

Expression pattern of glutaminase informs the dynamics of glutamine metabolism

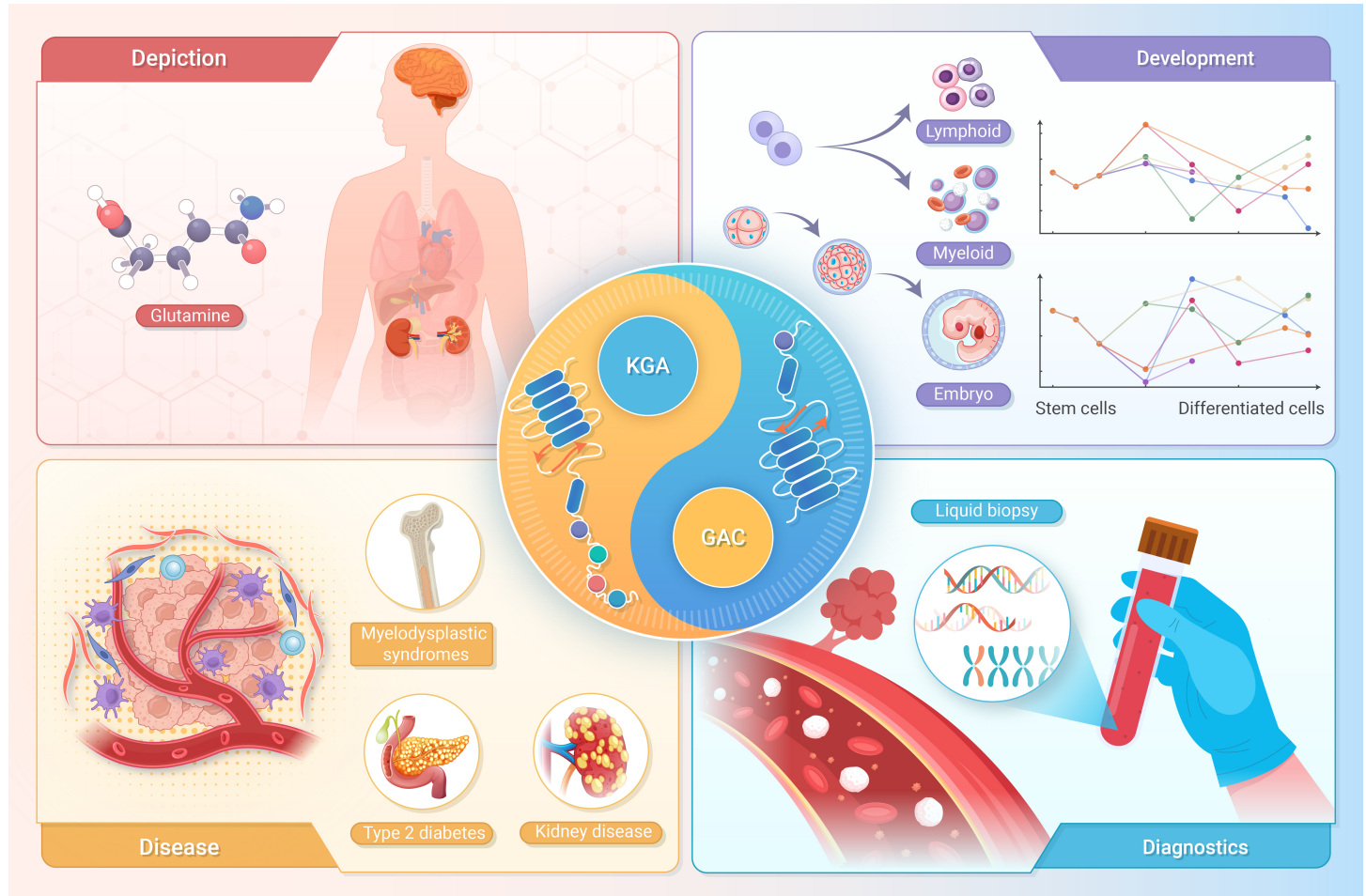
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- We propose a universal approach to quantify glutamine metabolism via glutaminase expression patterns.
- We reveal key timings and insights into glutamine metabolism dynamics in development, diseases, and cancer.
- Altered glutamine metabolism in cancer patients' plasma (PBMcs, platelets) aids noninvasive diagnosis.
- Glutaminase expression patterns track glutamine metabolism dynamics, demonstrating translational value.

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Glutamine is an essential nutrient that plays critical roles in both normal physiological processes and disease pathogenesis. In this study, we introduce a quantitative approach to trace the dynamics of glutamine metabolism by monitoring the expression patterns of glutaminase (GLS) and its two splicing isoforms, whose coding products exhibit distinct catalytic activities. This approach can be seamlessly integrated with quantitative PCR (qPCR), conventional bulk RNA sequencing, and emerging single-cell RNA sequencing technologies. We identified key temporal dynamics of glutamine metabolism during embryonic development and stem cell differentiation. Additionally, we characterized alterations in glutamine metabolism across various disease states and in response to drug treatments. In cancer, glutamine metabolism is highly variable across tumor types and is associated with T-cell exhaustion within the tumor microenvironment, as well as the metastatic potential of circulating tumor cells. Furthermore, aberrant glutamine metabolism was detected in various peripheral blood analytes from cancer patients, suggesting the potential for non-invasive cancer diagnosis. Our findings demonstrate the utility of quantitative glutamine metabolism tracing in investigating cellular processes, disease mechanisms, therapeutic responses, and its potential application in translational medicine.

INTRODUCTION

Glutamine is the most abundant free amino acid in the human blood and plays a pivotal role in numerous metabolic processes. In mammalian cells, glutamine is catabolized by glutaminases to produce glutamate and ammonia, which enter the citric acid cycle, serving as a critical energy source.¹ Beyond its role as a nutrient, glutamine also functions as a key regulator of gene expression, signaling pathways, and cellular stress responses, making it indispensable for maintaining homeostasis and adapting to physiological challenges.² For example, glutamine supports the proliferation and activity of lymphocytes and macrophages, enhancing the body's immune defense against pathogens.³ Consequently, glutamine is much more than a simple nutrient, with its metabolism playing a central role in human health.

In cell biology, the rate-limiting step in glutamine metabolism is its conversion to glutamate (i.e., glutaminolysis), a process catalyzed by glutaminases (primarily encoded by the GLS gene in human). The regulation of glutamine metabolism in humans involves complex interactions between hormones and stress signals. Disruption of this metabolic pathway is implicated in various pathological conditions, ranging from metabolic disorders to cancer. For instance, glutamine influences insulin sensitivity and glucose homeostasis,⁴ underscoring its relevance in metabolic disorders. In severe illnesses such as sepsis, the heightened demand for glutamine leads to depletion of plasma glutamine; if not adequately replenished, this depletion would impair immune function.⁵ Moreover, malignant tumors exhibit increased uptake and utilization of glutamine, a hallmark of the "Warburg effect", to support rapid proliferation and growth.^{6,7}

Currently, targeting glutamine metabolism or glutaminase has emerged as a promising therapeutic target for various diseases, including non-alcoholic fatty liver disease (NAFLD),^{8,9} Alzheimer's Disease (AD),¹⁰ myelodysplastic syndromes (MDS),¹¹ and cancer.¹²⁻¹⁴ For instance, in the context of NAFLD, glutaminase inhibitors show potential to enhance fatty acid oxidation capacity and regulate proline metabolism to alleviate nonalcoholic steatohepatitis in the liver.⁹ In cancer, a previous study demonstrated that prostate cancer tumors develop resistance to hormonal therapy by altering their glutamine metabolism, and therapeutic strategies targeting these metabolic alterations showed improved efficacy.¹⁵ Moreover, plenty of small molecule inhibitors targeting glutaminase, such as CB-839 and compound 968, have been developed and are currently under clinical trials. For instance, CB-839 can be utilized in combination with anti-PD1 or anti-CTLA4 antibodies to promote the infiltration of effector T cells into tumors by upregulating IFN γ -associated gene expression, thereby enhancing the antitumor activity of these checkpoint inhibitors;¹⁶ additionally, compound 968 inhibits ovarian cancer cell proliferation while enhancing granzyme B secretion in CD8⁺ T cells.¹⁷ Together, these studies highlight the translational significance of glutamine metabolism and glutaminase as potential therapeutic targets.

