Circulating oncometabolite 2-hydroxyglutarate (2HG) as a potential biomarker for isocitrate dehydrogenase (*IDH1/2*) mutant cholangiocarcinoma

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Running title:

Circulating 2HG as biomarker for IDH mutant cholangiocarcinoma

Abbrevivation list:

2HG: 2-hydroxyglutarate; IDH: isocitrate dehydrogenase; CCA: cholangiocarinoma; ccRCC: clear cell renal cell carcinoma;

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CONFLICTS OF INTEREST

No authors declared any conflicts of interest.

ABSTRACT

Isocitrate dehydrogenase (IDH) enzymes catalyze the decarboxylation of isocitrate to alpha-ketoglutarate. *IDH1/2* mutations preferentially convert αKG to R-2-hydroxyglutarate (R2HG), resulting in R2HG accumulation in tumor tissues. We investigated circulating 2-hydroxyglutate (2HG) as potential biomarkers for patients with *IDH-mutant (IDHmt)* cholangiocarcinoma (CCA).

R2HG and S-2-hydroxyglutarate (S2HG) levels in blood and tumor tissues were analyzed in a discovery cohort of *IDHmt* glioma and CCA patients. Results were validated in cohorts of CCA and clear cell renal cell carcinoma (ccRCC) patients.

The R2HG/S2HG ratio (rRS) was significantly elevated in tumor tissues, but not in blood for *IDHmt* glioma patients, while circulating rRS was elevated in *IDHmt* CCA patients. There were overlap distributions of circulating R2HG and total 2HG (t2HG) in both *IDHmt* and *wild-type* (*IDHwt*) CCA patients, while there was minimal overlap in rRS values between *IDHmt* and *IDHwt* CCA patients. Using the rRS cut-off value of 1.5, the sensitivity of rRS was 90% and specificity was 96.8%.

Circulating rRS is significantly increased in *IDHmt* CCA patients compare to *IDHwt* CCA patients. Circulating rRS is a sensitive and specific surrogate biomarker for *IDH1/2* mutations in CCA. It can potentially be used as a tool for monitoring *IDH*-targeted therapy.

Introduction:

Cholangiocarcinoma (CCA) is a heterogeneous group of hepatobiliary tumors that have a poor prognosis. Advanced CCA is traditionally sub-classified by anatomical sites into intrahepatic cholangiocarcinoma (iCCA) and extrahepatic cholangiocarcinoma (eCCA). Recently, genomic advances have partially unveiled the complex molecular landscape of CCA, shedding new light on novel therapeutic opportunities and ushering in the era of precision oncology for 40-55% of CCA patients. (1) Among these putatively actionable alterations, mutations in the isocitrate dehydrogenase (*IDH1/2*) genes are detected in 15% iCCA and <5% of eCCA.(2–4) *IDH1/2* mutations are also seen in other cancers including low-grade glioma (80%), acute myeloid leukemia (20%), and central chondrosarcoma (80%). (5,6) The majority of *IDH1 and IDH2* point mutations occur at residues arginine 132 (R132) or 172 (R172), respectively. *IDH* is an essential enzyme in the tricarboxylic acid cycle that catalyzes the decarboxylation of isocitrate to alpha-ketoglutarate (αKG). (6–8) However, *IDH1/2 mutations* preferentially convert αKG to R-2-hydroxyglutarate (R2HG). (9,10) The aberrant R2HG production from *IDH1/2* mutations results in R2HG accumulation, and its concentration in *IDH-mutant* (*IDHmt*) tumor tissues can reach 5-30 mM, far higher than its physiological concentrations(≈100μM). (11) Interestingly, the S-enantiomer (S2HG) concentrations remain low in both *IDHmt* and *wild-type* cancers.

Since 2HG is a membrane-diffusible small molecule, multiple attempts have been undertaken to explore the feasibility of circulating total 2-hydroxyglutarate (t2HG) or R2HG concentrations as alternative diagnostic tools for identifying *IDH1/2* mutations in various *IDHmt* malignancies. Studies have demonstrated elevated levels of circulating t2HG and/or R2HG in *IDHmt* cancers, including CCA. (12–20) We previously reported that tissue ratios of R2HG over S2HG (rRS) are a more sensitive biomarker for *IDH1/2* mutations in glioma patients than R2HG.(21) In this study, we investigated circulating R2HG, t2HG and rRS as potential biomarkers for *IDH1/2* mutations in CCA patients.

