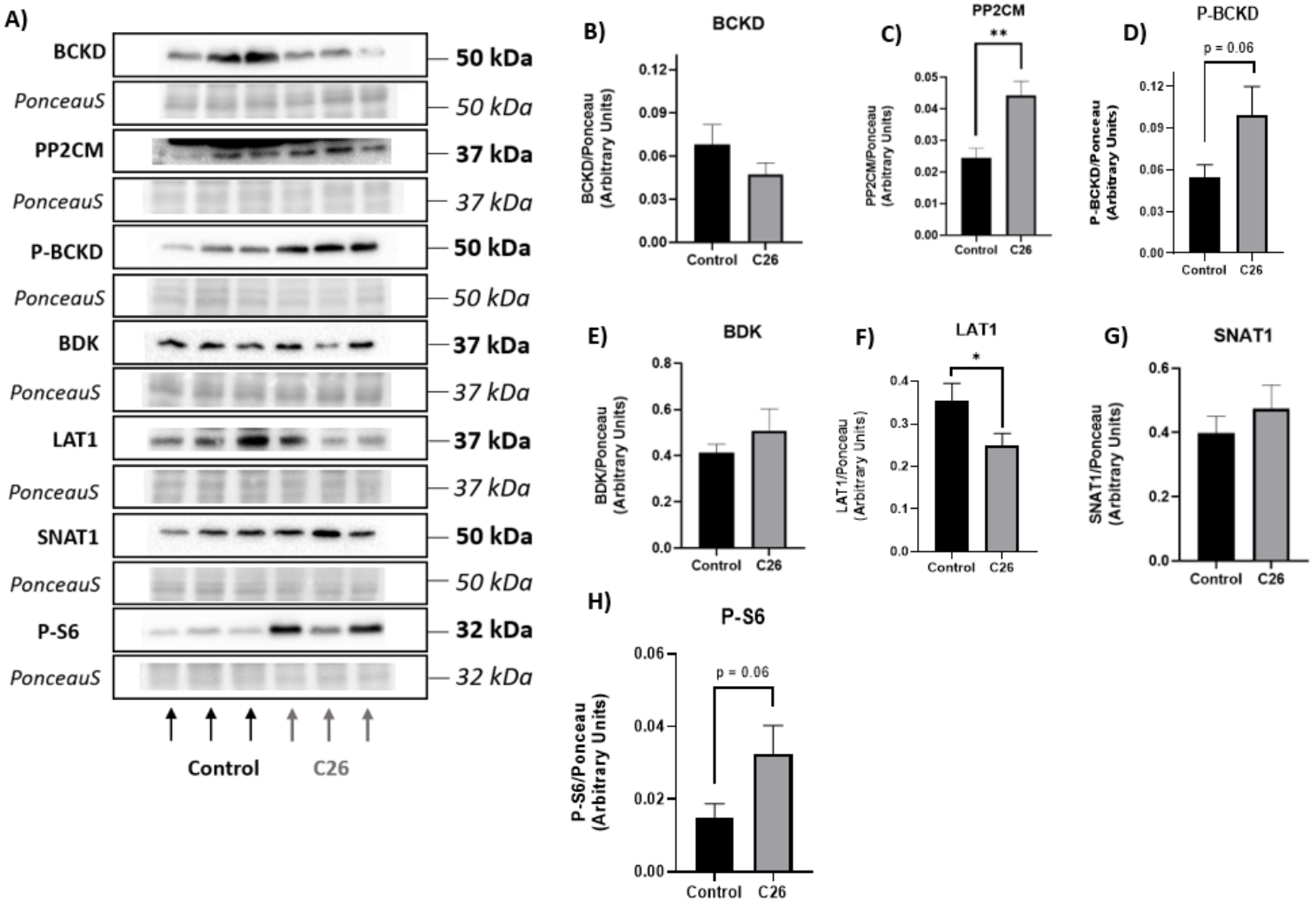

Figure 7: Upregulated PP2CM, P-BCKD, LAT1 and P-S6 expression in the liver tissue of C26 tumour-bearing mice Representative blots (**A**) and quantified protein expression of BCKD (**B**), PP2CM (**C**), P-BCKD (**D**), BDK (**E**), LAT1 (**F**), SNAT1 (**G**), and P-S6 (**H**) were measured by western blotting in liver tissue collected from control (n = 10) and C26 tumor-bearing (n = 10) mice. Protein levels were normalized to Ponceau staining. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. **$p < 0.01$, *$p < 0.05$.

5.3 Kidney

The kidney plays an important yet underappreciated role in systemic AA metabolism, including BCAA catabolism, ammonia production, and gluconeogenesis [129]. Although the

kidney is not typically central in cachexia research, its involvement in maintaining nitrogen balance, and AA homeostasis [226] suggests it may contribute to the systemic metabolic disturbances observed in cachexia. Emerging evidence also indicates that kidney metabolism may become metabolically altered in disease states such as cancer [172], warranting further exploration of its role in cancer-associated muscle wasting.

Decreased valine and alanine concentration is kidney tissue of C26 tumor-bearing mice

HPLC analysis showed a significant reduction in valine levels ($p < 0.01$) in the C26 group, while isoleucine, leucine, total BCAAs, and glutamate were unchanged (**Figure 8A–B**), suggesting selective loss of individual BCAAs in the kidney. Among other AAs, arginine showed a trend toward reduction ($p = 0.06$) and alanine was significantly decreased ($p< 0.05$) (Fig 8**C**). No other AAs measured were significantly different. BCKD activity was unchanged between groups (Fig 8**D**), indicating stable renal BCAA catabolism during tumor progression.

Tumor burden alters expression of BCKD regulators and AA transporters in kidney tissue

Western blotting analysis revealed that while BCKD (**Figure 9A-B**) and BDK (Fig 9A, **E**), levels remained unchanged. significant decreases in both PP2CM ($p < 0.001$) (Fig 9A, **C**) and P-BCKD ($p < 0.01$) (Fig 9A, **D**) were observed. These changes may reflect a regulatory balance that allows BCKD activity within the kidney to remain stable, as observed in **Figure 8D**. Furthermore, LAT1 expression was significantly decreased ($p < 0.01$) (**Fig 9A, F**), whereas SNAT1 was significantly upregulated (0.001) (Fig 9**G**), suggesting altered AA transport. P-S6 expression was unchanged (Fig 9A, **H**), indicating no major shifts in mTORC1 signaling at this time point.

Kidney Figure Result

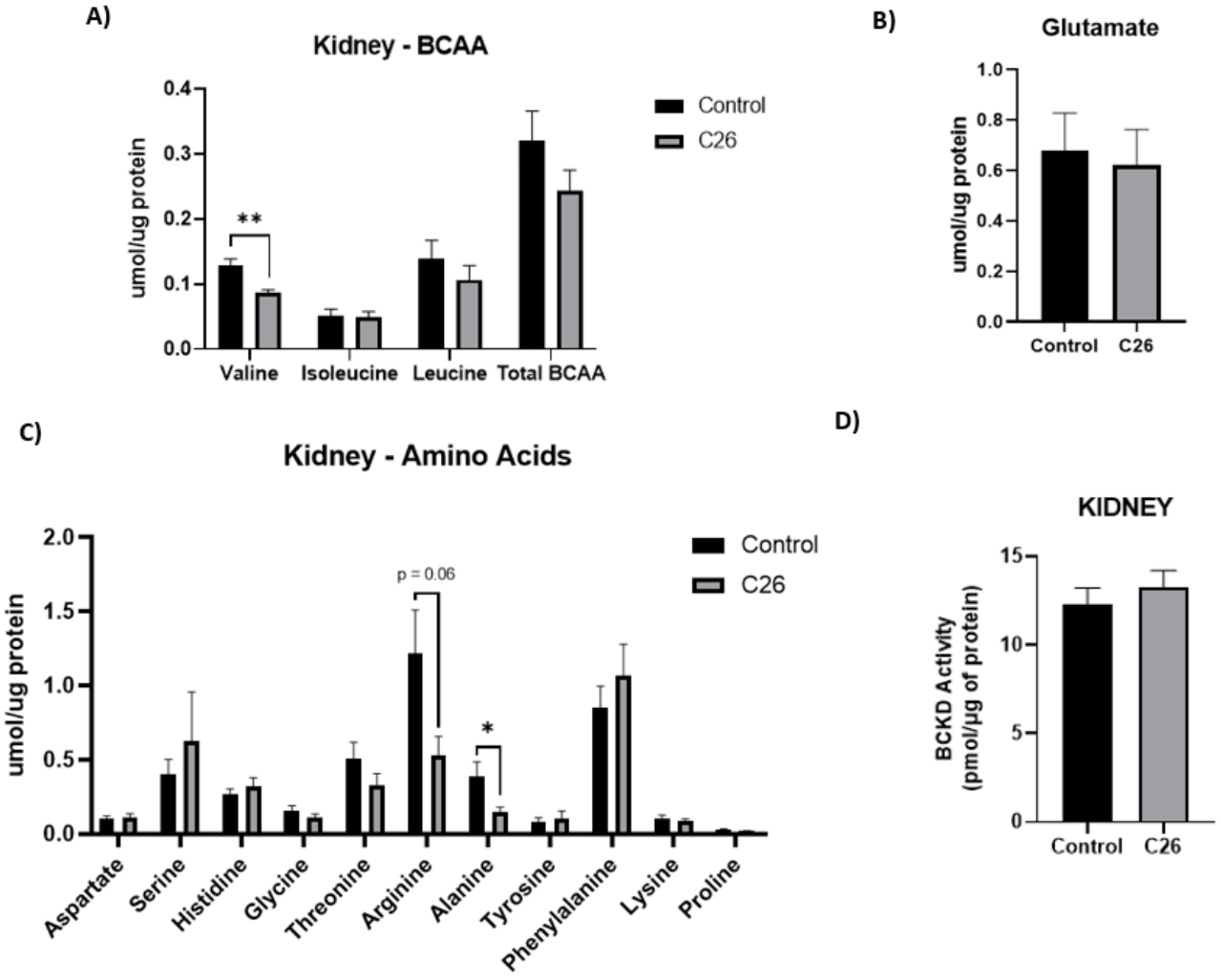

Figure 8: Decreased valine and AA concentrations in kidney tissue of C26 tumor bearing mice. BCAA concentrations (**A**), glutamate (**B**), other AAs (**C**), and BCKD activity (**D**) were measured in kidney tissue collected from control (n = 10) and C26 tumor-bearing (n = 9) mice. AA concentrations (**A–C**) are expressed in µmol per µg of protein. BCKD activity (**D**), measured using [^{14}C]-valine, is expressed as pmol of [^{14}C]-CO_2 released per µg of protein per minute. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. **$p < 0.01$, *$p < 0.05$.