Given the central role of glutamine metabolism in health, disease, and treatment, understanding its regulatory mechanisms and dynamics is critical for advancing biomedical research. However, conventional mass spectrometry-based approaches to studying glutamine metabolism are low-throughput, costly, and cannot be integrated with emerging single-cell technologies. Thus, in this study, we propose a quantitative approach to measure glutamine metabolism by modeling the expression pattern of glutaminase as a surrogate marker. We have evaluated this approach across a range of biological systems to validate its applicability and uncover novel insights into the role of glutamine metabolism in diverse biological processes and diseases.

MATERIALS AND METHODS

Sample processing and RNA-seq experiments

For each subject, 10 mL peripheral blood was collected using EDTA-containing tubes and centrifuged at 1,600 g, 4°C for 15 min, and then the blood cells were harvested and stored in liquid nitrogen until use. For bulk RNA-seq library preparation, total RNA was extracted from 1-2 million peripheral blood mononuclear cells (PBMCs) using TRIzol (Thermo, #15596018) and Direct-zol RNA miniPrep kit (Zymo, #R2052). The concentration and quality of RNA was analyzed on a Qubit fluorometer and an Agilent 2100 Bioanalyzer, respectively. 1 μ g total RNA was used for rRNA depletion according to instructions of the Ribo-off rRNA Depletion Kit (Vazyme, #N406-01). The rRNA-depleted RNA was used to construct a cDNA library following a protocol provided by VAHTS Universal V8 RNA-seq Library Prep Kit for MGI (Vazyme, #NRM605-01). The libraries were circularized (Vazyme, #NM201-01) and sequenced on a MGISEQ-2000 sequencer (MGI) in paired-end 100 bp mode.

Table 1. List of primers used in this study.

Target	Forward sequence	Reverse sequence
GLS	TGCATTCTGTGGCATGTAT	TTGCCATCTTATCCAGAGG
KGA	ACTGGAGATGTGTCTGCACTTCGAAG	CCAAAGTGCAGTGCTTCATCCATGGGAGTG
GAC	TTGGACTATGAAAGTCTCCAACAAGAAC	CCATTCTATATACTACAGTTGTAGAGATGCTCTC
ACTB	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG

Cell culture and RT-qPCR assays

HCT116 cells were cultured in RPMI-1640 medium (Thermo, #C11875500BT) supplemented with 10% FBS (Sigma-Aldrich, #F0193) at 37°C in a humidified 5% CO₂ atmosphere. We treated HCT116 cells with CPT-11, MG132, and DMSO (as control) for 0, 12, 24, and 48 hours. After treatment, cells were harvested and total RNA was extracted using TRIzol (Thermo, #15596018) and Direct-zol RNA miniPrep kit (Zymo, #R2052), and 1 µg of total RNA was reversed transcribed using HiScript III All-in-one RT SuperMix Perfect for qPCR (Vazyme, #R333-01). Quantitative real-time PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, #Q711-03) in triplicates on a QuantStudio5 real-time PCR system (Applied Biosystems). The threshold cycle (Ct) values obtained from different samples were compared using the $2^{-\Delta\Delta C_t}$ method with ACTB gene serving as internal reference. All experiments were repeated three times. The primers were listed in Table 1.

RNA-seq data analysis

For all bulk RNA-seq data, raw sequencing reads were first preprocessed to remove adapter and low-quality cycles using Ktrim software;¹⁸ PCR duplicates were removed using in-house programs.¹⁹ RSEM software was used to quantify gene- and isoform-level expressions against the RefSeq genes;^{20,21} samples with total expression of GLS higher than 1 TPM were picked up to calculate log2-scaled KGA/GAC ratio with a fix of 0.1 TPM. ScRNA-seq data generated using Smart-seq protocol was processed using the same pipeline as the bulk RNA-seq data, and cell type annotations were obtained from the corresponding studies. For scRNA-seq data generated using the 10x platform, the "count" command in cellranger pipeline (10x) was used to split the reads from each single-cell using barcodes, align the reads to human reference genome, and perform gene-level quantifications for each single-cell. The Seurat package was used to process the scRNA data (for both Smart-seq and 10x datasets),²² including data normalization, dimension reduction (using the UMAP algorithm), and clustering with default parameters. For studies with multiple scRNA-seq experiments, the samples were further integrated using Harmony package.²³ Cell clusters with less than 100 cells were discarded from downstream analyses. For human hematopoiesis dataset, cell type annotation was obtained from the Human Cell Atlas bone marrow single-cell interactive web portal,²⁴ and then served as reference to annotate the MDS dataset using SingleR package.²⁵ For the rest datasets generated using 10x platform, cell type annotations were either directly obtained from the corresponding studies or based on the marker genes used in the corresponding studies. For each annotated cell type in dataset generated using 10x platform, we extracted and counted the UMIs (Unique Molecular Identifiers) uniquely mapped to KGA or GAC isoforms from all the single-cells in this type, then a fix of 1 was added to calculate the log2-scaled KGA/GAC ratio.

GLS expression analysis in tumors

For each cancer type, we first extracted the GLS gene expression and KGA/GAC ratios in the adjacent normal tissues; then we used the Inter-Quartile Range (IQR) method to filter out outliers and determined the normal range of GLS gene expression and KGA/GAC ratios (i.e., the "box"). The tumors were divided into two groups based on whether their GLS gene expression and KGA/GAC ratios were located within the "box" or not. Hence, the tumors out of the "box" were considered to suffer from aberrant glutamine metabolism. Then, survival analysis was performed between the patients whose tumors were out of the "box" versus those within the "box" using Kaplan-Meier method, and a Cox regression was established to determine

the proportional hazards; a P-value lower than 0.05 obtained from Log-rank test was considered as statistically significant.

Statistical analysis

Kruskal-Wallis tests were used to determine whether there are statistically significant differences among three or more independent groups. Mann-Whitney U tests were used to determine whether there are statistically significant differences between two independent groups. For 10x scRNA-seq datasets, GLS expressions between two conditions were compared using DEsingle software (which reported adjusted P-values using Benjamini & Hochberg method);²⁶ Chi-Squared tests were used to compare KGA/GAC ratios. A P-value lower than 0.05 was considered as statistically significant.

RESULTS

Glutamine metabolism profiles in human tissues and cell lines

Human cells express two glutaminases: GLS (also known as GLS1) and GLS2. For this study, we focused on GLS as it is ubiquitously expressed in most tissues and cell types, while GLS2 is predominantly found in the liver. The human GLS gene consists of two major isoforms: KGA (kidney-type glutaminase) and GAC (glutaminase C), which differ in their terminal exons (Figure 1A). These isoform-specific exons allow for differentiation between KGA and GAC using PCR assays, conventional whole transcriptome sequencing (RNA-seq), or mainstream single-cell RNA-seq platforms, such as Smart-Seq and the 10x Chromium platform with 3' reagent kits.^{27,28} Notably, both KGA and GAC isoforms are highly conserved across many species, including the house mouse, a commonly used model organism (Figure S1). Previous studies had demonstrated that GAC exhibits much higher catalytic activity than KGA,^{29,30} suggesting that glutamine metabolism may alter if the KGA/GAC balance is disrupted. Hence, we utilized the overall expression of the GLS gene and the ratio between the KGA and GAC isoforms (i.e., the KGA/GAC ratio, log2-scaled such that positive values indicate KGA dominance, while negative values indicate GAC dominance) as a 2-dimensional, quantitative measure of GLS expression patterns to investigate glutamine metabolism.

