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Original Article

BRANCHED-CHAIN AMINO ACIDS DIFFERENTIALLY AFFECT PROLIFERATION, GLUCOSE-LACTATE METABOLISM AND BCKDHA EXPRESSION IN HUMAN LUNG AND PANCREATIC CANCER CELLS

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ABSTRACT

Branched-chain amino acids (BCAA) are increasingly discussed in the context of cancer metabolism and nutritional support, yet their effects on tumor cells remain insufficiently understood. This study determined how increased concentrations of leucine, isoleucine and valine affect proliferation, glucose–lactate metabolism, extracellular BCAA dynamics and expression of BCKDHA (the E1α subunit of the branched-chain α-keto acid dehydrogenase complex, BCKDC) in human lung (A549) and pancreatic (MIA PaCa-2) cancer cells and normal keratinocytes (HaCaT). Cells were cultured with individual BCAA or their mixtures at 1.5- and 2-fold higher concentrations than standard medium. Lower BCAA excess, particularly leucine, stimulated proliferation in both cancer cell lines, whereas higher concentrations, especially mixtures of all three amino acids, reduced cancer cell growth. A549 cells showed higher glucose consumption and lactate production than MIA PaCa-2 cells, indicating a more glycolytic phenotype, while pancreatic cancer cells displayed a distinct metabolic pattern despite comparable proliferation. Extracellular amino acid analysis revealed cell type-dependent differences in BCAA utilization, with A549 cells showing the highest depletion. Cancer cells generally exhibited lower BCKDHA expression than keratinocytes, suggesting reduced oxidative utilization of BCAA. These findings indicate that the biological effects of BCAA depend on concentration, composition and tumor type, and suggest that BCAA may support either anabolic or oxidative metabolic pathways depending on cellular context.

KEYWORDS: branched-chain amino acids, BCKDHA, cancer cell metabolism, glycolysis, amino acid catabolism

1. Introduction

Normal body cells and tissues need a stable supply of structural and energy substrates to maintain their physiological functions. Rapidly proliferating cancer cells require large amounts of these substrates, competing with normal cells for their availability [1]. Reprogrammed energy metabolism is now recognized as one of the hallmarks of cancer [2,3]. Cancer cells reprogram multiple metabolic pathways - including glucose, amino acid and lipid metabolism - to sustain rapid growth and adapt to microenvironmental constraints [3]. Amino acids provide crucial precursors for biosynthesis and play an essential role in cancer development and metastasis. Evidence has shown activation of several amino acid metabolic pathways in cancer cells, including glutamine, serine, glycine, proline, asparagine as well as branched-chain amino acids (BCAA) metabolism. The reprogramming of amino acid metabolism can modulate energy supply, redox homeostasis, and other metabolic pathways during cancer progression. It seems interesting how cancer cells influence systemic metabolic changes to gain an advantage in meeting nutritional and energy demand. Some malignant tumors, including pancreatic and lung cancer, strongly contribute to the development of

cachexia, including skeletal muscle wasting [4,5]. It is known that cancer cells stimulate the release of glutamine from skeletal muscles as an amino acid necessary for tumor growth namely for the synthesis of purines, pyrimidines and non-essential amino acids and energy in the process of glutaminolysis. Recent studies have demonstrated the existence of the alanine cycle between cancer cells and hepatocytes [6]. Importantly, circulating BCAA are not only nutrients but may also reflect early systemic metabolic remodeling in cancer. Increased plasma BCAA levels have been reported in individuals who later developed pancreatic cancer years before clinical onset [7], suggesting that BCAA-related pathways may be altered at very early stages of tumorigenesis. Nevertheless it remains unclear how cancer cells use BCAA as oxidative fuels via the second step of catabolism versus using them mainly as nitrogen donors and anabolic substrates. It is well-known that the BCAA like leucine (Leu, L), isoleucine (Iso, I), and valine (Val, V) are essential amino acids with a common two-step metabolic pathway [8]. Beyond their role as protein-building blocks, BCAA function as important nutrient signals regulating systemic metabolism [9]. The first step involves transamination, carried out by branched-chain amino acid transaminase (BCAT), which produces branched-chain α-keto acids and glutamate. Glutamate can serve as a nitrogen donor in the synthesis of nitrogenous compounds needed for the tumor growth, such as L-serine, L-aspartate, purines, and pyrimidines [10]. Apart from their metabolic role, BCAA may also exert signaling effects that depend on their transport into the cell and intracellular sensing mechanisms. Therefore, the response of cancer cells to increased BCAA availability may depend not only on catabolism itself but also on amino acid transport and nutrient-sensitive signaling pathways [11]. In the next step, the produced α-keto acids undergo oxidative decarboxylation by the branched-chain α-keto acid dehydrogenase complex. The activity of the BCKDC complex is tightly regulated by reversible phosphorylation of its E1α (BCKDHA) subunit, mediated by BCKDK kinase and PPM1K phosphatase [12]. The products of this complex reaction are incorporated as energy substrates into the Krebs cycle. BCAA metabolism has emerged as an important contributor to cancer cell biology, with both anabolic and oxidative roles documented across multiple tumor types [13]. Previous studies indicate that the first enzyme in this pathway, BCAT, is active in cancer cells. Overexpression of BCAT1 has been observed in patients with stomach cancer and is associated with higher mortality [14]. BCAT1 promotes proliferation, invasion, and angiogenesis of stomach cancer by activating the PI3K/AKT/mTOR pathway, thus acting as an oncogene in disease progression [15]. BCAT1 overexpression has also been demonstrated in IDH-wildtype glioblastomas, where it drives proliferation through amino acid catabolism [16]. In the study by Li et al. [17], it was shown that pancreatic ductal adenocarcinoma (PDAC) exhibits overexpression of the BCAT2 gene. Additionally, knockout of the BCAT2 gene inhibited the progression of pancreatic intraepithelial neoplasia (PanIN) in LSL-KrasG12D/+; Pdx1-Cre (KC) mice [18]. Furthermore, BCAA stimulated the growth of pancreatic ductal adenocarcinoma (PDAC) organoids in a dose-dependent manner. However, the effect of BCAT2 inhibition could be reversed by supplementation with branched-chain α-keto acids (BCKA) and purines and pyrimidines. A diet low in BCAA was also effective in inhibiting PDAC development in mice. Similarly, Zhang et al. [19] demonstrated that BCAA levels in both plasma and cancer tissue are elevated in breast cancer patients compared to healthy individuals. Knockdown of the BCAT1 gene resulted in inhibition of growth rates of breast cancer cells (MCF-7 and T47D). Very similar observations were made by Hattori et al. [20] who showed that BCAT1 was highly expressed in CML (chronic myeloid leukemia). In this context, instead of deaminating BCAA into BCKA, the overexpression of BCAT1 led to an increase in intracellular BCAA levels through the amination of BCKA. However, little is known about the second stage of BCAA metabolism and the influence of these amino acids on BCKDC activity, particularly in the context of different cancer types and varying substrate availability. This gap is relevant because the balance between transamination and oxidative decarboxylation may determine whether BCAA support energy production or preferentially feed nitrogen-dependent biosynthesis.

To address this question across distinct tumor types, we used A549 cells as a model of non-small cell lung cancer (NSCLC), the most common histological subtype of lung malignancy, and MIA PaCa-2 cells as a model of pancreatic ductal adenocarcinoma (PDAC) - a tumor type in which BCAA metabolism has been functionally implicated [17,18]. HaCaT immortalized keratinocytes were used as a non-cancerous reference line.