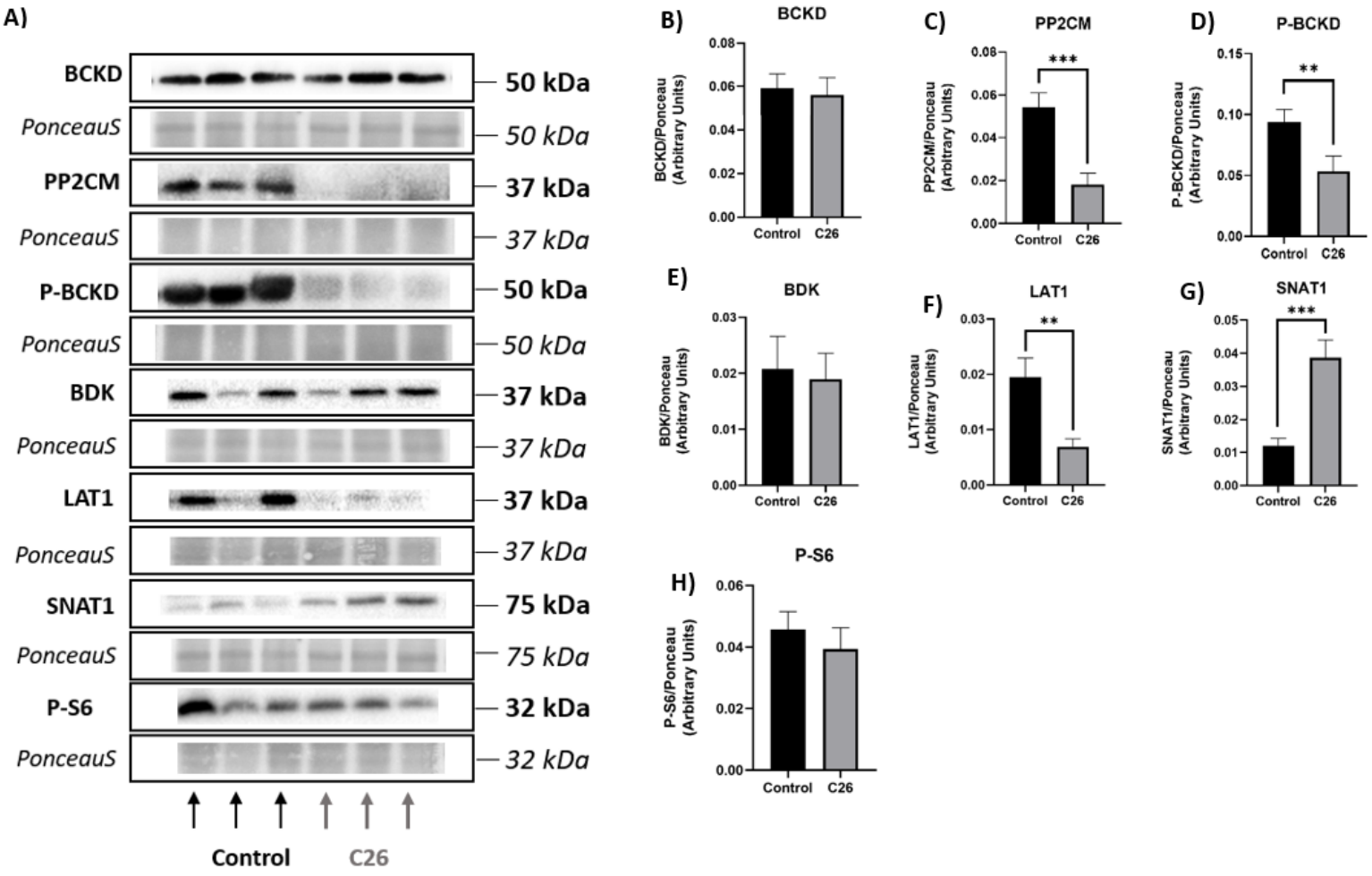

Figure 9: Decreased PP2CM, P-BCKD, LAT1 and increased SNAT1 expression in kidney tissue of C26 tumor-bearing mice. Representative blots (**A**) and quantified protein expression of BCKD (**B**), PP2CM (**C**), P-BCKD (**D**), BDK (**E**), LAT1 (**F**), SNAT1 (**G**), and P-S6 (**H**) were measured by western blotting in kidney tissue collected from control (n = 10) and C26 tumor-bearing (n = 9) mice. Protein levels were normalized to Ponceau staining. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. ***$p < 0.001$, *$p < 0.05$.

5.4 Adipose

Adipose tissue, beyond its role in energy storage, contributes to whole-body AA metabolism and inter-organ communication [132]. It also plays a role in cancer and cachexia, where inflammation and lipolysis [220] disrupt metabolic homeostasis and contribute to systemic

wasting [220]. Given the changes observed in tumor, liver, and kidney, we next assessed adipose tissue.

Decreased glutamate and altered AA concentrations in adipose tissues of C26 tumor-bearing mice.

BCAA concentrations were unchanged between groups (**Figure 10A**), while glutamate levels were significantly reduced ($p < 0.001$) in C26 adipose tissue (Fig 10**B**), suggesting altered nitrogen handling or transamination activity in response to tumor burden. Several other AAs were also affected as aspartate, glycine ($p < 0.001$), threonine ($p < 0.01$), alanine, and lysine ($p < 0.001$) were significantly decreased, whereas tyrosine was significantly increased ($p < 0.001$) and serine trended towards an increase ($p = 0.07$) (Fig 10**C**). These findings indicate broad dysregulation of AA metabolism in adipose tissue during tumor progression. BCKD activity was unchanged (Fig 10**D**), suggesting that adipose BCAA catabolism remains relatively stable despite these molecular changes.

Reduced LAT1 and P-S6 expression in adipose tissue of C26 tumor-bearing mice.

Expression of BCKD, PP2CM, and P-BCKD was not significantly different between groups (**Figure 11A–D**), while BDK expression was elevated in the C26 group, approaching statistical significance ($p = 0.06$) (Fig 11A, **E**). This increase in BDK may reflect a subtle regulatory shift, though P-BCKD remained unchanged (**Fig 11A, D**), suggesting that adipose BCAA oxidation is maintained despite altered enzyme expression. LAT1 (Fig 11A, F) and P-S6 (Fig 11A, H) expression were significantly reduced ($p < 0.01$) in the C26 group, while SNAT1 levels decreased by 30%, however, was not statistically significant (Fig 11A, **G**). These changes

suggest that tumor burden is associated with decreased AA transport and suppressed mTORC1 signaling in adipose tissue.

Adipose Figure Results

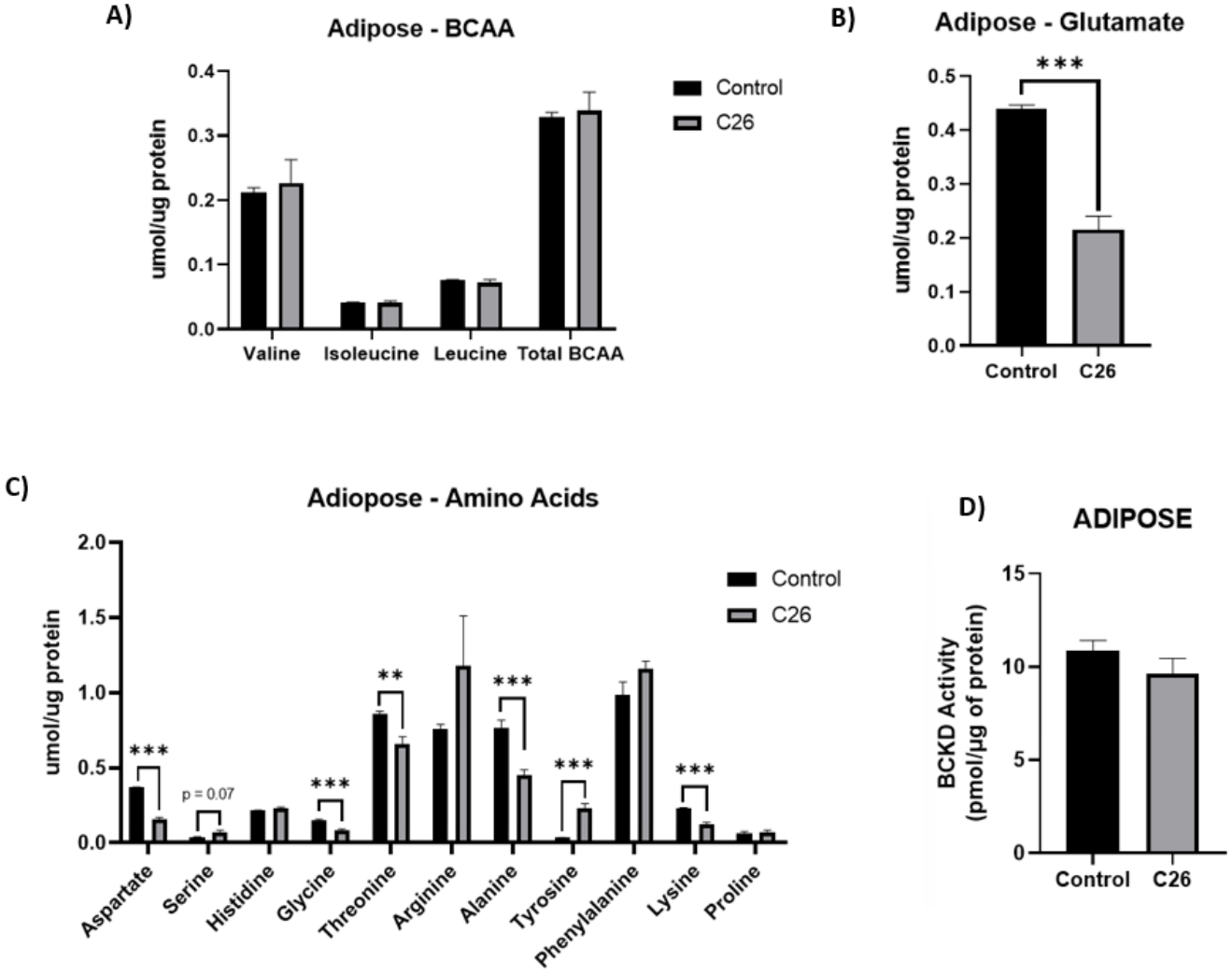

Figure 10: Decreased glutamate and altered AA concentrations in adipose tissues of C26 tumor-bearing mice. BCAA concentrations (**A**), glutamate (**B**), other AAs (**C**), and BCKD activity (**D**) were measured in adipose tissue collected from control (n = 10) and C26 tumor-bearing (n = 10) mice. AA concentrations (**A–C**) are expressed in µmol per µg of protein. BCKD activity (**D**), measured using [^{14}C]-valine, is expressed as pmol of [^{14}C]-CO_2 released per µg of protein per minute. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

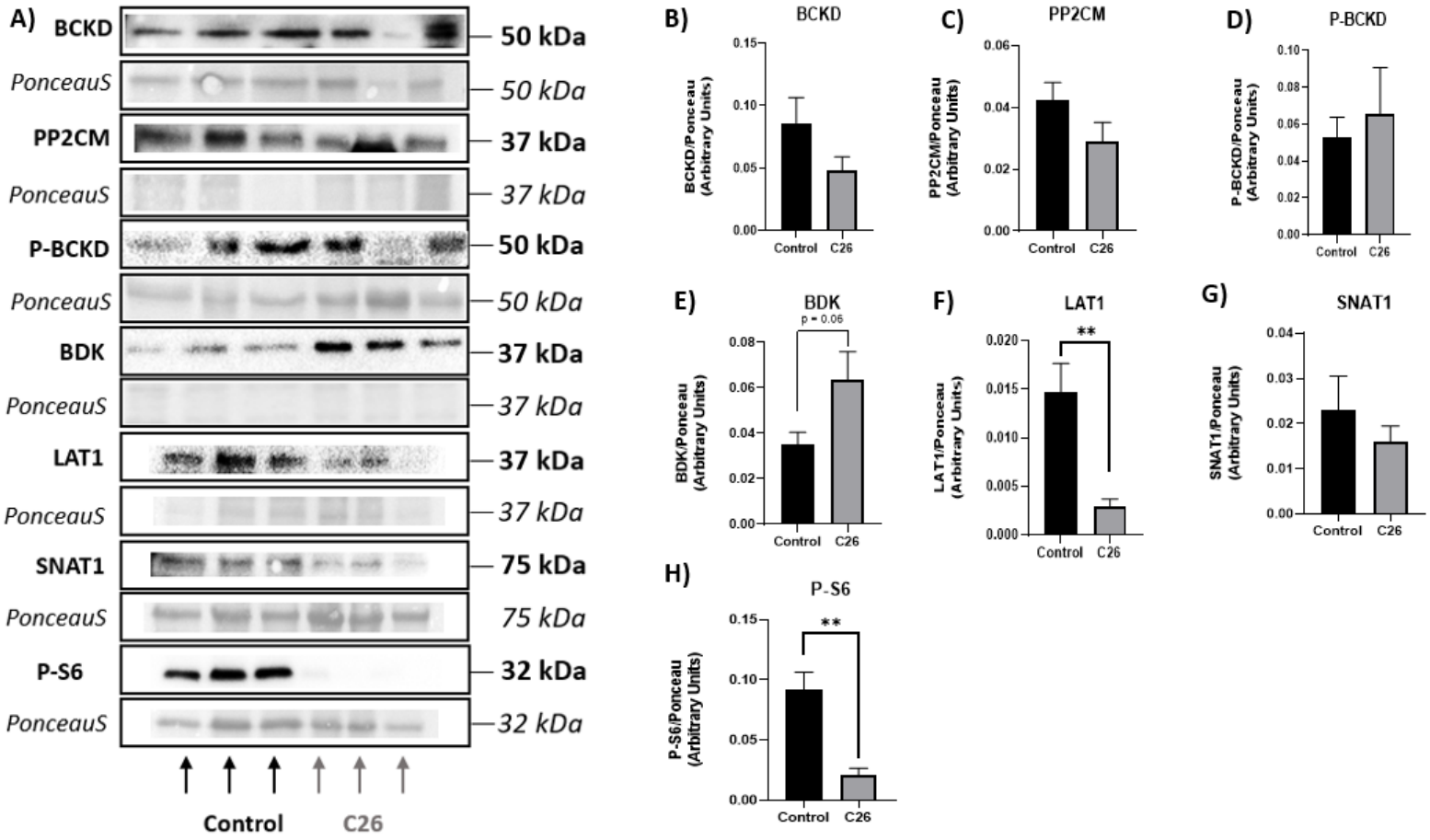

Figure 11: Decreased LAT1 and P-S6 expression in adipose tissue of C26 tumor-bearing mice. Representative blots (**A**) and quantified protein expression of BCKD (**B**), PP2CM (**C**), P-BCKD (**D**), BDK (**E**), LAT1 (**F**), SNAT1 (**G**), and P-S6 (**H**) were measured by western blotting in adipose tissue collected from control (n = 10) and C26 tumor-bearing (n = 10) mice. Protein levels were normalized to Ponceau staining. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. **p < 0.01.