Using this approach, we initially investigated normal human tissues profiled in the GTEx project,³¹ which includes 30 tissue types from 10 organ systems. As shown in Figures 1A-C, GLS gene expression levels exhibited significant variability among different tissues and organ systems, ranging from approximately 4 TPM (transcripts per kilobase million) in the liver to 88 TPM in the artery. In contrast, KGA/GAC ratios were relatively stable and close to zero in most tissues (indicating balanced expression of KGA and GAC), except in the brain and kidney, where KGA expression predominated. We then examined primary cells and cell lines profiled in the ENCODE project.³² As shown in Figure 1D, the expression patterns were similar to those observed in tissues, with cancer cell lines exhibiting significantly negative KGA/GAC ratios ($P < 10^{-10}$, t-test), suggesting a shift toward GAC dominance in these cells.³³

Glutamine metabolism in development and differentiation

Considering that glutamine metabolism is crucial for embryonic development and differentiation,³⁴⁻³⁶ we next investigated GLS expression patterns during these processes. In early human embryo development, both GLS gene expression and the KGA/GAC ratio peaked at the 8-cell stage, as validated by two time-series datasets (Figures 2A & S2A-C).^{37,38} A similar pattern was observed in mouse embryos,³⁹ where both GLS gene expression and the KGA/GAC ratio peaked at the late 2-cell stage (Figures 2B & S2D-E). These findings coincide with the timing of zygotic genome activation, marking the

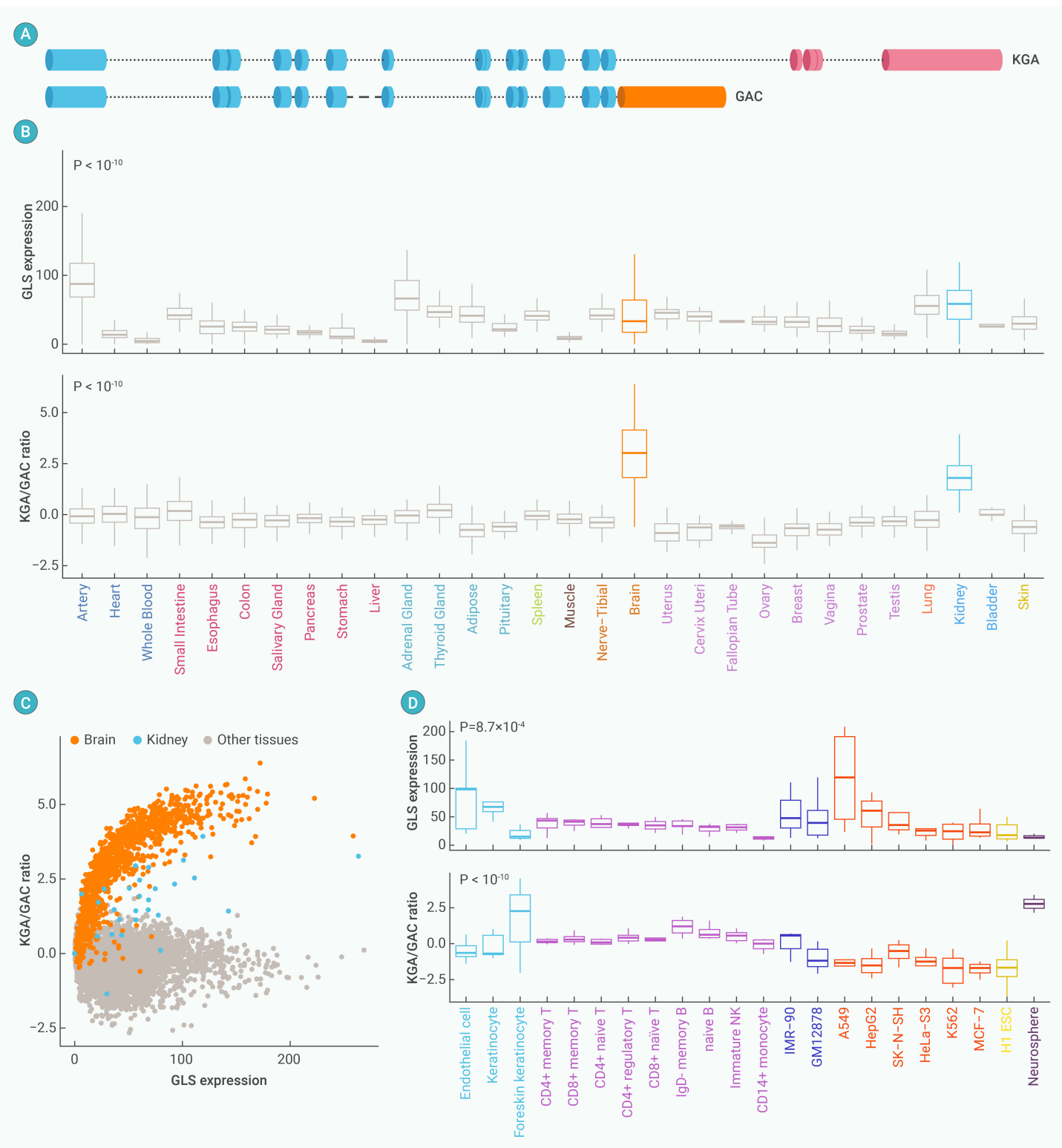


Figure 1. Glutamine metabolism patterns in human tissues and cell lines (A) Illustration of exon-intron structure of KGA and GAC isoforms of GLS gene. (B,C) GLS expression patterns (including GLS gene expression and log2-scaled KGA/GAC ratio) in human tissues, (D) primary human cells and cell lines. In (B,D), P-values were calculated using Kruskal-Wallis tests. In (C), each dot represents one sample.

loss of totipotency in human and mouse embryos at the 8- and 2-cell stages, respectively.⁴⁰⁻⁴² During subsequent embryonic organ development in mice, GLS gene expression exhibited two peaks at the E12.5 and E14.5 timepoints, while the KGA/GAC ratio gradually increased across most organs. Notably, the brain switched from GAC- to KGA-dominant expression at E13.5 (Figure 2C). In human placental tissue,⁴³ a GAC-dominant pattern was observed in both extravillous trophoblasts and cytotrophoblasts ($P < 10^{-10}$, t-test; Figure 2D). Moreover, GLS gene expression significantly increased, while

the KGA/GAC ratio significantly decreased in third-trimester extravillous trophoblasts compared to the first trimester. In contrast, no significant changes in GLS expression were observed in cytotrophoblasts, suggesting cell type-specific regulation of glutamine metabolism during placental development.