Methods:

Tissue and blood samples were collected from patients who were participating in studies approved by the Research Ethics Board at University Health Network, Toronto, Canada. Written informed consent was obtained from each patient before bio-specimen collection. These studies were conducted in accordance with the Declaration of Helsinki.

The study consisted of two cohorts of patients [**Figure 1]**. The discovery cohort comprised of patients with low-grade glioma or metastatic CCA. These patients were known to be *IDHmt.* The presence of *IDH1/2* mutations was determined via either immunohistochemistry for glioma patients or next-generation sequencing (NGS) for CCA patients as a part of standard clinical care. Matched tissue and blood samples were collected where available from these patients.

The validation cohort consisted of a second group of patients with metastatic CCA, whose blood samples were collected as a part of institutional biobanking initiatives. The mutational status of *IDH1/2* in this group of CCA patients was unknown except for one patient who was known to be *IDHmt* based on testing as part of routine clinical care. For patients who were suspected of harboring *IDH1/2* mutations based on the elevated circulating rRS results, mutational analysis using circulating tumor DNA (ctDNA) was performed in a CLIA-certified laboratory. In addition, a group of patients with clear cell renal cell carcinoma (ccRCC) was included as a control group since *IDH1/2* mutations have not been reported in the ccRCC population.

Tissue and blood samples collected were analyzed for R2HG and S2HG using a validated HPLC tandem mass spectrometry (HPLC-MS/MS) method as previously reported. (21)

Summary statistics were presented for R2HG, S2HG, t2HG, and rRS. The t2HG (t2HG = R2HG + S2HG) and rRS (rRS = R2HG/S2HG) values were calculated based on measured levels of S2HG and R2HG. R2HG, S2HG, t2HG, and rRS were compared using t-tests or one-way ANOVA between glioma and CCA patients, between

tissue and circulating 2HGs, or among cohorts using Prism (Version 9.5.1, GraphPad Software, Boston, MA). Results were considered statistically significant if $p \leq 0.05$.

Data Availability:

The data generated in this study are available upon request from the corresponding author.

Results:

In the discovery cohort, a total of 21 patients with *IDHmt* tumors were included (glioma=11, CCA=10). Blood samples were available for all 21 patients, while sufficient tissue samples were available for 10 glioma patients but only two CCA patients. The validation cohort included 32 CCA patients. There were no significant differences in demographic characteristics between CCA patients enrolled in the discovery and validation cohorts [**Table 1**].

In *IDHmt* glioma patients, S2HG levels were significantly lower in tumor tissues than in the blood, while R2HG levels were not statistically different [**Table 2, Figure 2**]. As a result, rRS was significantly higher in tumor tissue than in blood (46.4 ± 42.3 vs 1.1 ± 0.5, p=0.002). Due to limited tissue availability for CCA patients, comparisons between tissue and blood 2HG levels were not performed. For two CCA patients with sufficient tumor tissues for analysis, their tissue rRS levels were 144.8 and 244.9 respectively. Comparing glioma and CCA patients, circulating R2HG was significantly higher in CCA patients while there was no difference seen in S2HG [**Figure 2**]. Consequently, circulating t2HG and rRS were significantly higher in CCA patients than those in glioma patients.

Among 32 CCA patients in the validation cohort, one patient (Patient 460) was known to harbor *IDH1R132C* mutation [**Table 3**]. Circulating 2HG analysis revealed additional two patients with abnormally high R2HG and rRS levels (Patients 376 and 468).