5.5 Skeletal Muscle

Skeletal muscle is a major site of BCAA metabolism and a key tissue affected by cancer cachexia [38,39]. Given its central role in whole-body protein turnover and energy expenditure, skeletal muscle is a critical target for understanding how tumor burden impacts systemic AA handling and nutrient signaling. Cachexia-associated muscle loss is particularly detrimental to

quality of life and survival [80], making it essential to examine tissue-specific metabolic adaptations in different muscle types.

To investigate these effects, three skeletal muscles with varying fiber-type composition and metabolic profiles were selected: red gastrocnemius, soleus, and plantaris. These muscles offer insights into fiber-type-specific responses to tumor burden and their potential contributions to whole-body metabolic remodeling in cancer.

5.5.1 Red Gastrocnemius

The red portion of the gastrocnemius muscle is composed predominantly of oxidative, slow type I and fast type IIa fibers, which are rich in mitochondria and heavily involved in aerobic metabolism [173,174]. This fiber composition makes red gastrocnemius particularly relevant for examining metabolic shifts during tumor progression, due to its high mitochondrial content, role in BCAA metabolism and BCAA-sensitive mTORC1 signalling [221,222]. Together, these features make it a useful indicator of cancer-induced disruptions in nutrient and energy metabolism.

Reduced AA concentrations and BCKD activity in red gastrocnemius of tumor-bearing mice

HPLC analysis revealed significantly lower concentrations of valine, isoleucine, leucine, and total BCAAs ($p < 0.05$) in the red gastrocnemius of C26 tumor-bearing mice (**Figure 12A**), while glutamate levels were unchanged (Fig 12**B**). Among other AAs, threonine and arginine were reduced by 54% and 37% respectively, while alanine ($p < 0.01$) and lysine (0.001) were significantly decreased (Fig 12**C**). These changes highlight broader AA depletion in red gastrocnemius during cancer progression. BCKD activity was significantly reduced ($p < 0.01$) in the C26 group (Fig 12**D**), suggesting that the observed decline in BCAAs is not due to increased

local catabolism, however, may reflect systemic redistribution, towards tumor tissue. The red gastrocnemius may also be attempting to preserve its limited BCAA pool by downregulating BCKD activity.

Decreased LAT1 and P-S6 expression in red gastrocnemius muscle of C26 tumor-bearing

Western blotting revealed no significant differences in the expression of BCKD, PP2CM, P-BCKD, or BDK between groups (**Figure 13A-E**), suggesting that the reduction in BCKD activity observed in **Figure 12D** is not due to altered protein abundance or phosphorylation, however, may be due to the lower levels of BCAAs resulting in less activation of the BCKD complex. LAT1 expression was significantly reduced ($p < 0.001$) in the C26 group (Fig 13A, **F**), while SNAT1 remained unchanged (Fig 13A, **G**). P-S6 expression was also significantly lower ($p < 0.05$) in tumor-bearing mice (Fig 13A, **H**), indicating suppression of mTORC1 signaling. Together, these changes suggest that reduced BCAA uptake and suppressed anabolic signaling may contribute to skeletal muscle wasting during tumor progression.

Red Gastrocnemius Figure Results

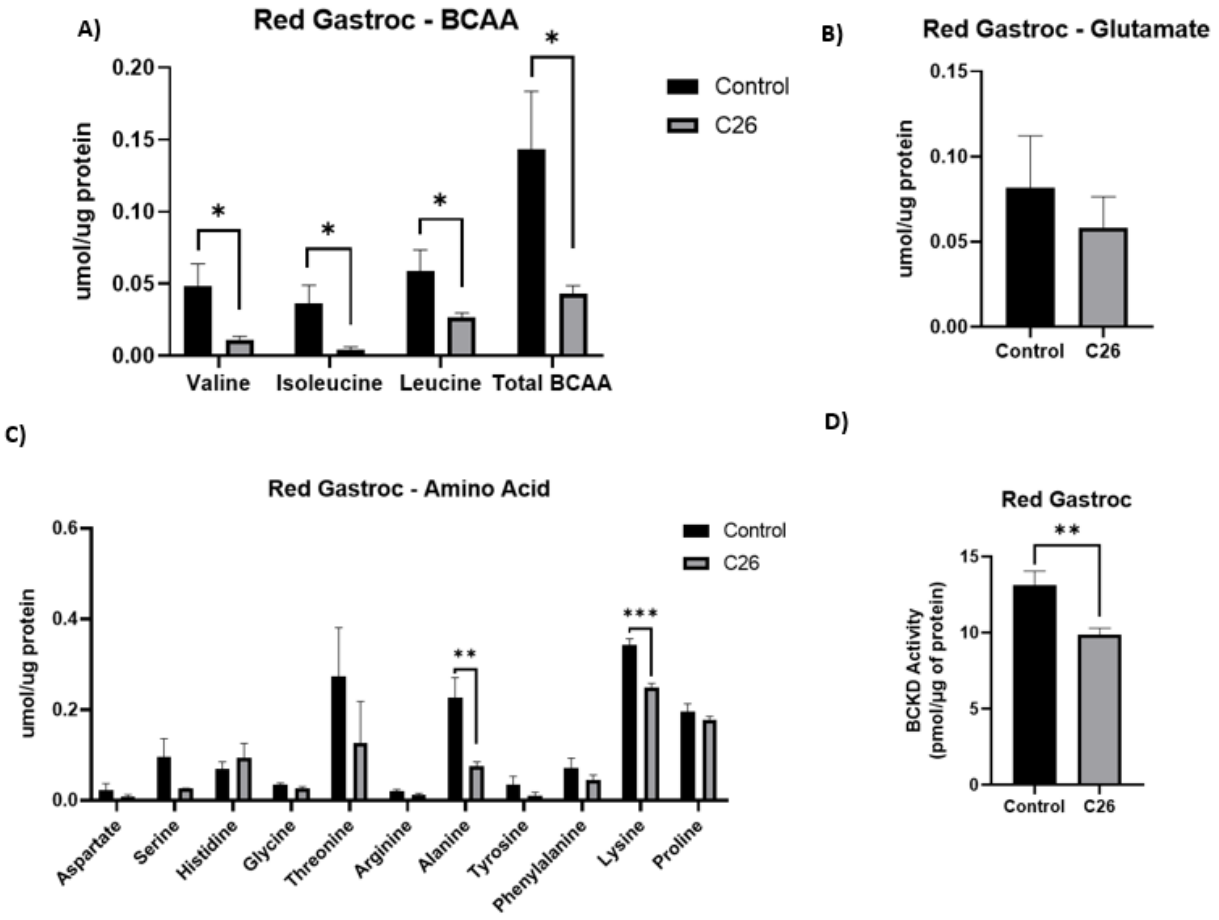

Figure 12: Decreased BCAA, AA concentrations and BCKD activity in red gastrocnemius muscle of C26 tumor-bearing mice. BCAA concentrations (**A**), glutamate (**B**), other AAs (**C**), and BCKD activity (**D**) were measured in red gastrocnemius muscle tissue collected from control (n = 10) and C26 tumor-bearing (n = 10) mice. AA concentrations (**A–C**) are expressed in µmol per µg of protein. BCKD activity (**D**), measured using [^{14}C]-valine, is expressed as pmol of [^{14}C]-CO_2 released per µg of protein per minute. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. ***p < 0.001, **p < 0.01, *p < 0.05.

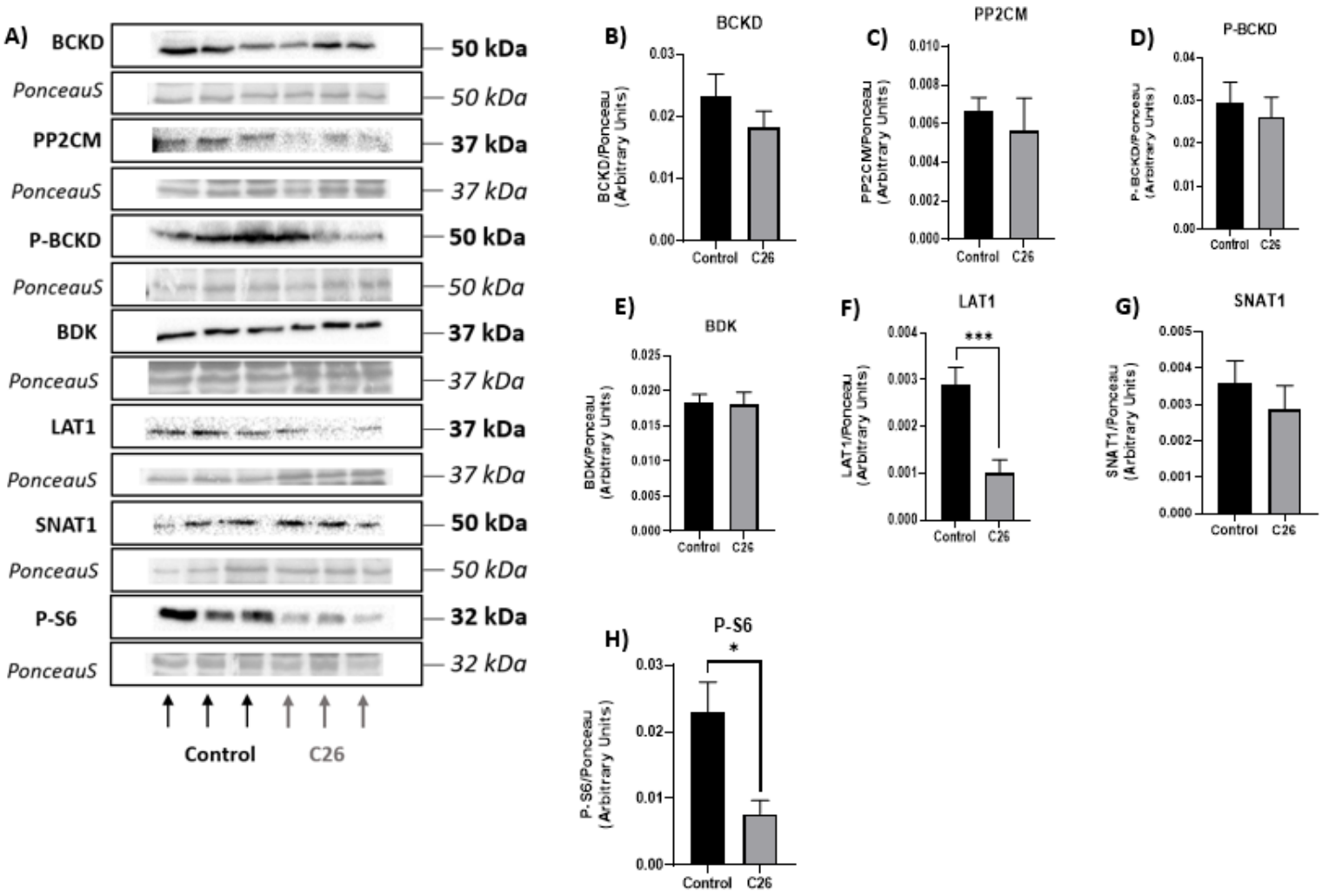

Figure 13: Decreased LAT1 and P-S6 expression in red gastrocnemius muscle of C26 tumor-bearing mice. Representative blots (**A**) and quantified protein expression of BCKD (**B**), PP2CM (**C**), P-BCKD (**D**), BDK (**E**), LAT1 (**F**), SNAT1 (**G**), and P-S6 (**H**) were measured by western blotting in red gastrocnemius muscle tissue collected from control (n = 10) and C26 tumor-bearing (n = 10) mice. Protein levels were normalized to Ponceau staining. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. ***$p < 0.001$, *$p < 0.05$.