We next analyzed glutamine metabolism dynamics during stem cell differentiation. In hematopoiesis, a widely studied model system of stem cell differentiation,²⁴ distinct dynamics in GLS expression were observed as

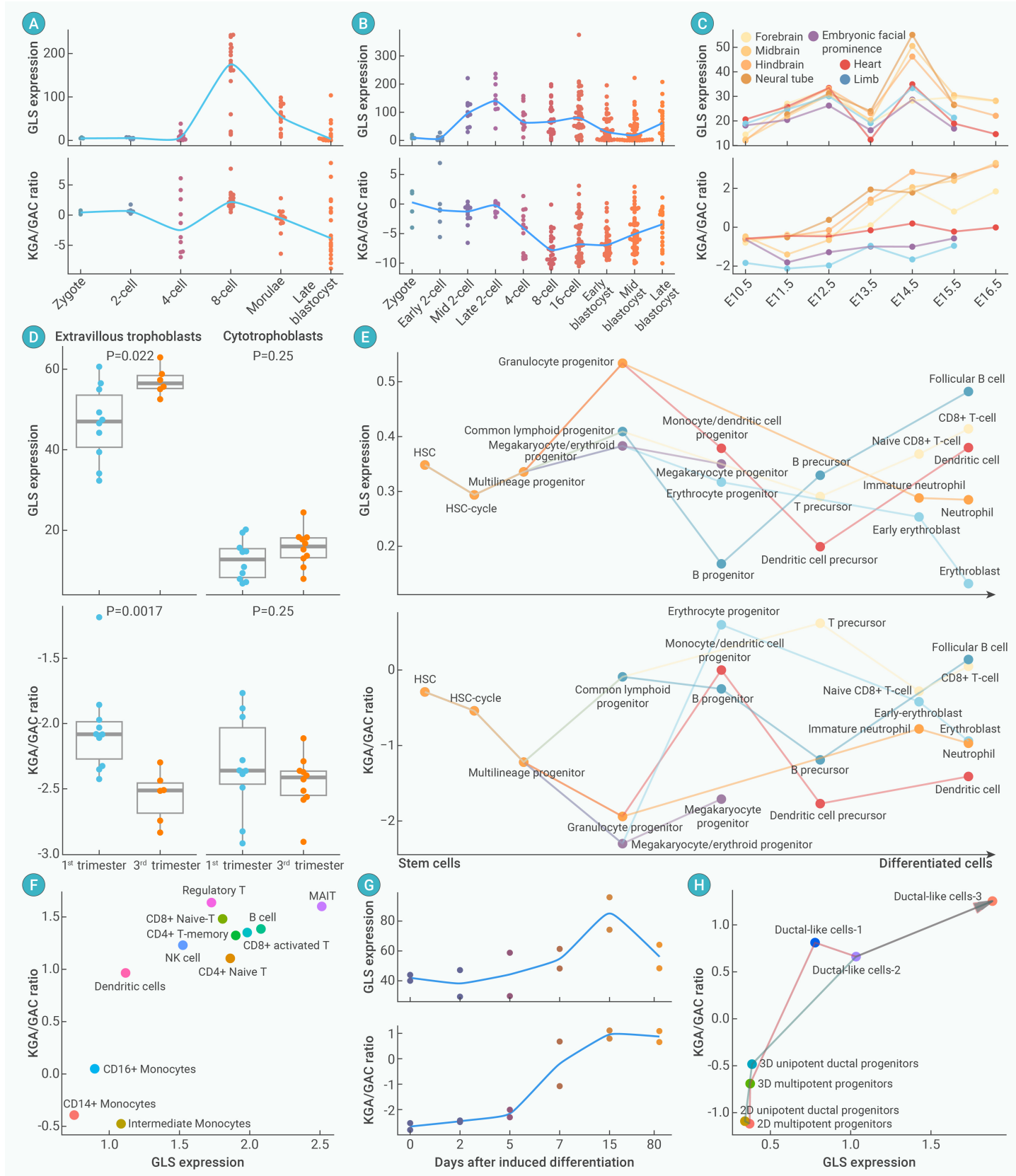
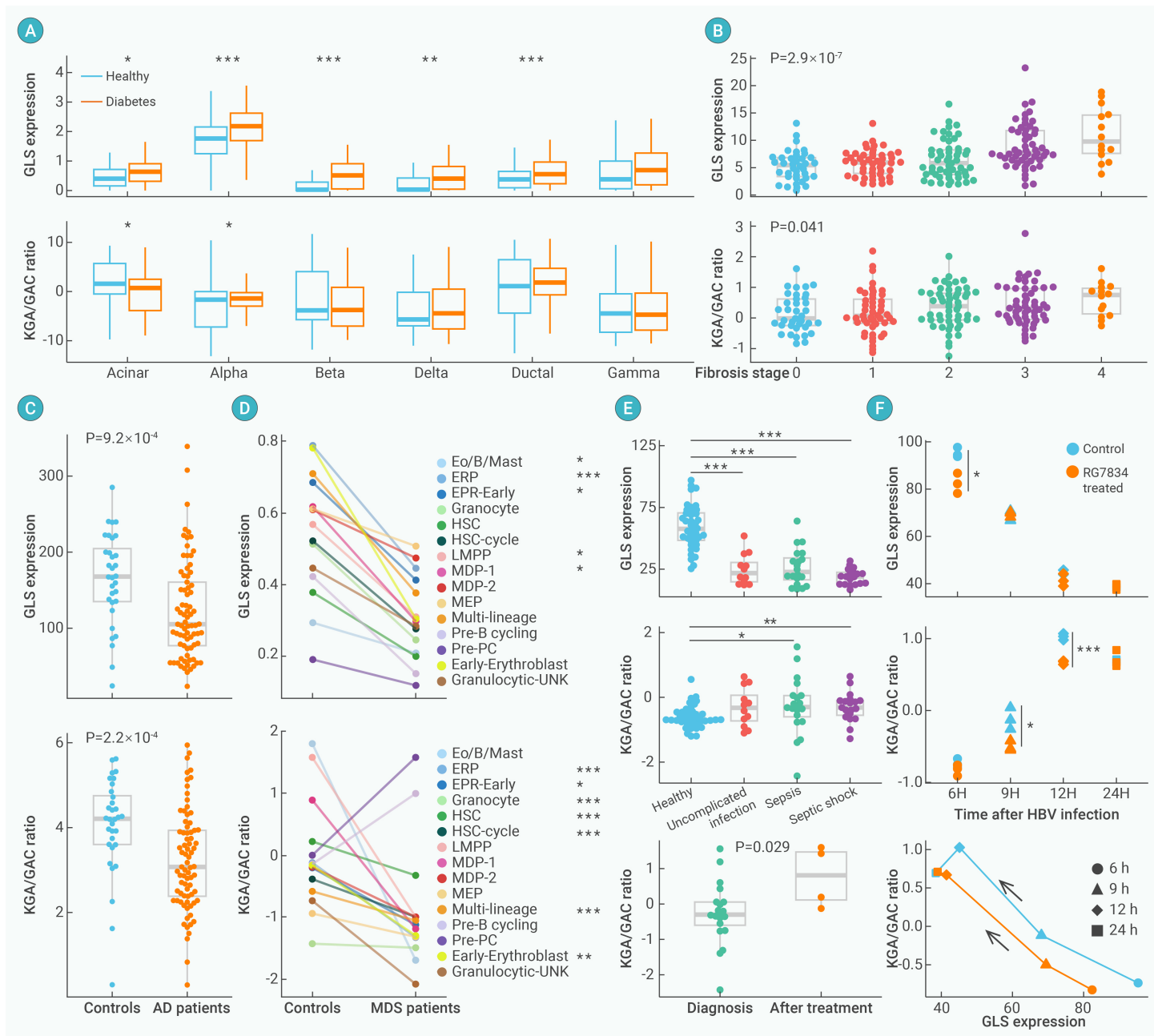


Figure 2. Glutamine metabolism dynamics in development and differentiation GLS expression patterns (A) during human and (B-C) mouse embryonic development, (D) in two types of trophoblast cells in placentae during gestation, (E) during hematopoietic differentiation and (F) in differentiated blood cells, (G) during cardiomyocyte differentiation, and (H) during pancreatic differentiation. In (A,B,D,G), each dot represents one sample, and the lines in (A,B,G) represent mean values for each time point. HSC: hematopoietic stem cell; MAIT: mucosal associated invariant T cell. In (D), P-value was calculated using Mann-Whitney U test.