Therefore, the aim of this study was to determine how supplementation with leucine, isoleucine and valine - administered individually and in combinations at increased concentrations - affects proliferation, glucose-lactate metabolism, extracellular BCAA dynamics and expression of the BCKDC E1 subunit (BCKDHA) in lung and pancreatic cancer cells compared with non-cancerous keratinocytes. These findings may contribute to a better understanding of how cancer cells regulate BCAA utilization and how the balance between anabolic and oxidative metabolic pathways differs across tumor types.

2. Materials and methods

2.1. Cell culture

A549 (human alveolar basal epithelial adenocarcinoma), MIA PaCa-2 (hypotriploid human pancreatic carcinoma) and HaCaT (immortalized human keratinocytes) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were propagated following ATCC handling recommendations and maintained in MEM (Biowest SAS, France) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, San Francisco, CA, USA) and 1% penicillin/streptomycin. Cultures were kept at 37°C in a humidified incubator with 5% CO_2 and routinely passaged at ~80–90% confluence using 0.25% trypsin–0.02% EDTA (Gibco BRL). Experiments were performed using passages 5–15 (from frozen stocks). All experimental variants followed the same scheme of branched-chain amino acid (BCAA) supplementation. Controls were cultured without extra amino acids in concentrations as in standard MEM formulation (for Leu and Ile 52 mg/L, for Val 46 mg/L). MEM with the addition of higher BCAA concentrations (1.5 and 2 times) was added in the following experimental models: Leu (L) at a concentration of 78 mg/L (L1.5) and 104 mg/L (L2),

Ile (I) at a concentration of 78 mg/L (I1.5) and 104 mg/L (I2), Val (V) at a concentration of 69 mg/L (V1.5) and 92 mg/L (V2), and a mixture of all tested compounds at lower and higher concentrations, designated as LV1.5, ILV1.5 and LV2, ILV2, respectively. The 1.5x and 2x BCAA concentrations relative to standard MEM represent a moderate stepwise increase over baseline culture conditions. Although BCAA concentrations in standard cell culture media exceed fasting human plasma levels, circulating BCAA levels in humans are subject to considerable physiological variation, rising 2- to 3-fold postprandially and up to 6- to 12-fold during intravenous amino acid infusion [21–23]. All experiments were performed in three independent biological replicates (n = 3).

To enable direct comparison of cellular responses to defined BCAA concentrations across all three cell lines, all cultures were maintained in the same base medium (MEM) rather than the cell-type-specific media recommended by ATCC (F-12K for A549, DMEM for HaCaT, DMEM with 2.5% horse serum for MIA PaCa-2). This unified approach was necessary because the recommended media differ substantially in their baseline amino acid composition, which would have confounded the interpretation of BCAA-supplementation effects. Cells were adapted to MEM for at least three passages before experiments to ensure stable growth in this medium. The choice of MEM as the common base medium is acknowledged as a methodological limitation and discussed in the Discussion section.

2.2. Trypan blue assay

For cell counting, cells were plated in 12-well plates (1×10^5 cells/well). After 24 h, the medium was replaced with fresh MEM containing the indicated BCAA conditions and cells were incubated for 72 h. Cells were detached with trypsin/EDTA, resuspended in PBS and mixed with trypan blue. Viable (dye-excluding) and non-viable cells were quantified using an automated counter (Countess, Invitrogen). Trypan blue exclusion reflects loss of plasma membrane integrity and does not detect early apoptotic or cytostatic changes. This limitation is acknowledged in the Discussion.

2.3. Glucose and lactate determination

Glucose and lactate in conditioned media collected at 24, 48 and 72 h were quantified using enzymatic colorimetric assays (Randox) according to the manufacturer's protocol. Absorbance was read at 500 nm (glucose) and 550 nm (lactate). Results were normalized to cell number.

2.4. Amino acid determination by HPLC

Separation was performed using a Kinetex C18 analytical column (100 mm × 4.6 mm, 3 µm; Phenomenex, Torrance, CA, USA). The column temperature was maintained at 25°C. Elution was carried out using mobile phase A (water:formic acid, 100:0.1, v/v) and mobile phase B (acetonitrile:formic acid, 100:0.1, v/v) under a one-step gradient program: 0–10 min, 1–10% B. The flow rate was set at 1 mL/min for both extract and standard analyses. A 5 µL aliquot of each sample was injected onto the column using an autosampler. The column was equilibrated for 4 min between injections. Calibration standards of L-leucine, L-isoleucine and L-valine were used to identify and quantify the corresponding amino acids in conditioned media. Conditioned media were cleared of cells and cellular debris by centrifugation prior to analysis. No internal standard was used.

The eluate was directly introduced to CAD instrument. A CAD response was set at 100 pA, high filter selected. Air from DynAir Silent Compressor (Shanghai Dynamic Industry, China), regulated at 35 psi, was introduced to detector. Data processing was carried out with Chromeleon® 6.8 SR15b (Dionex).

2.5. Western blotting

Protein extracts were prepared by lysing the cells in RIPA buffer supplemented with phosphatase and protease inhibitors. Total protein concentration was measured using the BCA method (Pierce BCA Protein Assay Kit, ThermoFisher Scientific). Samples containing 20 µg of protein were prepared for SDS-PAGE. Following electrophoresis, semi-dry transfer to nitrocellulose membranes was performed. After blocking in 5% nonfat dry milk in TBST buffer, the membranes were incubated overnight with the appropriate primary antibodies: antibodies Proteintech® anti BCKDHA antibody (Cat No. 30028-1-AP, 1:500, 1.6 µg/mL, 42-50 kDa). After incubation overnight at 4 °C nonfat dry milk, the secondary HRP-conjugated anti-rabbit IgG antibody (CellSignaling Technology®, Cat. No. 7074S) was applied for 1 h at room temperature.

Protein bands were visualized using Clarity Max Western ECL Substrate (Bio-Rad, Cat. No. 1705062), and chemiluminescent signals were detected using the Odyssey imaging system (LI-COR Biosciences).

For loading control normalization, membranes were stripped using Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, Cat. No. 46430) and subsequently incubated for 1 h at room temperature with an HRP-conjugated anti-ß-actin antibody (Sigma-Aldrich, Cat. No. A3854). After incubation in the presence of 5% nonfat dry milk, membranes were developed again using Clarity Max Western ECL Substrate, and signals were detected with the Odyssey imaging system.

For densitometric analysis of band intensities Image Studio software was used. BCKDHA protein levels were normalized to ß-actin, which served as a loading control.

Western blot experiments were performed in three independent biological replicates. BCKDHA protein expression was used as a proxy for the catalytic capacity of the BCKDC complex. It does not directly reflect enzymatic activity, which is additionally regulated by post-translational mechanisms including phosphorylation by BCKDK and PPM1K.

2.6. Statistical analysis

Statistical analysis was performed by using linear mixed models, on the basis of which the appropriate marginal means were determined and compared. This allowed for capturing the simultaneous impact of the analyzed factors on each of the studied parameters. Therefore, two forms of models were considered. In case of the number of cells and their viability, both measured only once during the study, the following explanatory variables were used: healthy or cancer line, amino acid combination and their interaction. The random factor was the measurement ID. For the parameters determining the level of glucose, lactate and HPLC for individual amino

acids, the explanatory variables were: healthy or cancer line, amino acid combination, moment of measurement and their interactions. The random factor was again the measurement ID.

Western blot data were analysed separately. For each cell line (HaCaT, A549 and MIA PaCa-2) we fitted a simple linear model with log-transformed normalized band intensity (BCKDHA/ß-actin) as the response and amino acid combination as the only explanatory variable. From these models we obtained estimated means on the log scale and then back-transformed them to get fold change values relative to the control, together with 95% confidence intervals. Each amino acid condition was compared with the control using contrast tests with Dunnett's correction for multiple testing.