5.5.2 Plantaris

Following the findings in red gastrocnemius, we next examined the plantaris muscle, which is located in the lower hindlimb and plays a role in locomotion [223]. The plantaris muscle is predominantly composed of fast-twitch fibers, including type IIB, IIX/IIB hybrids, and

IIX fibers, with a smaller proportion of oxidative type IIA fibers [173, 175]. This contrasts with muscles like the soleus, which are enriched in slow-twitch fibers (Type I and IIA) and are more fatigue-resistant [173,176]. The plantaris is therefore considered a fast muscle with a high glycolytic capacity and moderate oxidative potential [173,175]. Its mixed fiber-type composition makes it an informative tissue for evaluating intermediate responses to tumor-induced metabolic stress. By examining BCAA metabolism and related pathways in the plantaris, we aimed to determine whether the changes observed in red gastrocnemius were muscle-specific or reflected broader trends across skeletal muscle during cancer progression.

Decreased BCAA and proline concentrations in plantaris muscle of tumor-bearing mice

HPLC analysis revealed significantly lower levels of isoleucine ($p < 0.01$), leucine ($p < 0.05$), and total BCAAs ($p < 0.05$) in plantaris muscle of C26 tumor-bearing mice (**Figure 14A**). Although glutamate levels were not significantly different, there was a 157% increase in the C26 group (Fig 14**B**). Among other AAs proline was significantly decreased ($p < 0.05$), while threonine showed a non-significant reduction of 49%. Glycine and phenylalanine showed non-significant elevation of 173% and 166% respectively (Fig 14**C**). These findings suggest that BCAA depletion extends beyond red gastrocnemius and is accompanied by selective changes in other AAs in the plantaris during tumor progression.

Trends toward reduced LAT1 and P-S6K1 expression in plantaris muscle of tumor-bearing mice

No significant differences in the expression of BCKD, PP2CM, P-BCKD, or BDK between groups were found (Fig 15**A–E**), suggesting that the observed reduction in BCAAs in **Figure 14A** is not due to altered expression of BCAA metabolic enzymes. LAT1 (Fig 15A, F) and P-S6K1 (Fig 15A, H) expression were lower in the C26 group, however, did not reach

statistical significance (p = 0.09 and p = 0.08, respectively, while SNAT1 remained unchanged (Fig 15A, **G**). These trends are consistent with changes observed in red gastrocnemius and may reflect early suppression of AA transport and anabolic signaling in plantaris muscle during tumor progression.

Plantaris Figure Results

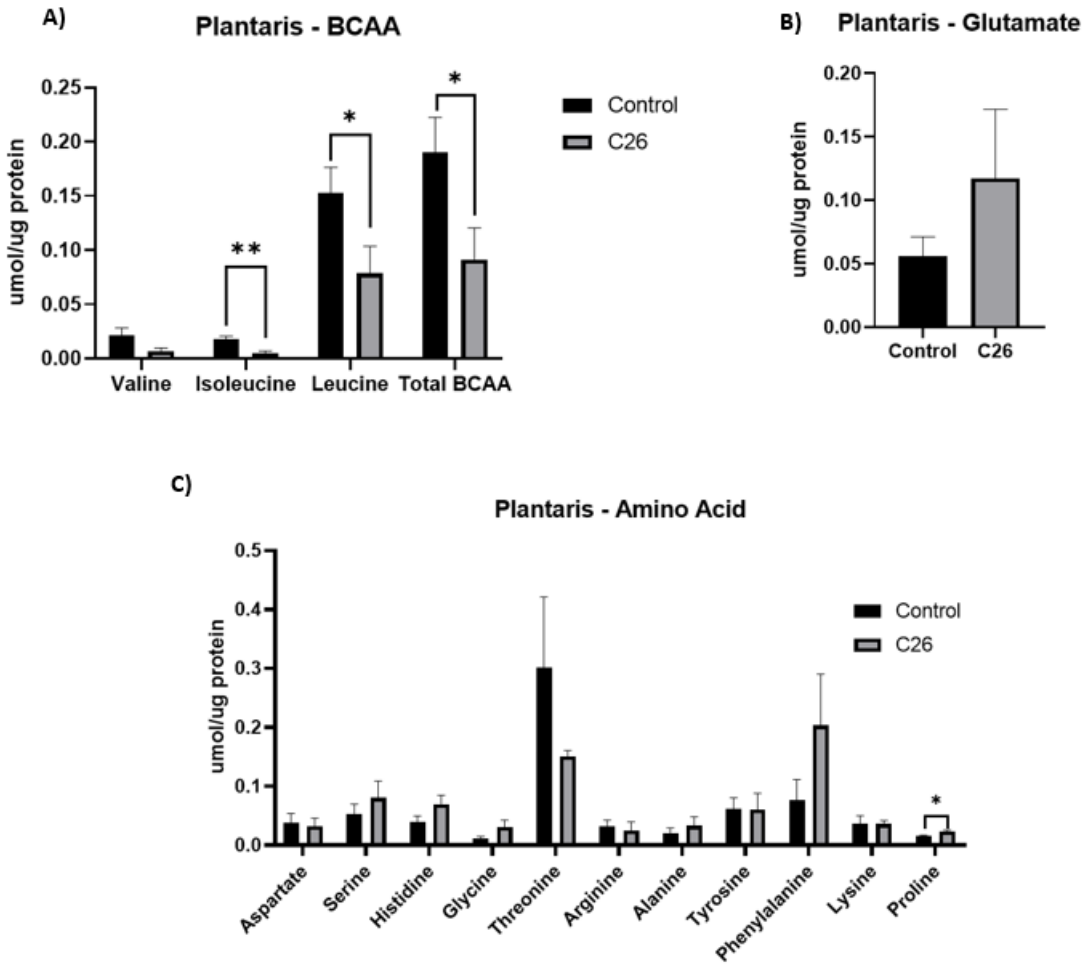

Figure 14: Decreased BCAA and proline concentrations in plantaris muscle of C26 tumor-bearing mice. BCAA concentrations (**A**), glutamate (**B**), and other AAs (**C**) were measured in plantaris muscle tissue collected from control (n = 10) and C26 tumor-bearing (n = 10) mice. AA concentrations are expressed in µmol per µg of protein. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. **p < 0.01, *p < 0.05.

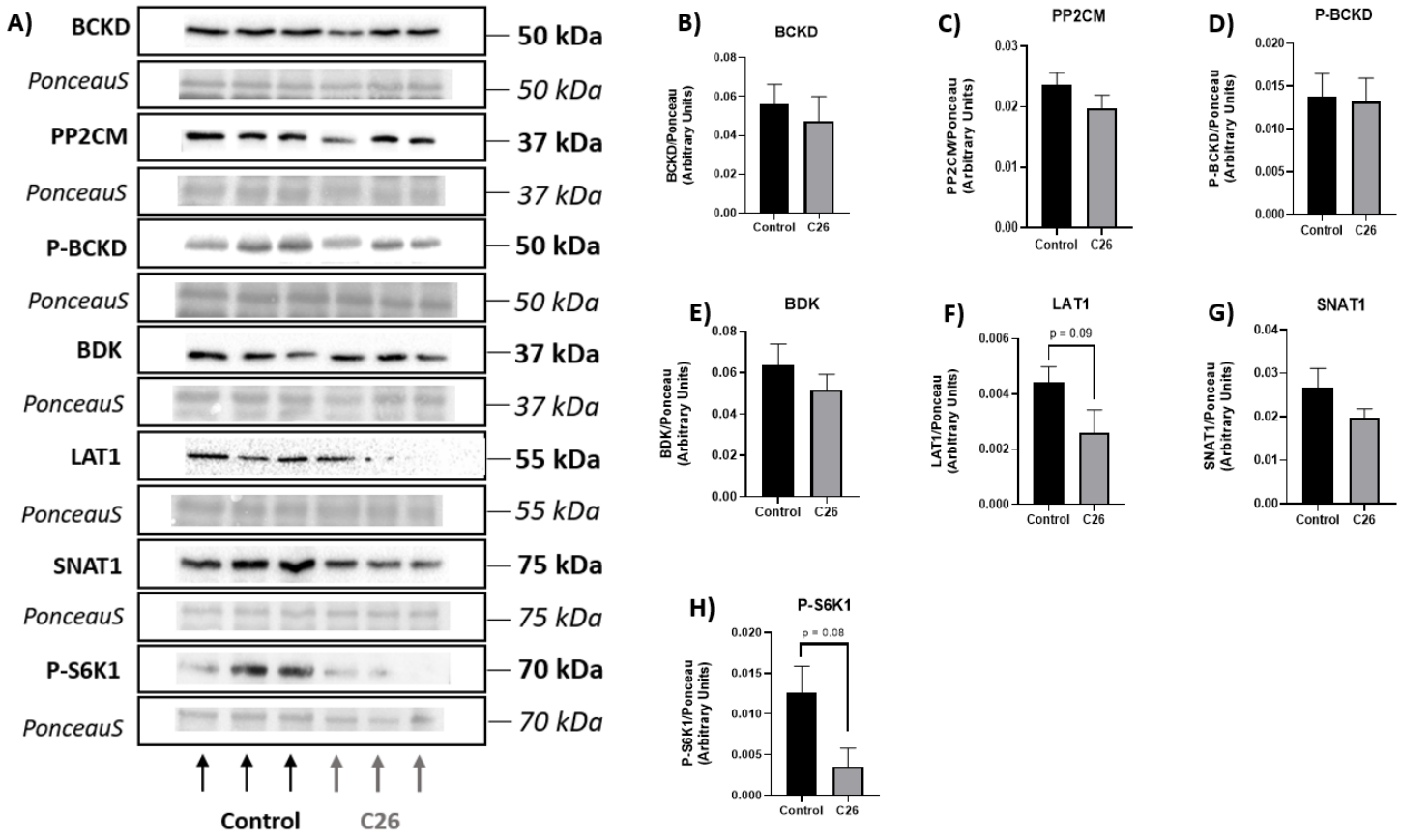

Figure 15: Trends for decreased LAT1 and P-S6K1 expression in the plantaris muscle of C26 tumor-bearing mice. Representative blots (**A**) and quantified protein expression of BCKD (**B**), PP2CM (**C**), P-BCKD (**D**), BDK (**E**), LAT1 (**F**), SNAT1 (**G**), and P-S6K1 (**H**) were measured by western blotting in plantaris muscle tissue collected from control (n = 10) and C26 tumor-bearing (n = 10) mice. Protein levels were normalized to Ponceau staining. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. **p < 0.01, *p < 0.05.

5.5.3 Soleus

Following the findings in the red gastrocnemius and plantaris muscle, we then wanted to look at the soleus muscle as the soleus muscle is predominantly composed of Type I (slow-twitch) muscle fibers [173,176]. This high proportion of oxidative fibers makes the soleus especially well-suited for sustained, low-intensity contractions, playing a critical role in postural control and endurance-based activities such as walking [176]. Owing to its metabolic profile and reliance on oxidative phosphorylation, the soleus is thought to be less susceptible to cancer-

induced wasting compared to more glycolytic muscles [71,177]. However, its high mitochondrial content and dependence on AA metabolism, including BCAAs [178], make it an important tissue for assessing systemic metabolic disruption.

Decreased BCAA concentrations and alterations in AA profiles in soleus muscle of tumor-bearing mice

HPLC analysis revealed significantly reduced levels of valine ($p < 0.05$), isoleucine ($p < 0.01$), and total BCAAs ($p < 0.05$) in the soleus muscle of C26 tumor-bearing mice, while leucine showed a trend toward reduction ($p = 0.07$) (**Figure 16A**). Glutamate levels trended higher in the C26 group ($p = 0.09$) (Fig 16**B**). Several other AAs were significantly decreased, including aspartate ($p < 0.05$), threonine, arginine ($p < 0.01$), and lysine ($p < 0.05$), while histidine showed a trend toward reduction ($p = 0.06$). In contrast, glycine, tyrosine ($p < 0.05$), and phenylalanine ($p < 0.01$) were significantly elevated (Fig 16**C**). These findings suggest complex AA remodeling in the soleus during tumor progression, with both depletions and accumulations indicating altered protein turnover and systemic redistribution.