The distribution of S2HG, R2HG, and t2HG was wide in both glioma and CCA patients [**Figure 3**]. Although circulating R2HG and t2HG were significantly higher in *IDHmt* CCA patients than those in *IDHwt* patients, there was considerable overlap in R2HG and t2HG concentrations in these patients. However, the circulating rRS distributed tightly

around 0.8 ± 0.2 (range: 0.45-1.55) in *IDHwt* patients [**Table 3**]. For the cohort of ccRCC patients, the mean rRS was 0.8 ± 0.3, ranging from 0.2-1.5.

rRS was significantly higher among *IDHmt* CCA patients in both discovery and validation cohorts than that for *IDHwt* CCA patients. In the discovery cohort, only one *IDHmt* CCA patient had a rRS value lower than 1.5 (1.3), while only one *IDHwt* patient in the validation cohort had a rRS value higher than 1.5 (1.55). In addition to Patient 460, two CCA patients in the validation cohort had rRS values above 1.5 [**Table 3**]. Subsequent ctDNA analysis confirmed that both patients harbored *IDH1R132C* mutations. Using a cut-off rRS value of 1.5, the sensitivity of rRS for predicting *IDH1/2* mutation status was 90%, and the specificity was 96.8%.

Discussion:

CCA is characterized by overlapping, low-penetrance genomic alterations that span diverse signaling pathways. (22). Identification of *IDH1/2* mutations has enabled targeted therapy in a subset of CCA patients. Numerous agents targeting *IDHmt* cancers, such as ivosidenib, vorasidenib, ceralasertib, olutasidenib, enasidenib, and BAY1436032, have either received regulatory approvals or are in various stages of clinical development. (23–25) The ClarIDHy trial evaluated ivosidenib in refractory *IDHmt* CCA patients. (24) Ivosidenib provides a modest, but statistically significant improvement in progression-free survival (PFS) of 1.3 months, compared to placebo. (24)

Obtaining tumor tissue samples for molecular profiling remains a clinical challenge in CCA patients. Some patients are diagnosed based on bile duct brushing during endoscopic retrograde cholangiopancreatography (ERCP). Even with core biopsies, there is a considerable failure rate in yielding sufficient tumor DNA content for accurate pathological diagnosis or comprehensive molecular profiling. (26,27) This limitation can be attributed to the challenging characteristics of CCA, including its desmoplastic and necrotic nature, as well as the presence of intra-tumoral heterogeneity. (28–30)

The intriguing aspect of *IDHmt* tumors is the aberrant production and accumulation of the circulating oncometabolite 2HG, specifically R2HG rather than S2HG. (7,9–11,31)

Under physiologic conditions, R2HG and S2HG exist at low but equal levels. Both are small molecules that can readily diffuse across cellular membranes. (11) There has been a significant interest in the evaluation of circulating 2HG as a biomarker for *IDH1/2* mutations in various hematologic and solid malignancies. (12–20,32) *Janin et al* found that elevated serum 2HG concentrations reliably diagnosed *IDHmt* AML, achieving 100% sensitivity and 79% specificity when compared to *wild-type* cases. (20) *Lombardi et al* showed urinary 2HG concentrations were not elevated in *IDHmt* glioma patients. (13) Similarly, *Capper et al* concluded that circulating 2HG were not elevated in *IDHmt* glioma patients. (14) For other solid tumors, circulating 2HG concentrations were reported to be a surrogate biomarker of *IDHmt*. (12,15,16) In addition, circulating 2HG levels were shown to correlate with tumor burden in *IDHmt* CCA patients. (16).

One possible explanation for the high levels of circulating 2HG levels in *IDHmt* CCA, but not in glioma patients, is due to the relative volumes of the circulatory system and brain tumor lesions. Any 2HG released from glioma lesions is diluted, making it harder to detect elevated 2HG in the peripheral blood of these patients.

We previously demonstrated that tissue rRS represents a highly sensitive and specific biomarker for *IDH1/2* mutations in glioma, compared to R2HG or t2HG levels. (21) The absolute levels of R2HG and/or t2HG are influenced by tumor volumes, cellularity and different point mutations in the *IDH1/2* genes. These limitations can be mitigated by using S2HG as a readily available internal standard since R2HG and S2HG share the same physiochemical properties except chirality. As demonstrated in this study, there is considerable overlap of circulating R2HG or t2HG between *IDHwt* and *IDHmt* CCA patients, while there is minimal overlap in rRS between *IDHwt* and *IDHmt* CCA patients. Therefore, the circulating rRS could offer a more sensitive and specific biomarker for identifying *IDH1/2* mutations in CCA patients, particularly in cases where traditional tissue-based methods are not feasible or practical.