CD34⁺ multi-lineage stem cells committed to myeloid or lymphoid lineages. A further notable change occurred when progenitor cells differentiated into premature cells (Figures 2E & S3). These dynamics were confirmed in a second

hematopoiesis transcriptome dataset (Figure S4).⁴⁴ Additionally, in differentiated hematopoietic cells, such as peripheral blood mononuclear cells (PBMCs), variations in both GLS gene expression and the KGA/GAC ratio



were observed across cell types, particularly between lymphocytes and granulocytes (Figures 2F & S5), suggesting lineage- and maturation-dependent glutamine metabolism dynamics. In a model of human embryonic stem cell (hESC) differentiation into cardiomyocytes,⁴⁵ both GLS gene expression and the KGA/GAC ratio increased gradually from day 0 (hESC) to day 15 (primitive cardiomyocytes), with a shift from GAC-dominant to KGA-dominant expression by day 7. As the primitive cardiomyocytes further differentiated into ventricular cardiomyocytes (day 80), GLS expression decreased while the KGA/GAC ratio remained relatively stable (Figure 2G), indicating a stage-specific role of glutamine metabolism in cardiac differentiation.⁴⁶ In a model of pancreatic progenitor differentiation using an organoid system,⁴⁷ both GLS gene expression and the KGA/GAC ratio increased gradually during differentiation (Figures 2H & S6). Notably, pancreatic progenitors differentiated into three types of ductal-like cells, each exhibiting distinct GLS expression

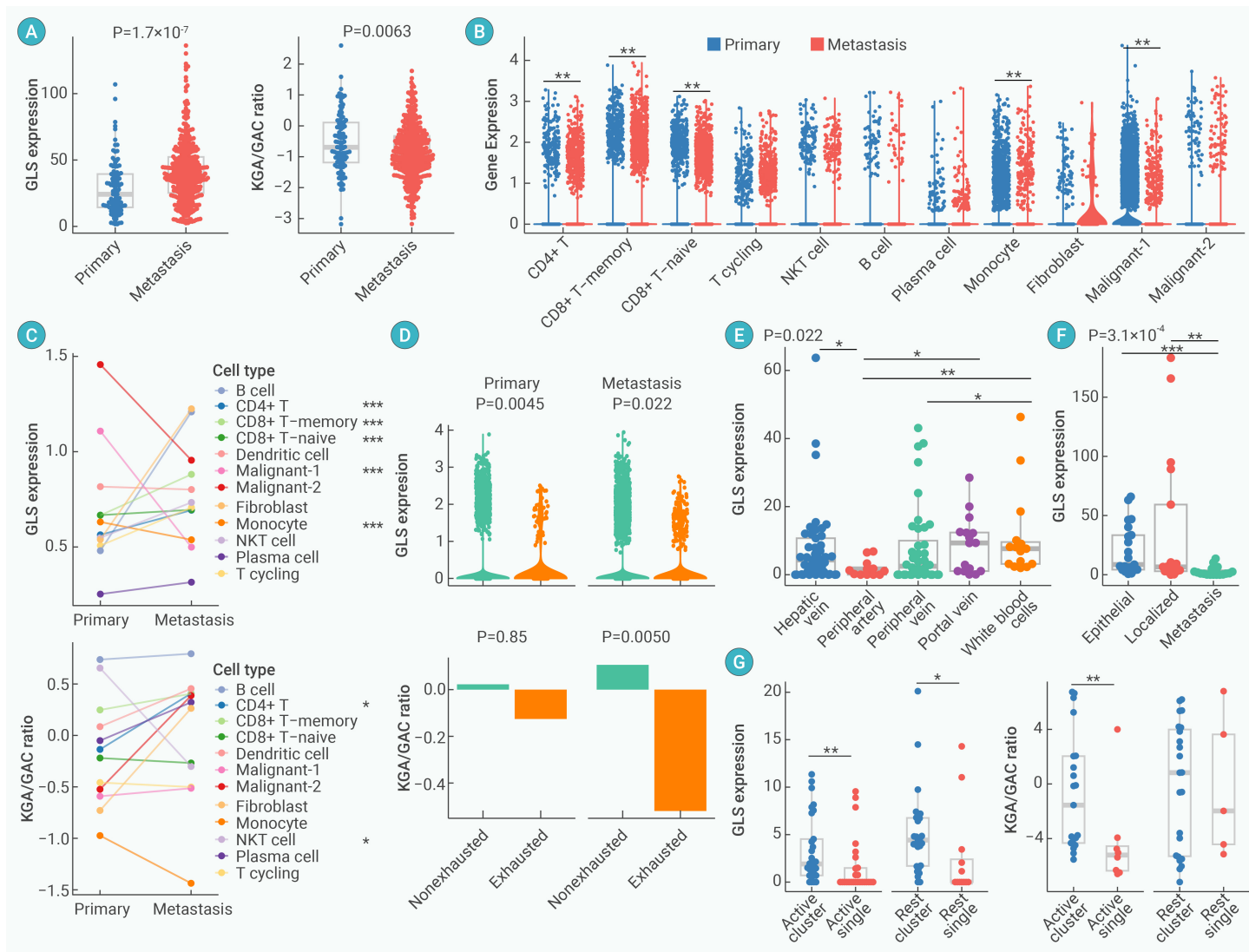
patterns, suggesting diverse glutamine metabolism profiles in these differentiated cell types.

Glutamine metabolism in human diseases

We then explored glutamine metabolism dynamics in various diseases.⁴⁸ First, we examined type II diabetes, a common metabolic disorder. Among six major pancreatic cell types,⁴⁹ GLS gene expression was significantly elevated in five of them in diabetic patients. Moreover, the KGA/GAC ratio was significantly decreased in acinar cells while increased in alpha cells (Figures 3A & S7). In non-alcoholic fatty liver disease (NAFLD),⁵⁰ a leading cause of cirrhosis and hepatocellular carcinoma (HCC), both GLS gene expression and the KGA/GAC ratio significantly increased with disease progression (Figure 3B). In contrast, GLS2, the other glutaminase expressed in the liver, showed no significant changes (Figure S8), suggesting distinct roles for GLS and GLS2 in



Figure 4. Glutamine metabolism in cancer (A) GLS gene expressions between tumors and adjacent normal tissues in pan-cancer. (B) Illustration of GLS expression patterns in tumors in kidney renal clear cell carcinoma (KIRC). The box indicated the normal range of GLS expression patterns in adjacent normal tissues, and tumors out of the box were considered as suffering from dysregulated, or aberrant, glutaminase metabolism. (C) Proportions of tumors with aberrant glutaminase metabolism among cancer types in TCGA. (D) Survival analysis between KIRC patients with aberrant or normal glutaminase metabolism. (E) GLS expression patterns in HCT116 cells after drug treatment. Two drugs were utilized. (F) GLS expression patterns between DU145 cells sensitive or resistant to paclitaxel. (G) GLS expression patterns A549 cells after methylmalonic acid (MMA) treatment. BLCA: bladder urothelial carcinoma; BRCA: breast invasive carcinoma, which includes infiltrating duct carcinoma (ILC) and lobular carcinoma (LIC); CHOL: cholangiocarcinoma; COAD: colon adenocarcinoma; ESCA: esophageal carcinoma; HCC: hepatocellular carcinoma; KICH: kidney chromophobe; KIRC: kidney renal clear cell carcinoma; KIRP: kidney renal papillary cell carcinoma; LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; PRAD: prostate adenocarcinoma; READ: rectum adenocarcinoma; STAD: stomach adenocarcinoma; THCA: thyroid carcinoma; UCEC: uterine corpus endometrial carcinoma; HR: hazard ratio. In (B,F,G), each dot represents one sample. In (A,F), P-values were calculated using Mann-Whitney U tests; in (D) P-value was calculated using Cox regression; in (E,G), P-values were calculated using t-tests. *: P < 0.05; **: P < 0.01; ***: P < 0.001.



hepatic metabolism.^{33,51} In Alzheimer's disease (AD),⁵² the most common cause of dementia in the elderly, both GLS gene expression and the KGA/GAC ratio were significantly reduced in the fusiform gyrus (Figure 3C), consistent with glutamatergic synaptic dysfunction in AD.⁵³ Interestingly, no significant differences were observed in the dorsolateral prefrontal cortex (Figure S9), suggesting region-specific dysregulation of glutamine metabolism in AD. In myelodysplastic syndromes (MDS),⁵⁴ which are characterized by ineffective hematopoiesis and clonal instability,^{55,56} both GLS gene expression and the KGA/GAC ratio were significantly decreased in multiple bone marrow cell types compared to controls (Figures 3D & S10).