Reduced LAT1 and P-BCKD expression and elevated P-S6 signaling in soleus muscle of tumor-bearing mice

Due to the small size of the soleus muscle, only a subset of proteins was assessed by western blotting, with limited tissue availability allowing for analysis of P-BCKD, LAT1, and P-S6 (Fig 17**A-D**). Findings revealed significantly decreased P-BCKD expression ($p < 0.01$) in C26 tumor-bearing mice (Fig 17A-**B**), suggesting increased BCKD enzymatic activity or perhaps alternate post-translation modifications. LAT1 expression was also significantly reduced ($p < 0.01$) (Fig 17A, **C**), indicating impaired BCAA transport. In contrast, P-S6 was significantly elevated ($p <$

0.05) in the C26 group (Fig 17A, **D**). These findings suggest that although AA transport may be reduced in the soleus, BCAA catabolism may be upregulated, and anabolic signaling via mTORC1 is elevated, potentially reflecting a compensatory mechanism to mitigate muscle loss or adapt to altered nutrient availability in this highly oxidative muscle.

Soleus Figure Results

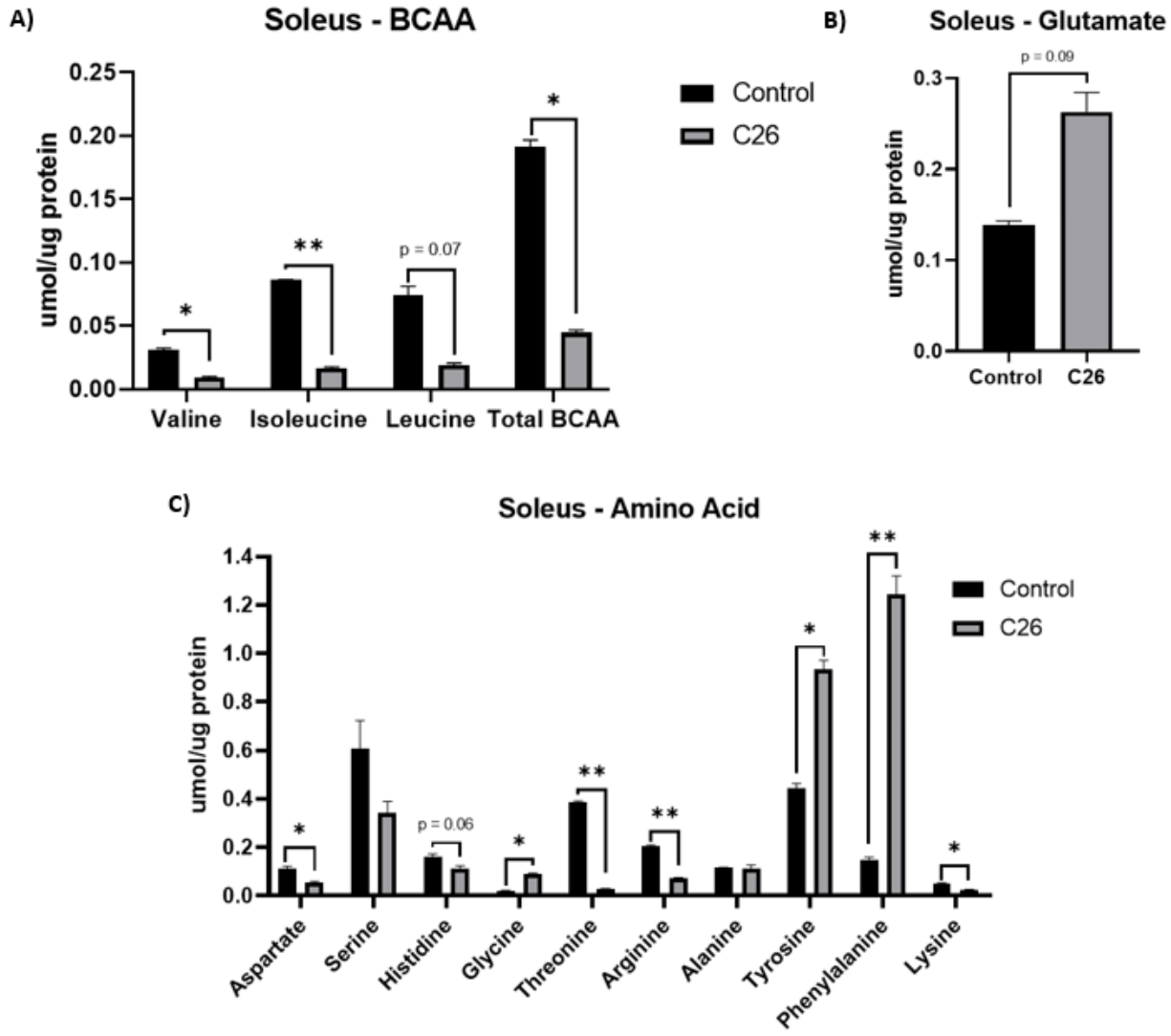

Figure 16: Decreased BCAAs and various alterations in AA concentrations in soleus muscle of C26 tumor-bearing mice. BCAA concentrations (**A**), glutamate (**B**), and other AAs (**C**) were measured in soleus muscle tissue collected from control (n = 5) and C26 tumor-bearing (n = 5) mice. AA concentrations are expressed in µmol per µg of protein. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. **p < 0.01, *p < 0.05.

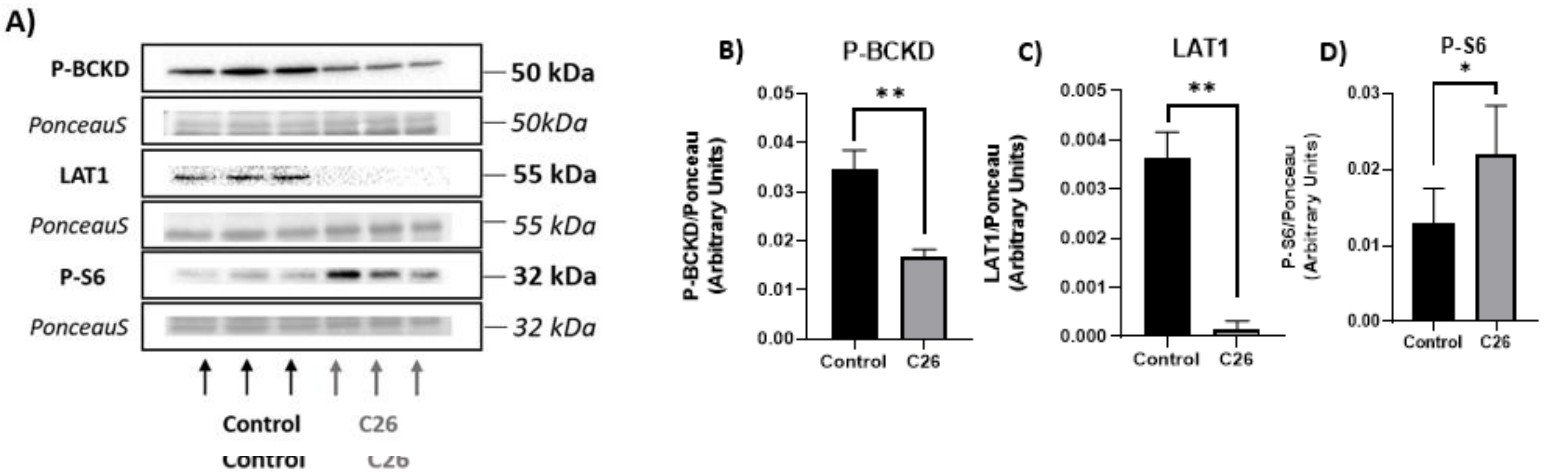

Figure 17: Decreased LAT1, P-BCKD and increased P-S6 expression in soleus muscle of C26 tumor-bearing mice. Representative blots (**A**) and quantified protein expression of P-BCKD (**B**), LAT1 (**C**), and P-S6 (**D**) were measured by western blotting in soleus muscle tissue collected from control (n = 5) and C26 tumor-bearing (n = 5) mice. Protein levels were normalized to Ponceau staining. Data are presented as mean ± SEM. Statistical significance was determined using unpaired t-tests. **$p < 0.01$, *$p < 0.05$.

5.6 Overview of Study Findings

Table 2 provides a comprehensive summary of all major/significant findings across organs and tissues analyzed in this study.

Table 2: Comprehensive Summary of Study Findings

Tissue	BCAA Levels	BCKD Activity	BCKD Regulatory Enzymes	AA Transporters/ mTORC Signaling	Other AA Changes
Tumor	↑	↑	↑ PP2CM	↑ SNAT1, P-S6	↔
Liver	↔	↔	↑ PP2CM, P-BCKD	↑ P-S6 (p = 0.06) ↓ LAT1	↑ Lys ↓ Asp, Arg, Ala
Kidney	↓ Val	↔	↓ PP2CM, P-BCKD	↑ SNAT1 ↓ LAT1	↑ Arg (p = 0.06) ↓ Ala
Adipose	↔	↔	↑ BDK (p = 0.06)	↓ LAT1, P-S6	↑ Ser (p = 0.07), Tyr ↓ Glu, Asp, Gly, Thr, Ala, Lys
Red Gastroc	↓	↓	↔	↓ LAT1, P-S6	↓ Ala, Lys
Plantaris	↓	N/A	↔	↓ LAT1 (p = 0.09), ↓ P-S6K1 (p = 0.08)	↑ Pro
Soleus	↓	N/A	↓ P-BCKD	↑ P-S6 ↓ LAT1	↑ Glu (p = 0.09), Gly, Tyr, Phe ↓ Asp, His (p = 0.06), Thr, Arg, Lys

↑ indicates increase in C26 group. ↓ indicates decrease in C26 group. ↔ indicates no change between groups. BCAA: Branched-Chain Amino Acids, AA: Amino Acids, BCKD: Branched-Chain Ketoacid Dehydrogenase, mTORC: Mammalian Target of Rapamycin, Ala: Alanine, Arg: Arginine, Asp: Aspartate, Glu: Glutamate, Gly: Glycine, His: Histidine, Lys: Lysine, Phe: Phenylalanine, Pro: Proline, Ser: Serine, Thr: Threonine, Tyr: Tyrosine

6.0 Discussion

Cancer cachexia is a debilitating condition marked by progressive skeletal muscle wasting, systemic inflammation, and metabolic dysfunction. Central to its pathology is the dysregulation of BCAA metabolism. While the role of BCAA metabolism in tumor cells is well documented [45,46,48,], far less attention has been paid to peripheral organs and tissues, such as skeletal muscle, liver, kidney, and adipose tissue. Thus, we investigated the effect of colon cancer (using the C26 tumor model) on BCAA metabolism in the tumor and peripheral sites to examine organ- and tissue-specific metabolic alterations and potential crosstalk between the various tissues in a cancerous environment, a perspective that remains largely undocumented in the field.

We found evidence of coordinated BCAA depletion in peripheral organs and tissues alongside tumor-specific accumulation and upregulation of BCAA transport and catabolism. However, these peripheral sites did not show a uniform compensatory response. While some, like the liver and red gastrocnemius, reduced BCKD activity, possibly as a conservation strategy or due to insufficient BCAA availability to drive BCKD activation, others, such as the kidney and adipose tissue, showed no changes. Transporter expression was also downregulated in several tissues despite low BCAA levels, suggesting impaired uptake or altered regulatory control. Notably, the soleus muscle maintained mTORC1 signaling despite reduced BCAA availability and transporter expression, indicating potential fiber-type-specific adaptations to preserve anabolic signaling.