The majority of previous studies on circulating 2HG only measured t2HG. We employed an analytical method that can quantitate R2HG and S2HG enantiomers separately, enabling the interpretation of rRS as a biomarker for *IDH1/2* mutations in CCA. Our discovery cohort in this study is limited by the small number, however, the difference

between tissue and serum 2HG levels in glioma patients and the elevated circulating rRS in *IDHmt* CCA patients is striking. Only two CCA patients in the validation cohort were selected for subsequent ctDNA analysis. This decision was made based on the cost of performing ctDNA analysis.

Recent advances in ctDNA technology have revolutionized genomic analysis by eliminating the need for tumor tissue samples. However, the analysis of circulating 2HG has advantages as it requires a small volume of blood (less than 0.5 mL) and it can be completed within 30 minutes. (33,34) The cost is also a fraction of the cost of ctDNA analysis. The ClarIDHy trial demonstrated that ivosidenib inhibited 2HG production and reduced circulating 2HG levels in responding patients. (35,36) Hence, circulating 2HG analysis represents a cost-effective method for serially monitoring responses to *IDHmt* targeted therapies.

Our results show that circulating R2HG, t2HG and rRS are elevated in *IDHmt* CCA patients, and rRS is a sensitive and specific biomarker for *IDHmt*, consistent with the biology of *IDH1/2* mutations. The potential of circulating rRS as a biomarker for response to *IDH1/2* targeted therapies should be incorporated in future studies of *IDHm*t targeting agents.

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Table 1: Characteristics of patients across cohorts

Characteristic	**Discovery Cohort**		**Validation Cohort**	
Histology	**Glioma**	**CCA**	**CCA**	**ccRCC**
Number of patients	11	10	32	31
Sex, male (%)	8 (73%)	5 (50%)	17(53%)	26 (84%)
Age, years				
Median	42.0	63.7	64.5	60
(range)	(23.2-66.6)	(48.0-78.9)	(42.3-78.1)	(30.0-81.0)

CCA: Cholangiocarcinoma; ccRCC: clear cell renal cell carcinoma

Table 2: Tissue and circulating S2HG, R2HG, t2HG, and rRS mean levels for glioma and cholangiocarcinoma patients in the discovery cohort

	Glioma			Cholangiocarcinoma	
Biomarkers	**Serum (ng/ml) (n=11)**	**Tissue (ng/g) (n=10)**	**p-value**	**Serum (ng/ml) (n=10)**	**Tissue (ng/g) (n=2)**
S2HG	60.3 ± 37.4	0.4 ± 0.5	**0.005**	82.3 ± 49.4	4.3 ± 3.0
R2HG	54.7 ± 19.3	30.3 ± 42.9	NS	458.8 ± 392.9	945.0 ± 883.9
t2HG	114.9 ± 52.1	30.7 ± 43.2	**NS**	541.0 ± 338.8	949.3 ± 886.9
rRS	1.1 ± 0.5	46.4 ± 42.3	**0.002**	7.6 ± 8.3	194.9 ± 70.8

S2HG: S-2-hydroxyglutarate; R2HG: R-2-hydroxyglutarate; t2HG: total of R2HG+S2HG; rRS: ratio of R2HG/S2HG

Table 3. Circulating R2HG, S2HG, t2HG, and rRS for cholangiocarcinoma (CCA) and clear cell renal cell carcinoma (ccRCC) patients in the validation cohort