The key elements of the analysis were the estimated marginal means determined on the basis of these models. They were compared using contrast tests, determining the differences in these means along with its 95% confidence interval. In the case of comparisons between amino acids, Dunnett's multiple testing correction was used, as we referred all amino acid combinations only to the control, but for comparisons between healthy or cancer lines, Tukey's correction was used. Marginal means and their corresponding 95% confidence intervals are presented in point-and-whisker plots, and for Western blot in bar plots with error bars. The level of significance was assumed to be $p<0.05$, however, it also indicates statistically significant results for the levels $p<0.01$ and $p<0.001$. Calculations were performed using R software version 4.3.2. The analysis was performed using the lmer function from the lme4 package (https://cran.r-project.org/web/packages/lme4/lme4.pdf) in R to fit models. Then, the emmeans package (https://cran.r-project.org/web/packages/emmeans/emmeans.pdf) was used to obtain marginal means.

3. Results

3.1. Proliferation rate and viability after supplementation with BCAA

For the A549 line, leucine, but also valine at 1.5 times the concentration, proved to be the most effective amino acid in enhancing proliferation of lung cancer cells. The combination of all three amino acids at concentration 1.5x increased rate of cell growth whereas their mix at higher concentration (2x) exhibited antiproliferative activity. Within the MIA PaCa-2 line, pro-proliferative potential was observed in the presence of excess of leucine 1.5x, and valine 1.5x and also with mix of both BCAA at 1.5x concentration but not at 2x concentration where the decrease of cell growth was showed. A similar antiproliferative trend was observed after addition of the combination of leucine and valine 2x ($p < 0.05$). It turned out that the most sensitive cell line for addition of extra BCAA turned out non-cancerous line HaCaT. All amino acids (AA) alone or in combination positively affected proliferation, with leucine and a mix of three amino acids having the leading impact. Statistically significant differences were observed in each of the comparisons regarding amino acid combinations ($p < 0.05$) (Fig. 1). In the case of the A549 line viability decreased in a statistically significant manner only after the addition of a mix of all three amino acids in 1.5x concentration. More of such significant comparisons were identified. These include comparisons of control with: leucine at both concentrations, isoleucine 2, the combination of leucine and valine 1.5, and all three BCAA at 1.5 ($p <0.05$), but all of them had their viability increased. Within the MIA PaCa-2 line, four statistically significant comparisons with the control were noted ($p <0.05$), namely for leucine 1.5x; isoleucine 1.5x; valine 2x, and the combination of leucine with valine 1.5, and they all meant a decrease in viability. Regarding HaCaT line, the only statistically significant difference detected was the combination of leucine and valine 1.5 compared to the control ($p <0.05$) (Fig. 2).

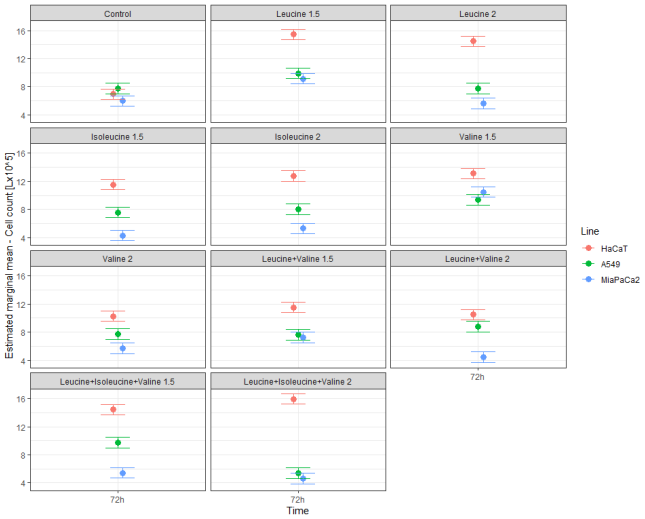

Fig. 1. Marginal means for cell count in normal (HaCaT) and cancer (A549, MIA PaCa-2) lines after BCAA supplementation

Fig. 2. Marginal means for cell viability (%) in normal (HaCaT) and cancer (A549, MIA PaCa-2) lines after BCAA supplementation

3.2. Influence of BCAA on glucose consumption and lactate production

Both glucose and lactate concentrations were analyzed at different timepoints (24 h, 48 h, 72 h).

3.2.1. Glucose breakdown

The glucose consumption by normal cells was notably lower than by cancerous cell lines ($p<0.005$). The lung adenocarcinoma used significantly more glucose than

pancreatic cancer cells (Fig. 3). During the first 24 hours, the A549 cells sparingly used glucose and this was most noticeable in the presence of isoleucine and valine at both concentrations, as well as in the mix of the three amino acids at 1.5x concentration. This trend persisted even after 48 hours of cell culture, but generally from this moment glucose consumption increased. It is also worth noting that this cell line exhibited lowest concentrations of glucose at 72 h timepoint for all BCAA combinations, except the one with all BCAA at double concentration. The least pronounced glucose consumption in this case was observed, which was most noticeable after 48 and 72 hours. Glucose consumption in pancreatic cancer cells MIA PaCa-2 appears to be more balanced and uniform. With each subsequent 24 h, the measured glucose levels decreased, although this was least pronounced in the presence of isoleucine and valine. Within the HaCaT line all comparisons with control indicated statistically significant differences between the temporal groups (p <0.001). The weakest differences were between 24 and 48 hours (Fig. 4).

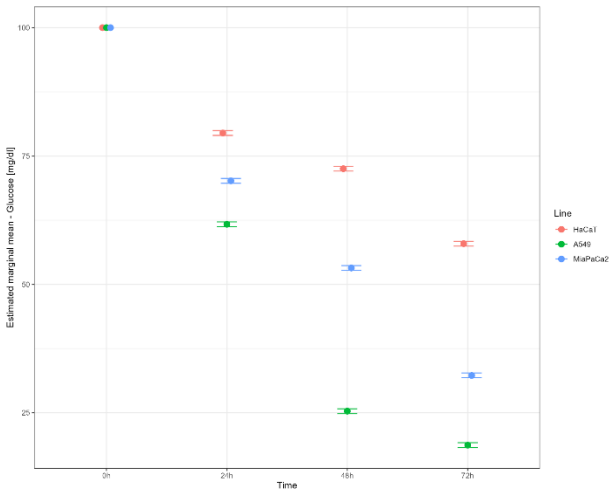

Fig. 3. Marginal means for glucose concentration in normal (HaCaT) and cancer (A549, MIA PaCa-2) lines after BCAA supplementation at different timepoints (24 h, 48 h, 72 h), without BCAA set combination factor

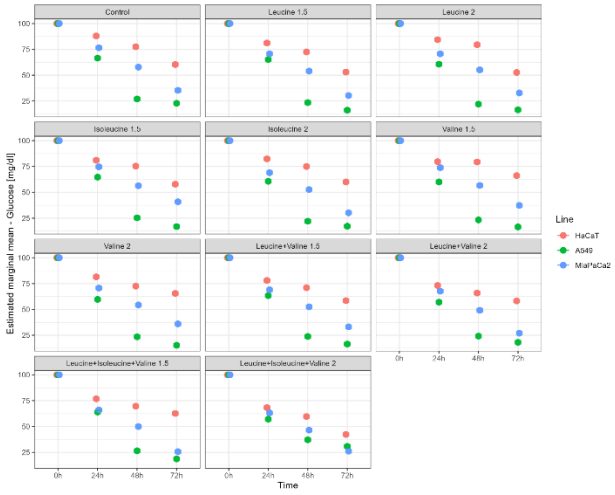

Fig. 4. Marginal means for glucose concentration in normal (HaCaT) and cancer (A549, MIA PaCa-2) lines after BCAA supplementation at different timepoints (24 h, 48 h, 72h), with BCAA set combination factor

3.2.2. Lactate synthesis

Similar to glucose, for each of the conducted comparisons, the result turned out to be statistically significant (p <0.001). Furthermore, analyzing the graph, we can observe that the highest lactate measurements at each time point were recorded for the A549 line. We can also note the arrangement of the measurements over time. For the A549 line, they had a linear character, while in the two other cases, they somewhat resembled a parabola, indicating decreasing increments (Fig. 5).