6.1 Tumor BCAA Accumulation and Systemic Redistribution

One of the main findings of this work was the marked accumulation of BCAAs and the upregulation of AA transporter, SNAT1, and catabolic enzymes in the tumor tissue in week 4

compared to week 2 supporting its role as a metabolic sink, actively drawing in and utilizing circulating AAs to sustain its growth and proliferation [45,46,179]. This aligns with previous studies showing that tumors increase their dependence on EAAs, particularly leucine [182] and glutamine [181], to support biosynthesis and energy demands [181,182,224]. The increased expression of LAT1, a large AA transporter [110,180,183] that is commonly overexpressed in solid tumors [193] including colorectal cancer [194], facilitates this influx of EAAs, stimulating mTORC1 for tumor growth [195], and has been associated with increased tumor aggressiveness and poor prognosis [190,206]. Simultaneously, the tumor maintains a highly active BCKD complex, promoting BCAA catabolism to generate substrates for the TCA cycle and lipid synthesis [131]. This strategy is not unique to colon cancer; as similar patterns have been observed in hepatocellular and breast carcinomas, where increased BCKD activity and upregulation of BCAT1 and BCAT2 promote enhanced BCAA catabolism to support tumor growth and proliferation [195,200].

Moreover, these tumor-specific metabolic shifts were paralleled by consistent reductions in BCAA concentrations across peripheral organs and tissues, particularly skeletal muscle (red gastrocnemius, plantaris, soleus) and kidney, suggesting a systemic redistribution of AAs toward the tumor. This pattern aligns with radiotracer studies showing increased leucine uptake in tumors [201,202], reinforcing the concept of nutrient hijacking in cancer cachexia. One possible mechanism contributing to this redistribution is the downregulation of AA transporters, as LAT1 expression was reduced in all peripheral sites examined (liver, kidney, adipose tissue, and skeletal muscle) of tumor-bearing mice. Given LAT1's role as a bidirectional antiporter facilitating the exchange of large neutral AAs across cell membranes [101,180,183], reduced expression may impair peripheral retention of BCAAs and shift the net flow of these substrates

toward circulation, ultimately favoring tumor uptake. This is consistent with prior studies done in our lab, in which chemotherapy-induced cachexia, had also identified LAT1 downregulation as a contributor to reduced intramuscular BCAA levels [18,238], suggesting the idea that transporter regulation may play a central role in cachexia-related AA imbalance, even in the absence of tumor competition [238]. Although a lot of literature has shown that LAT1 seems to be upregulated in a vast variety of cancers (including breast, lung and colon among others) [203,204,205,206], the downregulation of LAT1 in peripheral organs and tissues during cachexia appears to be a novel finding and perhaps represents a vulnerability during cachexia progression. In this context, LAT1 downregulation in peripheral sites may not simply be a consequence of tumor burden, but an active contributor to peripheral BCAA depletion and impaired anabolic signaling, one that could present an opportunity for therapeutic intervention.

The loss of peripheral AA levels and their accumulation in the tumor may be further amplified by the tumor-specific upregulation of SNAT1 likely enhancing glutamine uptake to drive LAT1 mediated BCAA import [184,185], supporting continuous nutrient acquisition. This mirrors findings in lung and colorectal cancers, where SNAT1 promotes glutamine metabolism and tumor progression [209]. Importantly, glutamine functions not only as a critical exchange partner for LAT1 transport, facilitating the uptake of EAAs like BCAAs, but also as key fuel for nucleotide synthesis, redox balance and the TCA cycle [186]. Elevated glutamine levels in week 4 tumors may therefore help sustain mTORC1 signaling and tumor growth [225]. This is why glutamine is at the forefront of oncology research and therapeutic interventions have shown promise in targeting tumor progression through glutamine [207,208]. Additionally, increased phenylalanine may reflect broader EAA uptake to meet biosynthetic and signaling demands

[187], highlighting the tumor's capacity to reprogram systemic AA metabolism in support of its proliferative needs.

6.2 Liver and Kidney

The next important finding was observed in the organs. Reduction in liver BCKD activity, despite concurrent upregulation of both P-BCKD and PP2CM, suggest a regulatory disconnect that may reflect the liver's attempt to restrain BCAA catabolism under conflicting metabolic cues. This could stem from insufficient substrate availability, as tumor-driven BCAA demand limits systemic supply, or from impaired enzymatic function. In particular, thiamine, a co-factor for the BCKD complex [210], is reduced in gastrointestinal cancers [244, 245], and other catabolic conditions such as sepsis and chronic inflammation including diabetes [211], which could potentially reflect reduced BCKD activity in the liver. Alternatively, the BCAA pool may also be preserved in the liver under metabolic stress conditions, such as fasting, trauma, or chronic disease, where skeletal muscle undergoes proteolysis and releases AAs, while the liver conserves key substrates for gluconeogenesis and nitrogen disposal [212]. Compared to muscle, which harbors a larger intracellular BCAA reservoir and exhibits greater depletion in this model, the liver may have less capacity but more urgency to retain BCAAs for vital functions consistent with our findings.

Another consideration in interpreting these liver findings is the concept of metabolic zonation within the hepatic lobule. Hepatocytes differ in function depending on their location, with periportal hepatocytes (near the portal triad) exposed to higher oxygen and nutrient levels, and perivenous hepatocytes (surrounding the central vein) operating under relatively lower oxygen and nutrient conditions [269,270]. These regions also differ in AA metabolism in which periportal hepatocytes are more active in oxidative pathways such as the urea cycle and

gluconeogenesis, whereas perivenous hepatocytes contribute more to glutamine synthesis, glycolysis, and xenobiotic metabolism [270,271]. Although BCAA catabolism has not been studied in detail across these zones, it is plausible that BCKD activity and regulation vary between them. This spatial heterogeneity may contribute to variability in hepatic BCKD activity that is not captured by whole-tissue measurements of phosphorylation or protein expression, and could therefore underlie some of the discrepancies observed in our study.

The kidney exhibited more overt signs of AA depletion, thus, even as AA concentrations decline, there was unchanged levels of BCKD activity. Further, the modest declines in NEAA, such as alanine and arginine, may reflect a selective release of AA that can be endogenously synthesized or are less critical under current metabolic demands. This pattern of selective conservation aligns with findings from fasting, acidosis, and systemic inflammation, where the kidney reduces oxidation of certain AA, particularly BCAAs, while prioritizing glutamine metabolism to sustain ammoniagenesis and acid-base balance [213,214]. Further, in models of chronic metabolic acidosis, glutamine uptake by the kidney increases markedly, while leucine and other BCAAs are spared from catabolism [215]. Although glutamine in the kidney was not increased, potentially due to the demand of the tumor, this adaptation enhances ammonia production, a key mechanism for neutralizing systemic acidity, and reflects the kidney's capacity to shift substrate use based on physiological need. Similarly, in the context of cancer-induced stress, the kidney in this study appears to adopt a comparable strategy. Taken together, these findings suggest that the kidney, like the liver, perhaps adopts a restraint based metabolic strategy to preserve core physiological functions in the face of tumor-induced nutrient stress.

Supporting this interpretation, the kidney does not simply filter AAs but actively regulates their reabsorption and metabolism along different nephron segments [275]. The

proximal tubules are particularly important, as they express neutral AA transporters such as LAT2 (with its heavy chain CD98hc) and SNAT2, which mediate the uptake and exchange of large neutral AAs across the basolateral membrane [272,273]. BCAT2 is also expressed in renal tissue and contributes to local BCAA transamination [274]. As mentioned earlier, under catabolic conditions these systems adapt by shifting glutamine handling and conserving BCAAs [213,214,215], a process that provides a mechanistic basis for our observation of stable BCKD activity despite overall AA depletion.

6.3 Adipose

Another interesting finding was that although adipose tissue BCAA levels remained unchanged, tumor-bearing mice exhibited clear signs of metabolic reprogramming within this tissue, as evidenced by decreases in other AAs, namely glutamate, alanine, aspartate, and glycine, as well as reductions in key metabolic markers LAT1 and P-S6. These changes may contribute to enhanced lipolysis and fat mass depletion, aligning with *Supplementary Figure 1*, where tumor-bearing mice exhibited significant losses in both body weight and adipose tissue mass [79]. Moreover, the reduced availability of glutamate and alanine, which support gluconeogenesis and nitrogen shuttling [115,118], may signal energy stress within adipocytes, activating AMP-activated protein kinase (AMPK), which is a key energy sensor that stimulates lipolysis under nutrient-deprived conditions [226], and promoting lipolytic pathways [191,192]. Glutamate also supports mitochondrial respiration and TCA cycle function in adipocytes and its depletion may impair oxidative metabolism, further shifting cells toward mobilizing lipid stores [232]. Additionally, reductions in LAT1 and P-S6 may impair protein turnover and mitochondrial integrity [192], accelerating adipose catabolism. Some of these findings are consistent with broader literature describing adipose tissue dysfunction under metabolic stress. For example,

alanine export during fasting contributes to hepatic gluconeogenesis [233], indicating a similar catabolic adaptation in cachexia. Additionally, reduced P-S6 signaling observed here mirrors suppressed mTORC1 activity seen in adipose tissue in other catabolic states. For example, in models where S6K1 is genetically ablated or pharmacologically inhibited, adipose tissue exhibits reduced mass and altered lipid handling, closely resembling the cachectic phenotype [234]. These findings contrast with obesity, where mTORC1 and P-S6 signaling are elevated to support adipose expansion and lipid synthesis [235], highlighting how nutrient sensing in adipose tissue is tightly linked to systemic energy demands.

Additionally, while reduced caloric intake may contribute to fat loss [141], tumor-derived factors such as zinc-α2-glycoprotein (ZAG) and lipid-mobilizing factor (LMF) directly stimulate lipolysis by activating hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), independent of food intake [41]. Notably, emerging evidence suggests that AA depletion may enhance adipose responsiveness to these signals. For instance, metabolically stressed adipocytes exhibit heightened lipolytic responses especially in tumor microenvironments [240] linking nutrient stress to exaggerated fat mobilization. In this context, AA dysregulation may not simply reflect adipose wasting, but actively potentiate it by impairing mitochondrial metabolism, blunting anabolic signaling, and priming adipose tissue for catabolic activation. Thus, AA metabolism may be intersecting with lipid mobilization as both a target and facilitator of tumor-induced wasting, underscoring its role as a central node in adipose tissue remodeling during cachexia.

6.4 Skeletal Muscle: Fiber-Type Specific Disruptions

In the red gastrocnemius, we observed pronounced reductions in BCAAs, BCKD activity, LAT1 expression, and P-S6 levels, indicating a strong catabolic shift and impaired AA

utilization. This aligns with previous cachexia models showing suppressed leucine signaling even in mitochondria-rich fibers [241,242]. The decline in BCKD activity, despite unchanged regulators, may reflect post-translational inhibition, such as impaired cofactor availability (such as thiamine) [244] or reduced substrate flux, mechanisms also implicated in other atrophy models [243]. The plantaris showed milder disruption, with partial reductions in BCAAs, LAT1, and P-S6, suggesting intermediate susceptibility.

In contrast, the soleus displayed a distinct and potentially protective profile. Despite reduced BCAAs and LAT1, it maintained muscle mass [79] and exhibited elevated P-S6, consistent with preserved mTORC1 signaling. This aligns with prior studies showing that muscle fiber type strongly influences susceptibility to cachexia, with fast-twitch muscles more prone to atrophy and oxidative, type I fiber-rich muscles more resilient [71,177]. In our model, this was reflected by the atrophy of red gastrocnemius and plantaris muscles and preservation of the soleus (*Supplementary Figure 2*). Type I fibers are characterized by high mitochondrial density, enhanced oxidative capacity, and elevated expression of PGC-1α, a key regulator of mitochondrial biogenesis and anti-atrophic signaling [197,198]. These features enhance energy efficiency and may suppress ROS accumulation and proteolytic signaling [177], while typically showing lower expression of MuRF1 and Atrogin-1 under cachectic conditions [199]. The elevated P-S6 in the soleus may therefore reflect a fiber-type–specific adaptive mechanism to preserve anabolic signaling and muscle integrity under metabolic stress. Interestingly, despite its oxidative profile, the red gastrocnemius did not exhibit the same protection, suggesting that oxidative phenotype alone may be insufficient. Other factors, such as fiber subtype composition, local metabolic demands, or muscle-specific function, may also determine susceptibility to tumor-induced atrophy. For instance, tyrosine was significantly increased in the soleus of tumor-

bearing mice and this AA, in particular, has been shown to help sustain anabolic signaling by enhancing leucine-induced mTORC1 activation [196]. This has been demonstrated in vitro and ex vivo, where tyrosine lowered the leucine threshold required for S6K phosphorylation and boosted mTORC1 engagement [227].