Finally, we examined infection, a common disease trigger that induces metabolic changes in both the immune system and host cells.⁵⁷ During bacterial infection (Figure 3E),⁵⁸ GLS expression was significantly decreased while the KGA/GAC ratio increased in PBMCs. Notably, in patients with sepsis, the KGA/GAC ratio was even higher after treatment. In another model,⁵⁹ HepaRG cells, a bipotent progenitor cell line capable of differentiating into biliary- and hepatocyte-like cells, were infected with Hepatitis B Virus (HBV). After infection, GLS gene expression decreased significantly, while the KGA/GAC ratio increased (Figure 3F). Treatment with RG7834, an anti-HBV dihydroquinolinone compound, altered both GLS expression and the KGA/GAC ratio compared to untreated cells, suggesting that antiviral drug

treatment can modulate glutamine metabolism during HBV infection.

Glutaminase metabolism in cancer

Dysregulated glutamine metabolism is a well-established hallmark of cancer, though the specific aberrations exhibit substantial heterogeneity across different tumor types.^{1,60–62} To characterize GLS expression patterns in cancer, transcriptome data from 18 cancer types within The Cancer Genome Atlas (TCGA) were analyzed.⁶³ Overall, GLS expression was significantly altered in most cancer types (Figure 4A). GLS gene expression was upregulated in 8 cancer types and downregulated in another 8; interestingly, the KGA/GAC ratio was elevated in only one cancer type but decreased in 10 others. To further explore the dynamics of glutamine metabolism in tumors, a "normal range" of GLS expression was defined based on non-cancerous adjacent normal tissues for each cancer type (Figure 4B). Tumors falling outside this normal range were presumed to exhibit dysregulated glutaminase metabolism. The proportion of such tumors varied widely across cancer types, ranging from approximately 20% in thyroid cancer (THCA) to nearly 100% in cholangiocarcinoma (CHOL; Figures 4C & S11). Prognosis analysis revealed that, in kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), and lung adenocarcinoma (LUAD), tumors outside the normal GLS expression range were associated with significantly worse

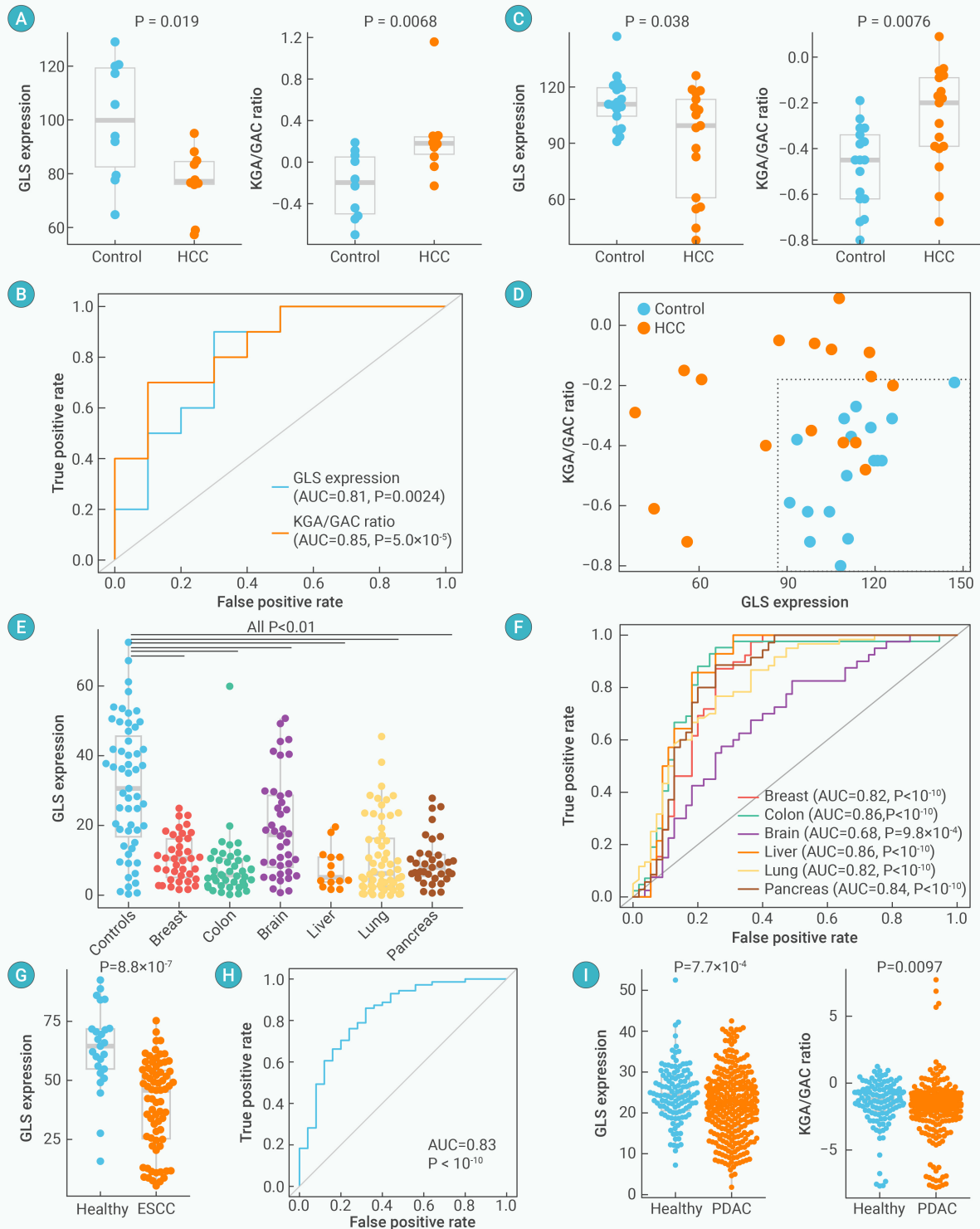


Figure 6. Glutamine metabolism in peripheral blood for non-invasive cancer diagnosis (A) GLS expression pattern in peripheral blood mononuclear cells (PBMCs) between Hepatocellular Carcinoma (HCC) patients and controls, and (B) corresponding Receiver Operating Characteristic (ROC) curves for diagnosis. (C) GLS expression pattern in PBMCs of HCC patients versus controls in an external dataset, and (D) cancer diagnosis through combination of GLS gene expression and KGA/GAC ratio. Blue and yellow dots represent control subjects and HCC patients, respectively. (E) GLS gene expression in platelets between cancer patients of various types and controls, and (F) corresponding ROC curves for diagnosis of each cancer type. (G) GLS gene expression in platelets between esophagus squamous cell carcinoma (ESCC) patients and controls, and (H) corresponding ROC curve for diagnosis. (I) GLS expression pattern in extracellular vesicles between pancreatic cancer (PDAC) patients and controls. AUC: Area under the ROC curves. In (A,C,D,E,G,I), each dot represents one sample. In (A,C,E,G,I), P -values were calculated using Mann-Whitney U tests; in (B,H), P -values were calculated using Z-tests.

overall survival compared to tumors within the normal range (Figures 4D & S12). These findings highlight the heterogeneity of glutaminase metabolism across cancers and suggest that dysregulated glutamine metabolism may influence prognosis in certain cancer types.