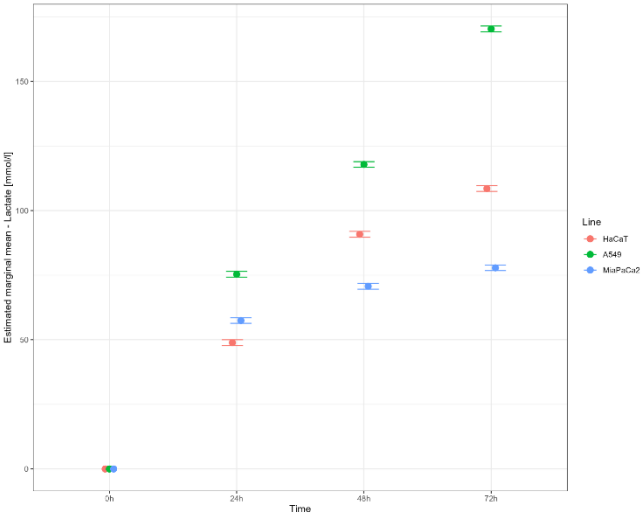

Fig. 5. Marginal means for lactate concentration in normal (HaCaT) and cancer (A549, MIA PaCa-2) lines after BCAA supplementation at different timepoints (24 h, 48 h, 72 h), without BCAA set combination factor

Comparing different amino acid combinations with control within the HaCaT line, statistically significant differences (p<0.05) were observed in the first measurement point (24 h) for all variants containing valine at both concentrations and the case of leucine at double concentration. In the next day (48 h), such differences were observed for all comparisons except for the combination of all three amino acids at double concentration, and in the last measurement point (72 h) isoleucine 1.5 and valine 1.5 also dropped from this group. In the A549 line, the only combination that differed significantly from the control regardless of the measurement point and concentration was leucine plus valine. On the other hand, in MIA PaCa-2 line, such prevalent combination was just valine. It is also evident that A549 line exhibited highest lactate concentrations at 72 h timepoint in every amino acid combination, in comparison to other lines (Fig. 6).

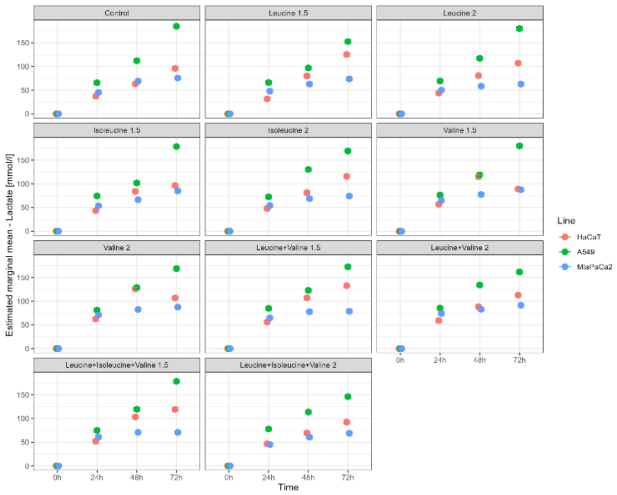

Fig. 6. Marginal means for lactate concentration in normal (HaCaT) and cancer (A549, MIA PaCa-2) lines after BCAA supplementation at different timepoints (24 h, 48 h, 72h), with BCAA set combination factor

3.3. Changes in BCAA concentrations

3.3.1. Leucine concentration

Statistically significant differences between the lines were observed for nearly all comparisons ($p<0.05$). The exception was the comparison of the healthy HaCaT line with MIA PaCa-2 on the first day. It should also be noted that A549 line strikingly diverged from the other lines over time, exhibiting lowest values of concentration at every timepoint in comparison to other lines (Fig. 7).

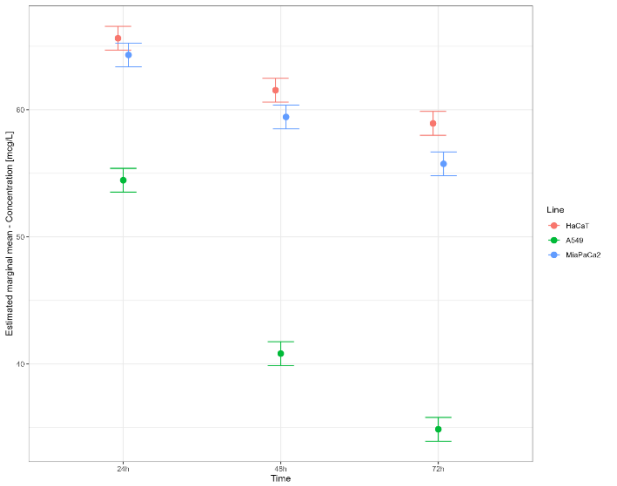

Fig. 7. Marginal means for leucine concentration in normal (HaCaT) and cancer (A549, MIA PaCa-2) lines after BCAA supplementation in different timepoints (24 h, 48 h, 72h), without BCAA set combination factor

Within the healthy cell line statistically significant ($p<0.001$) differences were observed for every combination containing leucine at both concentrations at every timepoint. In the A549 line, across all three measurements points, significant differences ($p<0.001$) occurred also between control and combinations containing leucine, but only at two times concentration ($p<0.05$). Surprisingly, MIA PaCa-2 line exhibited very similar behavior to HaCaT rather than A549, where statistically significant differences ($p<0.001$) were also observed for every combination containing leucine at both concentrations at every timepoint, with the exception of leucine 1.5 at 48 h and 72 h timepoint, which was less significant in comparison (Fig. 8).

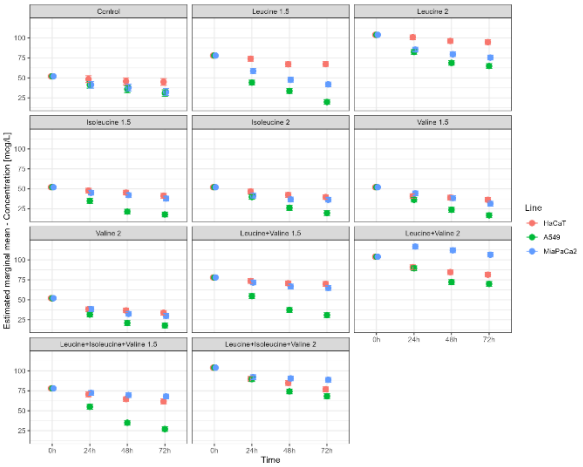

Fig. 8. Marginal means for leucine concentration in normal (HaCaT) and cancer (A549, MIA PaCa-2) lines after BCAA supplementation at different timepoints (24 h, 48 h, 72 h), with BCAA set combination factor

3.3.2. Isoleucine concentration

In the case of isoleucine, statistically significant differences between all three lines occurred for each comparison at timepoints 48 h and 72 h, but there were no statistically significant differences in the first day. It can be noted that at day one measurements for all lines are very similar and then begin to markedly diverge over time. Moreover, for each line, values decrease over time, with the lowest observed for the A549 line and the highest for MIA PaCa-2 at the 72h timepoint (Fig. 9).