6.5 Allosteric Regulation of BCKD

Another important finding of this work was the mismatch between BCKD activity and its phosphorylation status across several peripheral sites, suggesting additional regulation beyond the reversible phosphorylation of the E1α subunit by BDK and PP2CM [18,20]. Prior studies have shown that BCKD is also subject to allosteric control, whereby its activity can be directly inhibited by reaction products such as NADH and branched-chain acyl-CoAs, even under conditions of low phosphorylation [266]. Conversely, BCKAs, particularly KIC, have been identified as potent inhibitors of BDK, thereby reducing phosphorylation pressure and indirectly favoring BCKD activation [267,268]. These mechanisms provide possible explanations for our observations, as reduced BCKD activity in red gastrocnemius despite no change in regulatory protein expression may reflect product inhibition through NADH or acyl-CoA buildup under cachectic stress, while the maintenance of BCKD activity in adipose tissue despite increased BDK expression may suggest that BCKAs counteracted kinase activity to stabilize flux. Taken together, these findings highlight that BCKD function is shaped by the combined influence of phosphorylation and metabolite level regulation, underscoring the importance of considering allosteric mechanisms when interpreting tissue-specific activity expression relationships in cancer cachexia.

6.6 Limitations

While these findings point to potential direction and future research for intervention, several limitations must be considered when interpreting these results. Firstly, it important to note that while LAT1 and SNAT1 were selected for their established roles in BCAA uptake and mTORC1 signaling, particularly in skeletal muscle and tumor tissue [101,102,103,104], they are not the predominant transporters in all peripheral sites. In the liver, kidney, and adipose tissue, other transporters such as LAT2 (L-type amino acid transporter 2: SLC7A8), SNAT2 (sodium-coupled neutral amino acid transporter 2:SLC38A2), and B^{0}AT1 (sodium-dependent neutral amino acid transporter 1:SLC6A19) play more central roles in regulating amino acid exchange and flux [181,182]. Another limitation of this study includes that BCKD enzymatic activity was not measured in the plantaris and soleus muscles due to limited sample availability. As a result, conclusions regarding BCAA oxidation in these muscles are based solely on transporter and signaling data, which may not fully capture underlying enzymatic flux. This study also exclusively used male mice, limiting the ability to assess sex-based differences in tissue responses to cancer. Since sex hormones are known to influence AA metabolism, muscle wasting, and mTOR signaling [213,236,237], the findings may not be fully generalizable to females. Also, mice were relatively young at the time of sacrifice (10 - 12 weeks old), which may not accurately reflect the typical age-related context of colon cancer in humans. Younger animals are in a more anabolic state [204], potentially influencing how they respond to tumor burden. While aging the mice may have provided greater translational relevance, time and cost limitations made this approach unfeasible within the scope of this thesis project. In addition, the C26 colon cancer model itself presents limitations. Tumors in this model are implanted subcutaneously rather than arising in the colon, which bypasses the intestinal microenvironment

and nutrient absorption context characteristic of human colorectal cancer. The model is also non-metastatic [263], whereas colon cancer in patients is frequently metastatic, particularly to the liver [264], and therefore may not fully capture the systemic metabolic disturbances associated with advanced disease. Finally, the C26 model induces highly aggressive cachexia, with rapid muscle and adipose loss [265] that may not accurately reflect the slower and more variable progression observed in patients.

6.7 Reframing Cancer Cachexia

Taken together, the findings from this study point toward a coordinated, tissue-specific disruption in AA metabolism driven by the metabolic demands of the tumor. The tumor acted as a metabolically dominant tissue, showcasing increased transporter expression, elevated BCKD activity, and heightened mTORC1 signaling. Reflecting an increased capacity to import, oxidize, and utilize BCAAs and other AAs for growth and proliferation. Meanwhile, nearly all peripheral organs and tissues, including skeletal muscle, kidney, liver, and adipose, showed consistent trends of transporter downregulation, reduced AA retention, and suppressed mTORC1 signaling. In some cases, discrepancies between enzyme activity and regulatory protein expression further suggest additional layers of control, including allosteric regulation and regional heterogeneity within organs such as the liver and kidney. This pattern suggests that the tumor may be outcompeting host tissues for available nutrients, contributing to systemic metabolic stress, tissue wasting, and disrupted energy balance.

This imbalance helps explain why BCAA-targeted interventions have produced limited results in cachexia treatment [188,189]. While leucine and other BCAAs are essential for activating mTORC1 and maintaining muscle protein synthesis, reduced transporter expression and mTORC1 responsiveness in peripheral tissues may prevent these tissues from utilizing

supplemented BCAAs effectively. At the same time, the tumor, through increased AA transporters and mTORC1 activity, may be better equipped to take up and benefit from these nutrients, raising concerns that systemic supplementation may end up supporting tumor metabolism more than aiding recovery. Conversely, BCAA restriction, though it may limit tumor fuel, could further starve already compromised tissues. Altogether, these findings emphasize that cachexia is a whole-body response to tumor-driven nutrient high jacking, highlighting the need for targeted, tissue-specific strategies rather than single-agent nutritional interventions, and reinforcing the importance of thinking beyond the tumor itself and toward supporting the host.

7.0 Future Directions

Future investigations should include:

1. Expand to Additional Time Points

   This study examined metabolic alterations in peripheral organs and tissues only at a late-stage time point (week 4). Future research should include earlier time points, such as week 2, to capture the temporal progression of AA redistribution, transporter regulation, and signaling suppression. This time-course approach could clarify which peripheral sites are disrupted earliest, revealing early indicators of cachexia, and may also help distinguish reversible vs. irreversible stages of the syndrome.

2. Leverage Oxidative Fiber Resistance

   The preserved mass and elevated P-S6 expression observed in the soleus suggest that oxidative, slow-twitch fibers may be more resistant to cancer-induced atrophy. Future studies should explore whether enhancing anabolic signaling specifically in these fibers can help preserve muscle during cachexia. Although direct fiber-type targeting is challenging, certain strategies may offer specificity. These include exercise protocols that preferentially recruit type I fibers [228], pharmacological agents with selective uptake in oxidative muscle [229], and nanoparticle-based delivery systems designed to increase BCAA availability or promote LAT1 expression [230,231].

3. Investigate NEdd4-Mediated Regulation of LAT1 In Vivo

   Building on point 2 and previous in vitro findings from our lab [238], future studies should examine whether NEdd4 contributes to LAT1 degradation in tumor-bearing models of cachexia. In chemotherapy-treated myotubes, silencing NEdd4, an E3 ubiquitin ligase known to target LAT1 [239], restored intracellular BCAA levels,

enhanced mTORC1 signaling, and preserved myofibrillar protein content [238]. Expanding these findings to an in vivo context by depleting NEdd4 in tumor-bearing animals could help determine whether this regulatory mechanism also contributes to LAT1 loss and muscle wasting in cancer. If so, targeting NEdd4 may offer a therapeutic strategy to restore BCAA availability, support anabolic signaling, and mitigate muscle loss in cachexia.

4. Expand to Female Mice and Sex-Based Comparisons

   A novel aspect of this study was the examination of AA metabolism and signaling across multiple peripheral sites, including skeletal muscle, liver, kidney, adipose, and tumor, rather than focusing solely on muscle. Given this broader, systems-level approach, it is important to assess whether similar patterns emerge in females, who may exhibit greater resistance to cachexia due to protective hormonal or metabolic mechanisms [213]. Including female mice in future studies will allow for sex-based comparisons in tissue-specific AA metabolism, transporter expression, and mTORC1 signaling, and could help uncover sex-specific mechanisms and therapeutic windows, ultimately improving the translational relevance of preclinical cachexia models.

5. Investigate Zonation of Hepatic BCAA Metabolism

   Future studies should examine whether BCAA metabolism differs between periportal and perivenous hepatocytes, which are known to have distinct roles in AA metabolism, with periportal cells favoring oxidative pathways and the urea cycle, and perivenous cells contributing more to glutamine synthesis and glycolysis [269,270,271]. Approaches such as hepatocyte isolation could clarify whether this zonation contributes to variability in BCKD regulation observed in whole-liver measurements.

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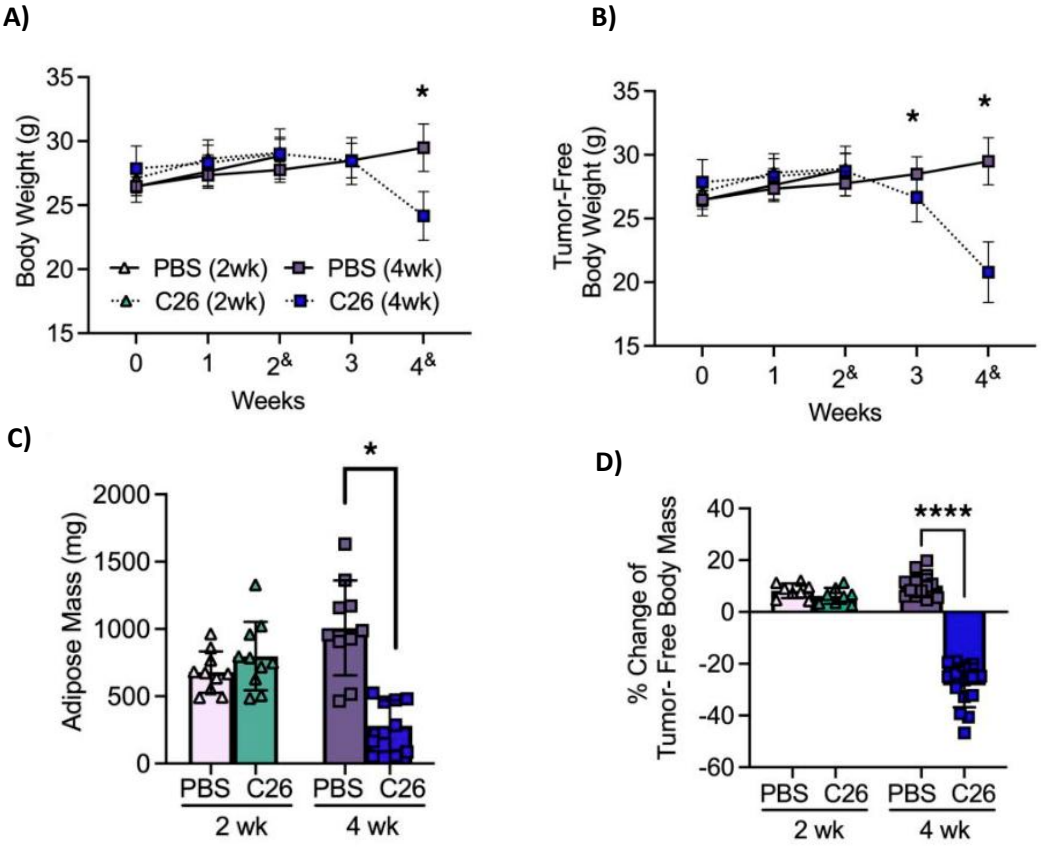
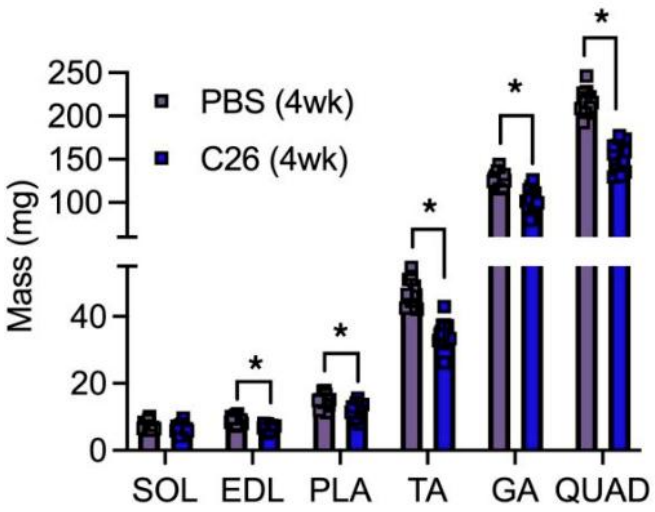
Supplementary Figures

The effects of C26 colon cancer cells' implantation on body weight, tumor-free body weight, adipose Mass, % change of tumor-free body mass, and muscle mass [79].