To investigate the effects of drug treatment and stimuli on glutamine metabolism in cancer, we treated HCT116 cells, a colorectal cancer (CRC) cell

line, with two commonly used drugs in CRC therapy: CPT-11 and MG132. RT-qPCR analysis revealed significant increases in both GLS gene expression and the KGA/GAC ratio following treatment, with distinct patterns observed between the two drugs (Figure 4E). In prostate cancer, treatment with docetaxel resulted in marked differences in GLS expression and the KGA/GAC ratio between docetaxel-sensitive and docetaxel-resistant DU145 cells

(Figure 4F).⁶⁴ In lung cancer, co-culture with methylmalonic acid (MMA) led to significantly elevated GLS expression and a reduced KGA/GAC ratio in A549 cells,⁶⁵ with more pronounced changes observed over longer treatment durations (Figure 4G). These changes may be associated with the tumor-promoting effects of MMA.⁶⁵

Next, we explored the dynamics of glutamine metabolism in cancer metastasis.⁶⁶ We first examined skin cutaneous melanoma (SKCM) data from the TCGA, which includes transcriptome data from both localized and metastatic tumors. As shown in Figure 5A, GLS expression was significantly elevated in metastatic tumors, while the KGA/GAC ratio was significantly decreased. Given that whole transcriptome sequencing captures a mixture of cell types (e.g., malignant, stromal, and immune cells),⁶⁷ we further analyzed single-cell RNA-seq data from colorectal carcinoma (CRC),⁶⁸ lung adenocarcinoma,⁶⁹ and pancreatic ductal adenocarcinoma (PDAC).⁷⁰ These datasets revealed significant alterations in GLS expression and the KGA/GAC ratio across various cell types, including malignant cells, immune cells, and fibroblasts (Figures 5B-C & S13-S15). Notably, exhausted PD1⁺LAG3⁺ CD8⁺ T-cells in primary and metastatic tumors exhibited markedly decreased GLS expression (Figure 5D).⁷¹ In metastatic tumors, exhausted T-cells switched from KGA- to GAC-dominant glutamine metabolism, suggesting that reprogrammed glutamine metabolism may influence immune responses in the tumor microenvironment.^{72,73} Additionally, we investigated circulating tumor cells (CTCs), key mediators of cancer metastasis, using scRNA-seq data from CTCs isolated from hepatocellular carcinoma (HCC) and PDAC patients.^{74,75} In HCC, CTCs from different anatomical locations exhibited variable GLS expression, differing significantly from white blood cells (Figure 5E). In PDAC, CTCs from metastatic patients showed significantly lower GLS expression compared to those from localized tumors or controls (Figure 5F). These findings suggest that changes in GLS expression in CTCs may be linked to their metastatic potential. Furthermore, studies have shown that CTCs in clusters exhibit higher metastatic potential.^{76,77} In a breast CTC-derived xenograft model, CTCs in clusters displayed higher GLS expression and KGA/GAC ratios compared to individual CTCs, with no significant differences between CTCs isolated during sleep or while awake (Figure 5G).⁷⁸ These results highlight the dynamic nature of glutamine metabolism in CTCs and its potential role in metastasis.

Glutamine metabolism patterns for non-invasive cancer diagnosis

Given that the immune system adapts its glutamine metabolism in response to various stimuli, particularly in cancer,^{72,73} we explored the potential of using glutamine metabolism dynamics in peripheral blood for non-invasive cancer diagnosis. We recruited 10 early-stage HCC patients and 10 age- and gender-matched controls (Table S1) and performed RNA-seq on their PBMCs to profile glutamine metabolism patterns. Significant decreases in GLS expression and increases in the KGA/GAC ratio were observed in HCC patients (Figure 6A). These changes enabled the differentiation of HCC patients from controls, as demonstrated by Receiver Operating Characteristic (ROC) analysis (Figure 6B). Validation in an independent PBMC dataset containing 17 HCC patients and 17 controls confirmed the findings (Figures 6C & S17);⁷⁹ moreover, combining GLS expression and the KGA/GAC ratio, we detected 12 out of 17 HCC samples (sensitivity = 70.6%, 95% confidence interval: 44.0-89.7%) with 100% specificity (Figure 6D).

To evaluate the broader applicability of glutamine metabolism dynamics in blood-based cancer diagnostics, we analyzed two platelet RNA-seq datasets. The first dataset, containing 228 samples from six cancer types and 55 controls,⁸⁰ showed significantly reduced GLS expression in all cancer types compared to controls (Figure 6E). ROC analysis demonstrated that GLS expression effectively distinguished cancer patients from controls, with area under the curve (AUC) values ranging from 0.68 to 0.86 across different cancers (Figure 6F). The KGA/GAC ratio exhibited an overall decreasing trend across cancer types, with significant differences observed in breast cancer (Figure S18). These findings were further validated in a second platelet RNA-seq dataset,⁸¹ which included 71 esophageal squamous cell carcinoma (ESCC) patients and 25 controls (Figures 6G-H & S19). In addition, altered GLS expression patterns were observed in extracellular vesicles from pancreatic cancer patients (Figure 6I), as analyzed in a dataset consisting of 284 PDAC patients and 117 controls.⁸² Together with the PBMC data, these

results underscore the high translational potential of glutamine metabolism dynamics in peripheral blood for non-invasive cancer diagnosis.

CONCLUSIONS

In summary, we propose and validate a quantitative approach for tracing the dynamics of glutamine metabolism. Given the widespread use of transcriptome profiling in biomedical research, this method provides a convenient and accessible tool for investigating glutamine metabolism dynamics in diverse biological processes and diseases, and for enhancing non-invasive cancer diagnostics.

DISCUSSIONS

In this study, we developed a quantitative method to trace the dynamics of glutamine metabolism by analyzing the expression pattern of the GLS gene, which encodes the major glutaminase in humans. We applied this method to various biological systems and diseases, demonstrating its utility by validating known physiological changes and uncovering novel biological insights. For instance, in most normal tissues, the KGA/GAC ratios were approximately 0, indicating a balanced expression of KGA and GAC; however, significantly elevated KGA/GAC ratios were observed in the brain and kidney (Figures 1B-C), suggesting a preference of KGA in these two tissues. Given the significantly distinct catalytic activities between KGA and GAC,²⁹ regulation of KGA/GAC ratio is crucial to prevent excess glutamate production and toxicity. Of note, the GAC isoform is recognized as excitotoxic within the brain: it can induce learning disabilities, synaptic dysfunction, neuroinflammation, and dementia.^{83,84} Similarly, increased expression of GAC is also associated with various kidney diseases, such as autosomal-dominant polycystic kidney disease (ADPKD),⁸⁵ which might elucidate the preference of KGA over GAC in the brain and kidney. In addition, characteristic GLS expression patterns were observed at key stages of early embryonic development (Figures 2A-B), specifically at the 8- and 2-cell stages in human and mouse embryos, respectively. These developmental stages coincide with zygotic genome activation onset and the loss of totipotency. In fact, murine 2-cell-like cells exhibit a distinct metabolic state compared to later-stage embryonic stem cells.⁸⁶ Biologically, glutamine metabolism plays a pivotal role in regulating the pluripotency transcription factor OCT4 as well as influencing pluripotent stem cell fate.^{87,88} Moreover, supplementation with glutamine in culture medium enhances human preimplantation embryo development *in vitro*, underscoring its significance during embryogenesis.⁸⁹ During brain development, silencing GLS in neuron progenitor cells results in a remarkable reduction of the neuronal marker microtubule-associated protein 2 (MAP-2) alongside decreased proliferating cell populations, suggesting an important regulatory role of GLS in neurogenesis.⁹⁰ Furthermore, during cell differentiation, significant changes in GLS expression were observed as stem cells transitioned into progenitor and precursor cells (Figures 2E-G), consistent with previous studies showing that glutamine metabolism regulates lineage allocation, myelomonocytic, erythroid differentiation, and lymphocyte activation during hematopoiesis.^{35,91-93} These findings underscore the critical role of glutamine metabolism in lineage determination during development and differentiation, suggesting that modulating glutamine metabolism could influence cell differentiation and reprogramming.