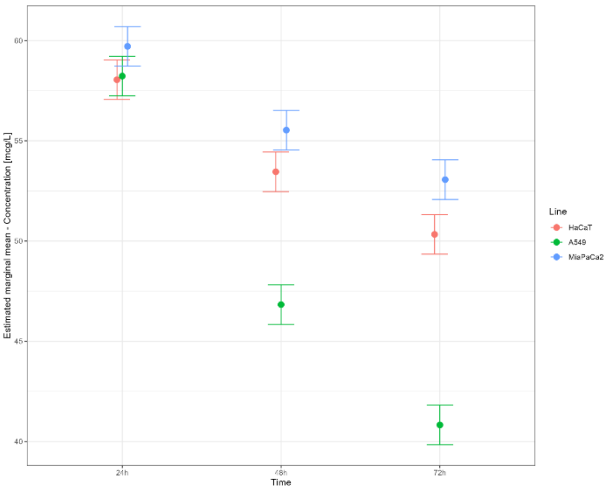

Fig. 9. Marginal means for isoleucine concentration in normal (HaCaT) and cancer (A549, MIA PaCa-2) lines after BCAA supplementation in different timepoints (24 h, 48 h, 72h), without BCAA set combination factor

Within the healthy line, it can be observed that statistically significant differences occurred for isoleucine at both concentrations in each measurement point. Additionally, the only other combination that its statistically significant difference ($p<0.001$) from the control remained is the mix of all three BCAA at two times concentration. Within the A549 line, statistically significant differences ($p<0.05$) compared to the control were present at each measurement point for isoleucine and a mix of all BCAA at both concentrations, although

not all of them kept their p-value below 0.001 over time. Similarly, within the MIA PaCa-2 line, statistically significant differences ($p<0.001$) were maintained over time between control vs. isoleucine and control vs. three BCAA at both concentrations (Fig. 10).

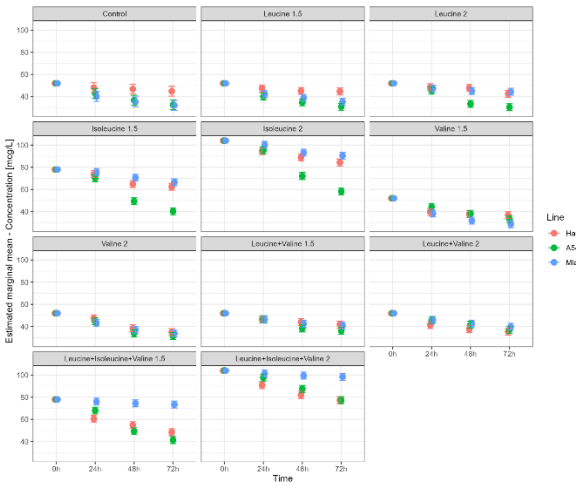

Fig. 10. Marginal means for isoleucine concentration in normal (HaCaT) and cancer (A549, MIA PaCa-2) lines after BCAA supplementation at different timepoints (24 h, 48 h, 72 h), with BCAA set combination factor.

3.3.3. Valine concentration

Statistically significant differences between HaCaT and A549 lines were observed for every timepoint. Such difference was also observed for the comparison between both cancer lines, but only at timepoints 48 h and 72 h. Interestingly, there were no statistically significant differences at any measurement point for MIA PaCa-2 vs. healthy line. Moreover, A549 line once again exhibited the lowest value of concentration at 72 h timepoint in comparison to other lines (Fig. 11).

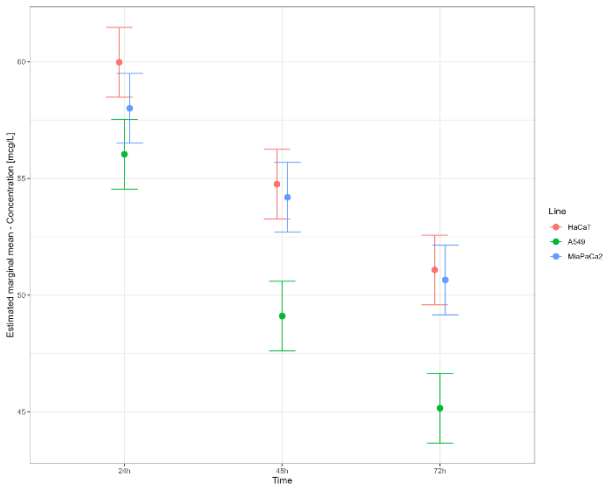

Fig. 11. Marginal means for valine concentration in normal (HaCaT) and cancer (A549, MIA PaCa-2) lines after BCAA supplementation at different timepoints (24 h, 48 h, 72 h), without BCAA set combination factor

Within the HaCaT line at all time points, statistically significant comparisons ($p<0.05$) of the control with all combinations containing valine were noted. Although, only the combinations containing valine at 2 times concentration uphold the p-value below 0.001 at every timepoint. Similarly, within the A549 line, significant differences with control ($p<0.001$) were observed for every combination containing valine at 2x concentration. On the other hand, for the MIA PaCa-2 line, such significant differences ($p<0.001$) were also observed for every combination containing valine, but at both concentrations: 1.5x and 2x (Fig. 12).

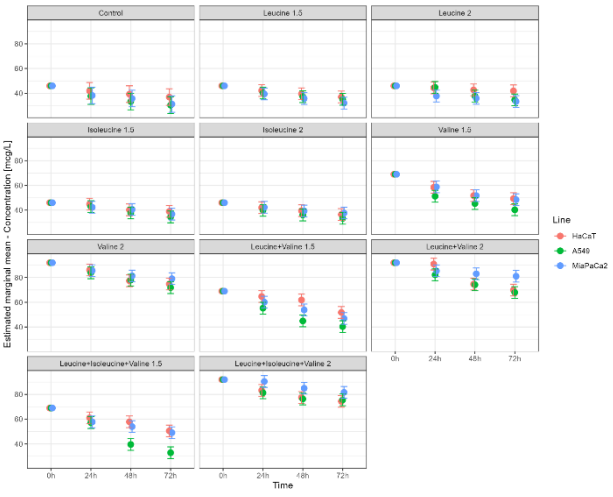

Fig. 12. Marginal means for valine concentration in normal (HaCaT) and cancer (A549, MIA PaCa-2) lines after BCAA supplementation in different timepoints (24 h, 48 h, 72 h), with BCAA set combination factor

3.4. Effect of BCAA on BCKDC expression on protein level

To assess whether BCAA supplementation affects BCKDC expression, we measured protein levels of its catalytic E1α subunit (BCKDHA) by Western blot in all three cell lines. The weakest expression of this protein was observed in the A549 cell line, while pancreatic cancer cells and the non-cancerous, normal cell line HaCaT exhibited moderately high expression. For A549 line, in all studied sets the BCKDC expression was lower than in control, while only in the presence of AA sets (L+V; L+I+V) in higher concentration (2x) a significant fall in enzyme expression was observed. The differences in the effect of AA concentration on expression were limited to the above-mentioned mixtures. Contrary to that, pancreatic cancer cell line exhibited moderate increases in BCKDC expression concerning all studied sets in lower concentration excluding Leu. However, higher concentrations of single AA and mix of AA consequently resulted in reduction of enzyme expression. It seems that Val has revealed the strong influence on protein level. In normal, noncancerous cells the expression of this protein was higher after treatment with single AA in 1.5x concentration and consequently decreased in a concentration-dependent manner for all studied individual BCAA. Meanwhile, expression declined in HaCaT cells treated with mix of AA in both concentrations. The best inducer of protein expression was Iso in lower concentration 1.5x (Fig. 13).

A summary of the direction and magnitude of the observed effects across cell lines is presented in Table 1.