Supplementary Figure 1: These Analysis' were completed by then PhD Student Luca Delfinis [79]. Analysis of CD2F1 mice with subcutaneous C26 implantations or with PBS were performed. Body weights (A, n = 8–16) and tumor-free body weights (B, n = 8–16) were analyzed every week ($2^{\&}$ mice were measured at a 14- to 17-day window, and $4^{\&}$ mice were measured on a 26- to 29-day window). Subcutaneous fat from the inguinal fat depot was weighed (C, n = 10) Percentage change in tumor-free body weights were analyzed from day 0 to endpoint (D, n = 8–16). Results represent mean ± SD; 2-tailed t tests were used to determine the difference between PBS(2wk) vs. C26(2wk) and PBS (4wk) vs. C26(4wk). One-way ANOVA was used to determine the difference between PBS(4wk) vs. C26(4wk) tumor growth. *P < 0.05 PBS(4wk) vs. C26(4wk); ****P < 0.0001 PBS(4wk) vs. C26(4wk) [79]



Supplementary Figure 2: These Analysis' were completed by then PhD Student Luca Delfinis [79]. Analysis of CD2F1 mice with subcutaneous C26 implantations or with PBS were performed. Evaluations of hind limb muscle wet weights were made at 4-week cohort (J, n = 16). One-way ANOVA was used to determine the difference between PBS(4wk) vs. C26(4wk) tumor growth. *P < 0.05 PBS(4wk) vs. C26(4wk) [79].

Appendix A – In-Vivo BCKD Activity Assay

Materials

Buffer 1

- Potassium phosphate buffer (KPi) (30mM, pH 7.5)
- EDTA (3mM)
- DTT (5mM) - (Research Organics, #2190D-A101X)
- Valine (1mM) - (Sigma Aldrich, V0513)
- FBS (3%) - (Gibco, #12483-020)
- Triton X-100 (5%) - (MP Biomedicals, LLC, #M2528)
- Leupeptin (1 μM) - (Sigma Aldrich, #L2884)

Buffer 2

- HEPES (50 mM) - (Research Organics, #6003H)
- KPi (30 mM)
- CoA (0.4 mM) - (Sigma Aldrich, #4282)
- NAD+ (3 mM) - (Sigma Aldrich, #N0632)
- FBS (5%) - (Gibco, #12483-020)
- Thiamine (2 mM) - (Sigma Aldrich, #T1270)
- Magnesium Chloride2 (2 mM) - (Sigma Aldrich, 7786-30-3)
- Radiolabeled U-14C-Valine (7.8 μM) - (American radiolabelled chemicals,
- #ARC0678)

Procedure

Step 1: Make Potassium Phosphate Buffer KPi Buffer (30mM)

- To make 100mls of KPi Buffer (30mM):
    - Add 80mL of ddH2O in a 200mL beaker
    - Add 107.96mg of potassium phosphate monobasic - (KH2PO4 salt, BioShop, #PPM302.1)
    - Add 384.376mg of potassium phosphate dibasic - (K2HPO4 salt, BioShop #PPD555.1)

Step 2: Make Buffer 1

- To make 25mL solution of buffer 1:
    - Add 15mL of your KPi buffer into a 50mL test tube
    - Add EDTA (3mM) = 150μL
    - DTT (5mM) = 125μL
    - Valine (1mM) = 147.92μL
    - FBS (3%) = 750μL
    - Triton X-100 (5%) = 1250μL
    - Leupeptin (1uM) = 11.90μL

- Once all the above have been added, top up the buffer to 25mls using KPi (30mM) buffer

Step 3: Make Buffer 2

- To make 30mL of buffer 2:
  - Add 15 mL of KPi buffer into a 50mL test tube
  - Add HEPES (50mM) = 357.465mg
  - Add CoA (0.4mM) = 9.2mg
  - Add NAD+ (3mM) = 61.75mg
  - Add FBS (5%) = 1.5mLs
  - Add Thiamine (2mM) = 20.24mg
  - Add Magnesium Clhoride2 = 12.2mg
  - Once all the above have been added, top up the buffer to 30mLs using KPI (30mM) buffer.

Step 4: Homogenize

- On ice, homogenize (using hand help homogenizer PRO200) ~35mg of tissue sample at max speed in 250µL of icecold Buffer 1. Homogenize for around 30 seconds at max speed in a 15mL homogenization tube (Sarstedt, #62.515.006 – must cut some of the tube off as they are too long for homogenizer)
- Transfer full homogenate into a 1.5mL eppendorf tube.

Step 5: Centrifuge

- Centrifuge the homogenized sample in each eppendorf tube for 10 minutes at 10,000g, 4°C

Step 6: Calculate [^{14}C] needed

- During this centrifuge time, calculate the total amount of buffer 2 that will be needed. Here is a sample calculation for 20 samples:
  - For each sample, we need 300µL x 20 (samples) = 6000µL total.
  - Per sample, we want to add 0.16µL of radioactive valine.
  - *That means in 6000µL add 3.2µL (20 x 0.16) of radioactive valine.*
- After doing this, add 300µL of buffer 2 with the radioactive valine into 20 separate eppendorf tubes (as you have 20 samples).

Step 7: Add supernatant to buffer (active)

- After the centrifuging is complete, remove 50µL of the supernatant and add to the 300µL of Buffer 2 in each 1.5mL eppendorf tube.
- **Note:** During this time, BCKD is active, therefore the next couple of steps need to be done fast.

Step 8: Taping Wicks

- Add 60µL of 2M NaOH to a tape a raised wick trap over the lid of the eppendorf tube, cap the tube, and tape over to make sure there is a tight seal.
- **Note:** work fast as we do not want any CO2 from the BCKD reaction to escape

Step 9: Incubation

- Place all samples in a non-shaking incubator at 37°C for 30minutes.
- The 14CO2 released from the BCKD reaction binds to the NaOH on the wick, forming
- NaHCO3

Step 10: Scintillation vials and counter

- After the 30 minutes, remove the wick from each eppendorf tube and place in a scintillation vial with 3.5mLs of scintillation fluid. Count this value in a liquid scintillation counter.
- take 50µL of the liquid from each eppendorf tube and place in a separate scintillation vial with 3.5mLs of scintillation fluid, this will give us a read of total radioactivity in each tube. Count this value in a liquid scintillation counter as well.

Step 11: Protein Assay

- With the remaining liquid left in each tube, perform a protein assay

Step 12: Calculating BCKD activity

- After you get the scintillation value for the wick, for the total radioactivity and protein concentration in each sample, BCKD activity will be calculated by dividing CPM by the total radioactivity and then dividing that value by total protein concentration. This will then be further divided by the conversion factor related to the specific activity of the radioactive substrate [^{14}C] that was used. This conversion factor is 17760.

Appendix B – High Pressure Liquid Chromatography (HPLC) Protocol – AAs

Materials

- Column (YMC-Triart 75 mm x 3.0 mm OD x 1.9 µm particle size)
- Metal Ferrule (IDEX health & science, #VHP-321)
- Metal tubing (IDEX Health & Science, #U-154)
- Vials and lids (Supelco, #27080-U)
- Vial Inserts (Supelco, #24716)
- Phthaldialdehyde solution (OPA) – needs to be made and kept on ice
    - Phthaldialdehyde salt (Sigma Aldrich, #P1378)
    - HPLC grade methanol (Fisher Chemical, #A-4524)
    - Brij 35 (Sigma Aldrich, #B4184)
    - 2-mercaptoethanol (Sigma Aldrich, #M6250)
    - 0.04 M sodium borate buffer
- Micropipette (20-200µL) and tips (20-200µL)
- Samples (keep on ice)
- Saturated Potassium Borate Buffer (30 g of potassium borate into 800 mL of ddH2O, bring up to 1L, then add potassium borate until no more will dissolve)
- Standards (made from Sigma Aldrich, #AAS18)
- Phosphate buffered saline (PBS, Wisent Inc, #311-010-CL))
- 10% Trichloroacetic Acid (TCA)
- 0.1N hydrochloric acid (HCl)
- Sodium hydroxide (NaOH)
- Buffer A (made in 1 L erlenmeyer flask in lab)
    - Potassium phosphate dibasic (K2HPO4 salt) (BioShop, PPD555.1)
    - Potassium phosphate monobasic (KH2PO4 salt) (BioShop, PPM302.1)
    - ddH2O
- Buffer B (made in 1 L erlenmeyer flask in lab)
    - HPLC grade methanol (Fisher Chemical, #A-4524)
    - HPLC grade acetonitrile (Sigma, Aldrich, #34998)
    - ddH2O

Making OPA Reagent (Light sensitive) – can only be used same week it was made

- To make 5mLs (in a 15mL test tube wrapped with aluminum foil)
    - Add 0.02g of OPA in 500µL of HPLC grade methanol
    - Add 120µL of Brij 35
    - Add 20mL of 2-mercaptoethanol
    - Add 4.48mL of 0.04M Sodium borate buffer (15.234g of sodium borate in 900mL of ddH2O, and then adjust to pH 9.5 with sodium hydroxide (NaOH)/hydrochloric acid (HCl) with pH reading machine in the lab and bring up to 1L).

Making Buffer A: 20mmol/L (potassium) phosphate buffer (pH6.5)

To prepare 1L:

1. Add 800mL of ddH2O in a 1L Erlenmeyer flask.
2. Add 1.175 g of K2HPO4 to the solution.
3. Add 1.804 g of KH2PO4 to the solution.
4. Add ddH2O until volume is 1L.

Making Buffer B: HPLC-grade Acetonitrile/HPLC-grade Methanol/Water

To prepare 1L:

1. 450mL of Acetonitrile
2. 400mL Methanol
3. 150ml of ddH2O

Sample/Standard Preparation:

Sample Preparation

1. Centrifuge homogenized samples at 2.3g for 15minutes in 4°C
2. Next take supernatant and dilute in another set of test tubes as shown below

Sample Dilution

Prepare sample by diluting in a ratio of 1:2:1:8:

1: Sample
2: Saturated Potassium Borate Buffer
1: 0.1N HCL
8: ddH2O

Ensure that samples are filtered before they are run through the column to extend life of column and health of HPLC machine. They can be filtered using a 0.2µM 1ml syringe filter (83.1826.001 from Sarstedt). In the filtering process you lose a lot of samples so prepare for this.

Standard Preparation and Dilution

Dilute samples similarly to samples in a ratio of 1:2:1:8

1: Standard (Sigma, #AAS18) - See next steps
2: Saturated Potassium Borate Buffer
1: 0.1N Homogenization Buffer*
8: ddH2O

*AA standard in HCl so add filtered homogenization buffer, into standards, while samples are harvested with homogenization buffer, so add 0.1 HCl into samples

Making Standards

Make sure to have 4 standard curve points (0, 0.25, 0.5, 1.0)

- **For 0 standard curve point:**
  - 1: ddH2O
  - 2: saturated potassium buffer
  - 1: 0.1N homogenization buffer
  - 8: ddH2O
- **For 0.25 standard curve point:** first dilute amino acid standard 1-part amino acid standard, 3-part ddH2O. Then:
  - 1: diluted amino acid standard
  - 2: saturated potassium buffer
  - 1: 0.1N homogenization buffer
  - 8: ddH2O
- **For 0.5 standard curve point:** first dilute amino acid standard 1-part amino acid standard, 1-part ddH2O. Then:
  - 1: diluted amino acid standard
  - 2: saturated potassium buffer
  - 1: 0.1N homogenization buffer
  - 8: ddH2O
- For 1.0 standard curve point:
  - 1: undiluted amino acid standard
  - 2: saturated potassium buffer
  - 1: 0.1N homogenization buffer
  - 8: ddH2O

Protocol Parameters:

- **Gradient Elution**
- **Flow rate:** 0.8mL/min
- **Column temperature:** 35 degrees Celsius
- **Cell Temp:** 4°C
- **Excitation-Emission:** 350-450
- **Maximum Pressure:** 18000psi
- **Injection volume:** 1mL
- **Preferred Column:** YMC-Triart 75mm x 3.0mmOD x 1.9um particle size

Pretreatment: Go to Reagent and add 40mL (this is the OPA that'll be put into sample in each vial – 1:1 ratio of OPA to sample, so add 40mL into each sample