We also observed altered GLS expression patterns indicative of dysregulated glutaminase metabolism in several diseases, including type II diabetes, AD, MDS, as well as infections (Figure 3). Alterations in glutamine metabolism exhibit high heterogeneity across diseases, which are relevant to tissue type, underlying genetic factors, and microenvironment.^{5,48,94,95} In NAFLD, overexpression of GLS was detected in steatotic hepatocytes in both pre-clinical and clinical samples, indicating disrupted glutamine metabolism homeostasis; however, the pathophysiology remains incompletely understood; some studies suggested associations with urea cycle dysfunction and immune system alterations.^{8,96} In AD, glutamate-mediated toxicity has been implicated as a potential mechanism contributing to neuronal loss,^{97,98} within which GAC participates in pathogenesis through regulating microglial activation and pro-inflammatory exosome release,⁸⁴ therefore its up-regulation leads to the decrease of KGA/GAC ratio in AD (Figure 3C). Moreover, recent studies suggest that GLS plays a crucial role in MDS; however, the mechanisms underlying its dysregulation within MDS bone marrow remain elusive

and may be associated with imbalanced metabolites resulting from defective/mutant IDH1 enzyme in this disease.⁹⁹

Additionally, alterations in GLS expression were commonly observed in various cancers.¹⁰⁰ Interestingly, dysregulated glutamine metabolism exhibited substantial variability across cancer types, with approximately 20% of thyroid cancer cases and up to 100% of cholangiocarcinoma cases showing aberrant GLS expression. In breast and prostate cancers, the oncogenic transcription factor c-Myc up-regulates GAC, while not KGA.^{15,101} Besides c-Myc, other regulators, including c-Jun,¹⁰² RBF1,¹⁰³ and PGC-1alpha,¹⁰⁴ could also regulate GLS expression, suggesting that the dynamics of glutamine metabolism in tumors is associated with activation statuses of various regulators. Furthermore, tumors with dysregulated glutamine metabolism were associated with significantly worse prognosis in kidney and lung cancers. In response to drug treatment, malignant cells reprogrammed their glutamine metabolism, which supports the previous findings in prostate cancer and suggests that glutamine metabolism dynamics may be linked to acquired drug resistance.¹⁵ In cancer metastasis, circulating tumor cells (CTCs) collected from different anatomical locations showed diverse glutamine metabolism patterns (Figure 5E). Moreover, CTCs traveling in clusters, which have higher metastatic potential, exhibited significantly different glutamine metabolism compared to those traveling singly (Figure 5G), highlighting the importance of glutamine metabolism for CTC survival and metastatic potential. Altered glutamine metabolism was also observed in a high proportion of infiltrating immune cells (Figures 5B-D). Glutamine metabolism plays a pivotal role in immune cell function, supporting lymphocyte proliferation, cytokine production, macrophage activity, and neutrophil bacterial killing.⁵ Together, these results highlight the significance of glutamine metabolism in cancer, metastasis, and immune responses.

In addition to immune cells within the tumor microenvironment, we also investigated the dynamics of glutamine metabolism in circulating immune cells in peripheral blood mononuclear cells (PBMCs). Significant alterations in glutamine metabolism were observed in PBMCs under various physiological conditions, including infection (Figure 3E) and cancer (Figures 6A-D), suggesting that immune cells may alter their glutamine metabolism in response to infections and tumors,⁷² or that tumors may “educate” immune cells by modulating their glutamine metabolism, similar to their effects on platelets.⁸⁰ Thus, quantitative measurement of glutamine metabolism in PBMCs could serve as a valuable tool for monitoring immune responses in various diseases. Moreover, our approach demonstrates significant potential for non-invasive cancer diagnosis, as it allows for the analysis of glutamine metabolism in PBMCs and other peripheral blood analytes, such as platelets and extracellular vesicles (Figure 6). Although altered GLS expressions were more frequently observed in blood analytes with valuable diagnostic potential, however, co-analysis with KGA/GAC ratio exhibited improved performance (Figure 6D), suggesting the translational merit of both parameters in cancer diagnosis. In addition, currently the most widely used non-invasive diagnostic biomarker for HCC is serum/plasma alpha-fetoprotein (AFP) with limited sensitivity.¹⁰⁵ Hence, it would be worthwhile to explore the correlation/synergy between GLS expression patterns and AFP towards high-performance assays in non-invasive HCC diagnosis in following studies. Our work demonstrates that PBMCs, an abundant yet often overlooked analyte, can be informative for cancer diagnosis and may complement current liquid biopsy methods that focus on cell-free DNA.¹⁰⁶⁻¹⁰⁹

On the other hand, there are several additional enzymes involved in glutamine metabolism besides glutaminase, such as glutamine synthetase (GLUL), which catalyzes the conversion of glutamate to glutamine. A co-analysis of these enzymes alongside GLS may enhance the characterization and measurement of glutamine metabolism, therefore deserves investigations in future studies.

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AUTHOR CONTRIBUTIONS

Conception and design: K.S.; Study supervision: H.H., C.D., K.S.; Development of methodology: D.H., W.W., H.H., C.D., K.S.; Patient recruitment and clinical data analysis: X.Z., K.Z., Z.Z., Y.W.; Cell culture and experiments: W.W., Y.A., M.Y., Y.S.; Acquisition of data: all authors; Analysis and interpretation of data: all authors; Writing the manuscript: D.H., W.W., K.S.; Review and edit of the manuscript: H.H., C.D., K.S. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

H.H. and K.S. had filed patent applications based on the method developed in this work; K.S. is a consultant to and has ownership interest in OmixScience Inc. The remaining authors declare no conflict of interests.

ETHICAL STATEMENT AND PATIENT CONSENT

This study had been approved by the Ethics Committee of Shenzhen Bay Laboratory (approval number: YL2021-002-GZ02) and Ethics Committee of The Third People's Hospital of Shenzhen. Participants were recruited from The Third People's Hospital of Shenzhen with written informed consents. All participants were fully informed about the study's purpose and voluntarily agreed to participate.

DATA AND CODE AVAILABILITY

Raw RNA-seq data for PBMCs generated in this study had been deposited to Genome Sequence Archive in National Genomics Data Center (GSA-Human) under accession number HRA007717. Public bulk RNA-seq datasets were downloaded from Gene Expression Omnibus (GEO; accession numbers: GSE173323, GSE116862, GSE135251, GSE95587, GSE133684, GSE154918, GSE101575, GSE127001, GSE197514, GSE68086, and GSE144561) and Sequence Read Archive (SRA; project number PRJNA739257). Preprocessed gene- and isoform-level expression quantifications from bulk RNA-seq experiments were downloaded from UCSC Xena database (<https://xena.ucsc.edu/>; GTEx and TCGA datasets), ENCODE data portal (<https://www.encodeproject.org/search/?type=Experiment>; expression profiles for human primary cells or cell lines released between January 2013 and December 2022 were collected), and BLUEPRINT project (<http://blueprint-data.bsc.es/>). For TCGA datasets, cancer types with less than 5 adjacent normal tissues were removed from the analysis. ScRNA-seq datasets were downloaded from GEO (accession numbers: GSE36552, GSE45719, GSE44183, GSE162547, GSE169426, GSE81547, GSE140440, GSE75688, GSE123902, GSE154778, and GSE178318), China National GeneBank DataBase (CNCBdb; accession number: CNP0000095), 10x website (<https://www.10xgenomics.com/resources/datasets/10k-human-pbmcs-3-v3-1-chromium-controller-3-1-high>), and Human Cell Atlas (HCA) (<https://explore.data.humancellatlas.org/projects/cc95ff89-2e68-4a08-a234-480eca21ce79>). Computational programs and scripts to reproduce the results had been deposited at <https://github.com/hellosunking/Glutamine-metabolism-dynamics>.

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-life.2024.100128>

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