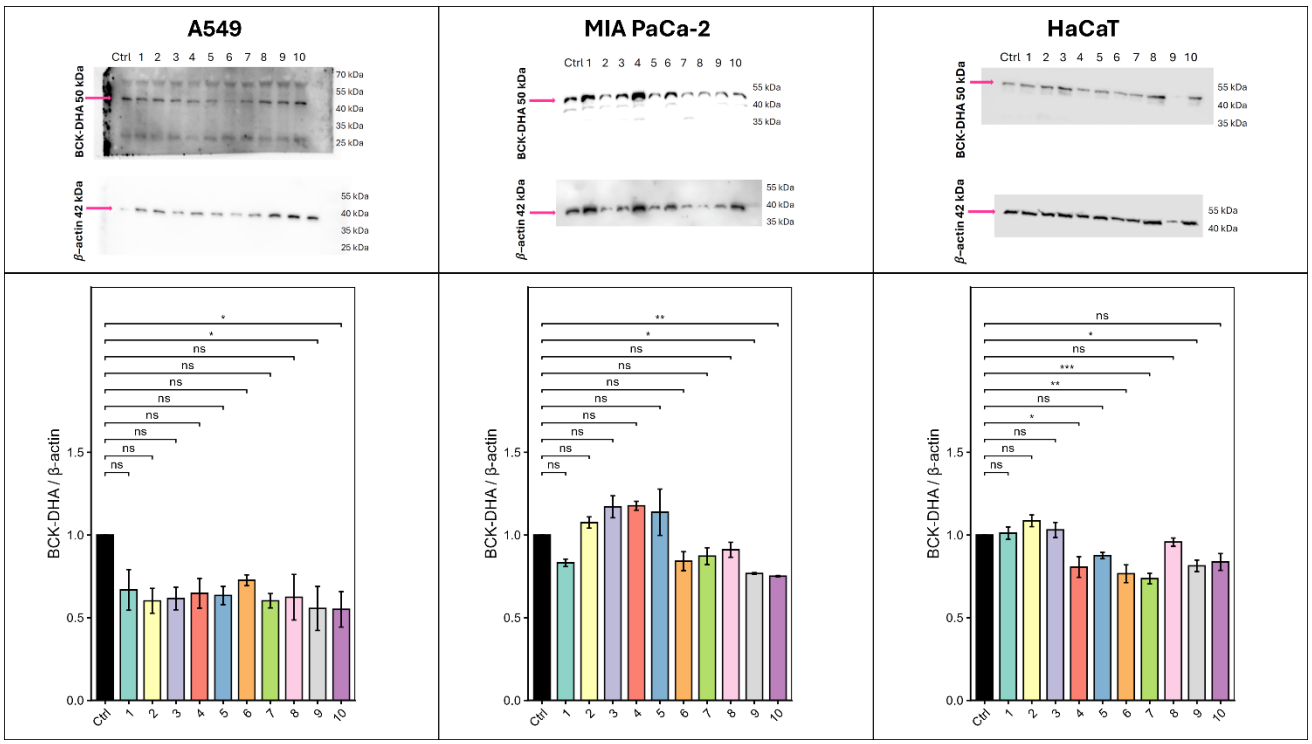

Fig. 13. Effect of BCAA on BCKDHA expression in A) lung cancer cells; B) pancreatic cancer cells and C) normal keratinocytes. Western blot analysis with Proteintech® anti BCKDHA antibody was performed as described in Materials and methods. Comparable amounts of protein (20 µg) were loaded per lane. A representative blot from three independent experiments is shown. Lines: Ctr – Control, 1 – leucine 1.5x; 2 – isoleucine 1.5x; 3 – valine 1.5x; 4 – leucine+valine 1.5x; 5 – leucine+isoleucine+valine 1.5x; 6 – leucine 2x; 7 – isoleucine 2x; 8 – valine 2x; 9 – leucine+valine 2x; 10 – leucine+isoleucine+valine 2x. Abbreviations: BCKDHA, E1α subunit of the branched-chain α-keto acid dehydrogenase complex.

Table 1. Directional changes relative to control are indicated as increases (↑), decreases (↓) or no major change (↔). Arrows reflect overall trends observed across experimental conditions based on statistical modeling and graphical analysis. BCKDHA expression refers to normalized protein levels determined by Western blot analysis.

Cell line	Proliferation response	Glucose consumption	Lactate production	BCAA utilization	BCKDHA expression
A549	↑at moderate BCAA; ↓ at high mixtures	↑↑	↑↑	↑↑	↓
MIA PaCa-2	↑ at moderate BCAA; ↓at high concentration	↑	↔/↓ vs A549	↑	↔ / ↓
HaCaT	↑ in most conditions	↓	↑	↑	↑single AA; ↓ mixtures

4. Discussion

The findings of this study suggest that branched-chain amino acids do not act solely as growth-promoting nutrients in cancer cells but may also regulate metabolic routing in a concentration- and cell-type-dependent manner. Moderate increases in BCAA availability promoted proliferation, particularly in the presence of leucine, whereas higher concentrations, especially mixtures of all three amino acids, exerted inhibitory effects in cancer cells. At the metabolic level, lung cancer cells displayed a more glycolytic phenotype than pancreatic cancer cells, with higher glucose consumption and lactate production. Importantly, differential expression of the BCKDC E1 subunit suggests that cancer cells may utilize a smaller fraction of BCAA for oxidative energy metabolism compared with non-cancerous keratinocytes. BCAA are multifunctional metabolites involved in both anabolic and energetic pathways. As essential amino acids they must be supplied exogenously and serve not only as substrates for protein synthesis but also as sources of nitrogen and carbon skeletons supporting biosynthesis and energy production [21,24]. Among them, leucine is considered the most potent anabolic signal, largely due to its capacity to activate mTOR signaling pathways associated with cell growth and proliferation [25,26]. Our findings are consistent with this concept, as leucine supplementation at moderately elevated concentrations induced proliferation in both cancer cell lines and keratinocytes. However, higher concentrations produced the opposite effect, suggesting the presence of non-linear dose-response relationships. This effect may be related not only to metabolic imbalance but also to mechanisms controlling amino acid transport and intracellular sensing. Uptake of large neutral amino acids, including BCAA, is mediated by transporters such as LAT1 (SLC7A5), which exchanges intracellular amino acids, particularly glutamine, for extracellular leucine [11]. In parallel, leucine is a well-established activator of the mTORC1 pathway, with

Sestrin2 acting as a key intracellular leucine sensor [27]. Therefore, lower BCAA excess may favor growth-promoting signaling, whereas higher concentrations - especially amino acid mixtures - may disturb intracellular amino acid balance and limit the availability of other essential substrates required for proliferation [11]. This mechanism may help explain why the mixture of all three amino acids exerted a stronger inhibitory effect than leucine alone. Such bidirectional responses may reflect metabolic imbalance or adaptive regulation induced by excessive amino acid availability. The distinct effects observed between individual amino acids may also be related to differences in metabolic fate. Leucine and isoleucine contribute to acetyl-CoA production, whereas valine is exclusively glucogenic, which may influence cellular energy balance and biosynthetic capacity [28,29]. The strong inhibitory effect observed with mixtures of all three amino acids at higher concentrations may reflect combined metabolic pressure exceeding the adaptive capacity of cancer cells. Importantly, these effects were not associated with cytotoxicity, indicating metabolic rather than toxic mechanisms.

The BCAA concentrations used in this study (78–104 mg/L for leucine and isoleucine, 69–92 mg/L for valine) fall within a range relevant to several physiological and clinical scenarios. Plasma BCAA levels can rise 2- to 3-fold postprandially after high-protein meals, increase several-fold during oral or intravenous BCAA supplementation used in clinical settings (e.g., parenteral nutrition, management of hepatic encephalopathy), and reach up to 12-fold elevations during therapeutic amino acid infusions [21–23]. Elevated circulating BCAA have also been reported in patients who later develop pancreatic cancer [30]. The concentrations applied in our experimental system therefore approximate physiologically and clinically achievable elevations rather than supraphysiological pharmacological doses.

Our metabolic analyses confirmed preferential glycolytic metabolism in A549 cells compared with MIA PaCa-2 cells, consistent with metabolic heterogeneity between tumor types [31,32]. Lung cancer cells exhibited both higher glucose consumption and lactate production, indicating a classical Warburg phenotype [33,34]. In contrast, pancreatic cancer cells produced less lactate despite measurable glucose utilization, suggesting differences in substrate routing and possibly greater reliance on mitochondrial metabolism. Such differences may reflect tumor-specific adaptations to microenvironmental conditions, including nutrient availability and stromal architecture characteristic for pancreatic tumors [35]. Significant differences were also observed in BCAA utilization between cell types. A549 cells demonstrated a markedly higher demand for both glucose and BCAA, whereas pancreatic cancer cells appeared metabolically more restrained despite comparable proliferation rates. These observations support the concept that different cancers may adopt distinct metabolic strategies rather than relying uniformly on increased nutrient consumption. One of the most relevant findings of this study concerns the expression of the BCKDC complex. Cancer cells showed generally lower expression of BCKDHA compared with normal keratinocytes, suggesting reduced oxidative catabolism of BCAA. Supplementation with amino acids further modulated BCKDHA expression in a dose-dependent manner, indicating adaptive regulation of BCAA utilization pathways. These observations are consistent with a shift in BCAA metabolism toward anabolic nitrogen utilization rather than mitochondrial oxidation, although direct metabolic flux measurements would be required to confirm this interpretation. These findings suggest that BCAA metabolism may represent a metabolic decision point between anabolic nitrogen utilization and oxidative energy production (Fig. 14).

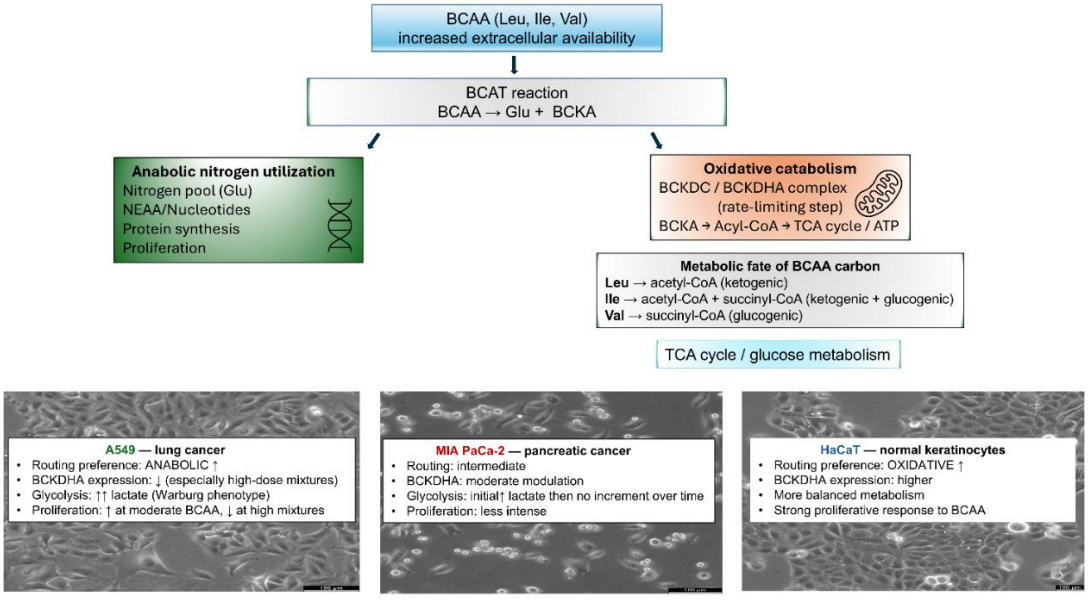

Fig. 14. Proposed metabolic routing of branched-chain amino acids and the role of the BCKDH complex in BCAA catabolism. Branched-chain amino acids are transaminated by BCAT to form branched-chain keto acids and glutamate. Glutamate contributes to nitrogen metabolism, whereas oxidative decarboxylation of BCKA by the BCKDH complex generates acyl-CoA derivatives entering central metabolic pathways. The BCKDH complex represents a key regulatory step controlling the oxidative utilization of BCAA-derived carbon. Abbreviations: BCAA, branched-chain amino acids; BCAT, branched-chain aminotransferase; BCKA, branched-chain α-keto acids; BCKDH, branched-chain α-keto acid dehydrogenase; BCKDC, BCKDH complex.

The balance between these pathways appears to depend on both substrate availability and cellular context, with cancer cells exhibiting a tendency toward anabolic routing, whereas non-cancerous cells demonstrate more complete oxidative utilization. Although the present study focused primarily on extracellular metabolite dynamics and BCKDHA protein expression, the observed patterns strongly suggest differential regulation of BCAA catabolic pathways in cancer and non-cancer cells, and further studies integrating metabolic flux analyses will help to clarify the underlying mechanisms. From a translational perspective, the observation that elevated circulating BCAA levels precede the diagnosis of pancreatic cancer by several years [7,30] suggests that systemic alterations in amino acid metabolism may occur early during tumor development. Our results indicate that increased BCAA availability does not uniformly stimulate cancer growth but instead produces dose-dependent and cell-specific effects, which may help explain the complex relationship between circulating amino acid levels and cancer risk. Following cellular uptake, branched-chain amino acids undergo transamination by branched-chain amino acid transaminase (BCAT) to generate branched-chain keto acids (BCKA) and glutamate, forming a metabolic decision point between anabolic nitrogen utilization and oxidative mitochondrial catabolism. BCKA may be further oxidized by the branched-chain α-keto acid dehydrogenase complex (BCKDC), whose E1 subunit is encoded by BCKDHA, producing acyl-CoA derivatives that enter the tricarboxylic acid cycle. Our findings are consistent with the hypothesis that cancer cells may channel BCAA-derived metabolites toward anabolic processes, whereas non-cancerous keratinocytes show patterns suggestive of greater oxidative utilization. Direct validation of this hypothesis will require metabolic flux analyses in follow-up studies. The balance between these pathways is cell-type specific and depends on BCAA availability, with dose-dependent effects observed particularly under high-concentration amino acid mixtures.

5. Study limitations

The present study has several limitations that should be considered when interpreting the results. All three cell lines were cultured in unified MEM rather than the media recommended by ATCC. While this choice was necessary to standardize the baseline amino acid environment, it may not represent optimal growth conditions for individual lines and could influence their baseline metabolic phenotypes. HaCaT cells, although widely used as a non-cancerous reference line, are immortalized and therefore do not fully represent normal epithelial physiology. The use of a single cell line per cancer type also limits the generalizability of our findings to broader tumor populations.

Viable cell numbers were assessed by Trypan blue exclusion, a method that reflects loss of plasma membrane integrity but does not detect early apoptotic or cytostatic responses. Complementary assays such as MTT or Annexin V/PI staining would provide a more comprehensive picture of cellular responses to BCAA supplementation. In a similar way, BCKDHA protein expression was measured as a proxy for BCKDC capacity, but it does not directly reflect enzymatic activity, which is additionally regulated by post-translational mechanisms. Future studies incorporating direct activity assays and isotope tracer-based flux analyses (e.g., ^{13}C-leucine tracing) would substantially strengthen mechanistic interpretation.

It is also worth noting that, although mTOR signaling and the LAT1 transporter are discussed in the context of our observations, these pathways were not directly assessed in our experimental system and remain hypotheses to be tested in follow-up studies. Finally, the BCAA concentrations used (1.5x and 2x above standard MEM) represent moderate elevations consistent with postprandial and infusion-induced fluctuations reported in humans, but do not fully reproduce the dynamic metabolic environment encountered by tumor cells in vivo.

6. Conclusions and future directions

This study demonstrates that the effects of branched-chain amino acids on cellular metabolism and proliferation are strongly dependent on concentration, amino acid composition and cell type. Moderate increases in BCAA availability promote proliferation, whereas higher concentrations, particularly amino acid mixtures, may exert inhibitory effects in cancer cells. These findings indicate that BCAA do not act solely as growth-promoting nutrients but may influence cellular metabolism in a context-dependent manner. The observed differences between lung and pancreatic cancer cells confirm the metabolic heterogeneity of tumors and suggest that individual cancer types may adopt distinct metabolic strategies in response to nutrient availability. A key observation is the generally lower expression of the BCKDC complex in cancer cells compared with non-cancerous keratinocytes, which is consistent with a possible preference for anabolic nitrogen metabolism over oxidative energy production. This interpretation remains to be validated by direct flux and enzymatic activity analyses.

Understanding how cancer cells regulate BCAA metabolism may provide new insight into metabolic reprogramming and potential metabolic vulnerabilities associated with cancer progression. Further investigations integrating metabolic flux analysis, regulatory mechanisms of the BCKDC complex and additional tumor models will be necessary to clarify the biological and clinical relevance of these pathways, including their potential role in systemic metabolic alterations and cancer-associated cachexia.

Appendix

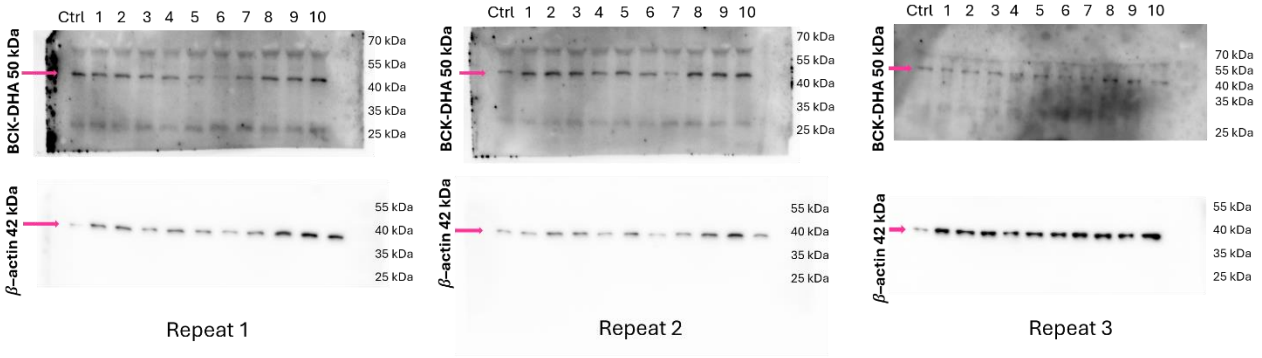

Fig. A1. Western blot analysis - A549

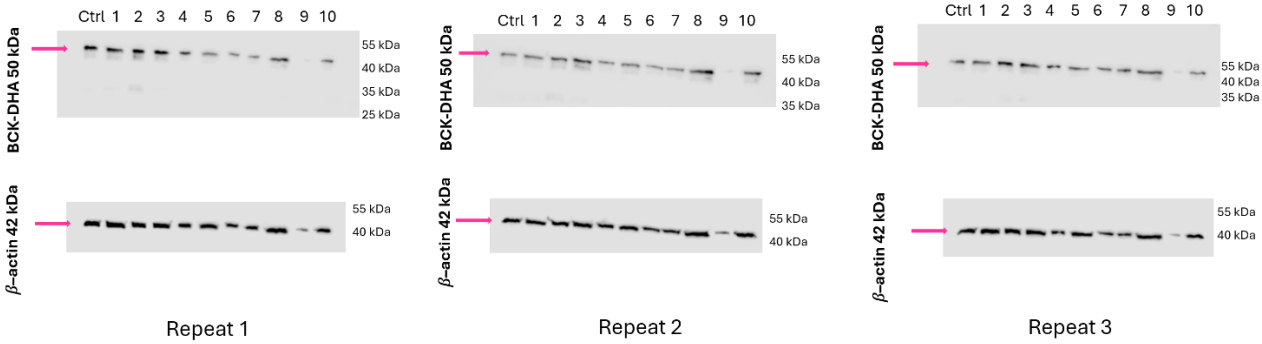

Fig. A2. Western blot analysis - HaCaT

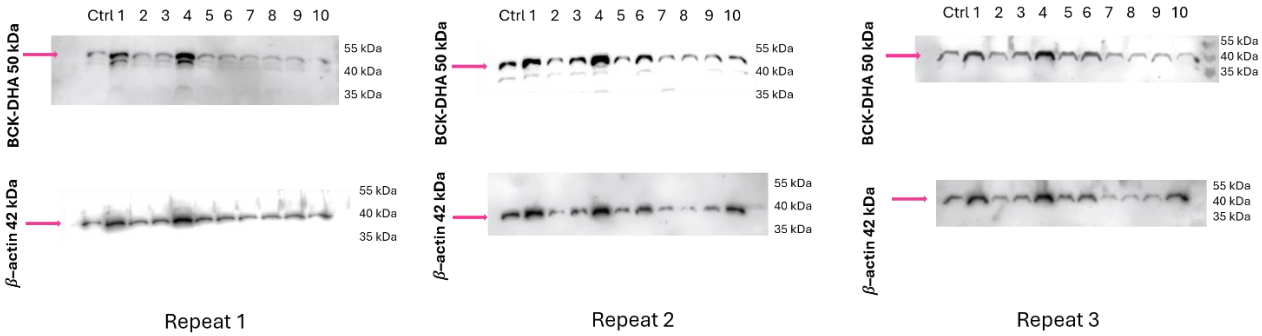

Fig. A3. Western blot analysis - MIA PaCa

Abbreviations: AA - amino acid; BCAA - branched-chain amino acid(s); BCAT - branched-chain aminotransferase; BCKA - branched-chain α-keto acid(s); BCKDC - branched-chain α-keto acid dehydrogenase complex; BCKDHA - E1α subunit of BCKDC; FBS - fetal bovine serum; HPLC - high-performance liquid chromatography; Ile/I - isoleucine; LAT1 - large neutral amino acid transporter 1; Leu/L - leucine; mTOR - mechanistic target of rapamycin; MEM - minimum essential medium; NSCLC - non-small cell lung cancer; PDAC - pancreatic ductal adenocarcinoma; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis; Val/V - valine.

Data Availability: All data generated or analyzed during this study are included in this published article. Any additional inquiries can be directed to the corresponding author.

Ethics Statement: Ethical approval was not required for this study, as it was conducted exclusively on established human cell lines obtained from ATCC and did not involve human participants or animals.

Author Contributions: Conceptualization, J.Ch. and W.G.; methodology, J.Ch., W.G. and S.G.; formal analysis, J.Ch. and W.G.; investigation, J.Ch., M.L., A.Z., B.P. and S.G.; writing—original draft preparation, J.Ch. and W.G.; writing—review and editing, J.Ch., W.G., M.S. and A.C.-J.; supervision, A.C.-J.; funding acquisition, J.Ch. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable. This study was conducted exclusively on established human cell lines obtained from ATCC and did not involve human participants.

Conflicts of Interest: The authors declare no competing interests.

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