Characteristics	S2HG (ng/ml)	R2HG (ng/ml)	t2HG (ng/ml)	rRS
All CCA patients (n=32)				
Mean ± SD	166.1 ± 74.4	316.8 ± 625.1	482.9 ± 654.6	1.7 ± 2.9
(range)	(79.7-356)	(67.4 -2570)	(148.8 -2747)	(0.5-14.5)
CCA with *IDH1R132C* mutations (n=3)				
Patient 376	242	2290	2532	9.5
Patient 468	177	2570	2747	14.5
Patient 460	324	1730	2054	5.3
Mean ± SD	247.7 ± 73.7	2196.7 ± 427.7	2444.3 ± 354.7	9.8 ± 4.6
(range)	(177-324)	(1730-2570)	(2054-2747)	(5.3-14.5)
***IDH wild-type* CCA (n=29)**				
Mean ± SD	157.7 ± 70.4	122.3 ± 41.4	280 ±103	0.8 ± 0.2
(range)	(79.7-356)	(67.4-226)	(148.8-538)	(0.5-1.6)
All ccRCC patients (n=31)				
Mean ± SD	89.0 ± 70.9	60.2 ± 19.8	149.2 ± 85.4	0.8 ± 0.3
(range)	(35.1-365)	(34.7-124)	(72.1-451.2)	(0.2-1.5)

S2HG: S-2-hydroxyglutarate; R2HG: R-2-hydroxyglutarate; t2HG: total of R2HG+S2HG; rRS: ratio of R2HG/S2H

Figure 1. Study flow diagram

CCA: Cholangiocarcinoma; ccRCC: clear cell renal cell carcinoma; 2HG: 2-Hydroxylutarate; rRS: ratio of R2HG/S2HG; ctDNA: circulating tumor DNA; *IDH*mt: *IDH* mutated; *IDH*wt: *IDH* wild type

Figure 2. **A**) S2HG, **B**) R2HG, **C**) t2HG, and **D**) rRS in tissue and blood samples from the discovery cohort

S2HG: S-2-hydroxyglutarate; R2HG: R-2-hydroxyglutarate; t2HG: total of R2HG+S2HG; rRS: ratio of R2HG/S2HG. Y-axis is in logarithmic scale.

Figure 3. Circulating **A**) S2HG, **B**) R2HG, **C**) t2HG, and **D**) rRS for cholangiocarcinoma patients in the discovery and validation cohorts.

S2HG: S-2-hydroxyglutarate; R2HG: R-2-hydroxyglutarate; t2HG: total of R2HG+S2HG; rRS: ratio of R2HG/S2HG. Y-axis is in logarithmic scale.

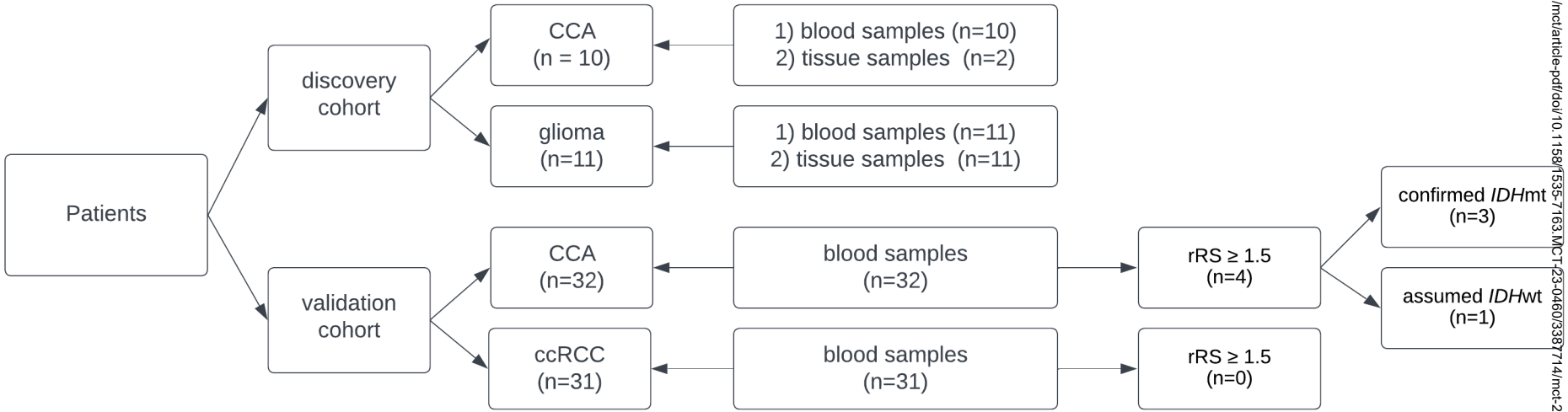

Figure 2

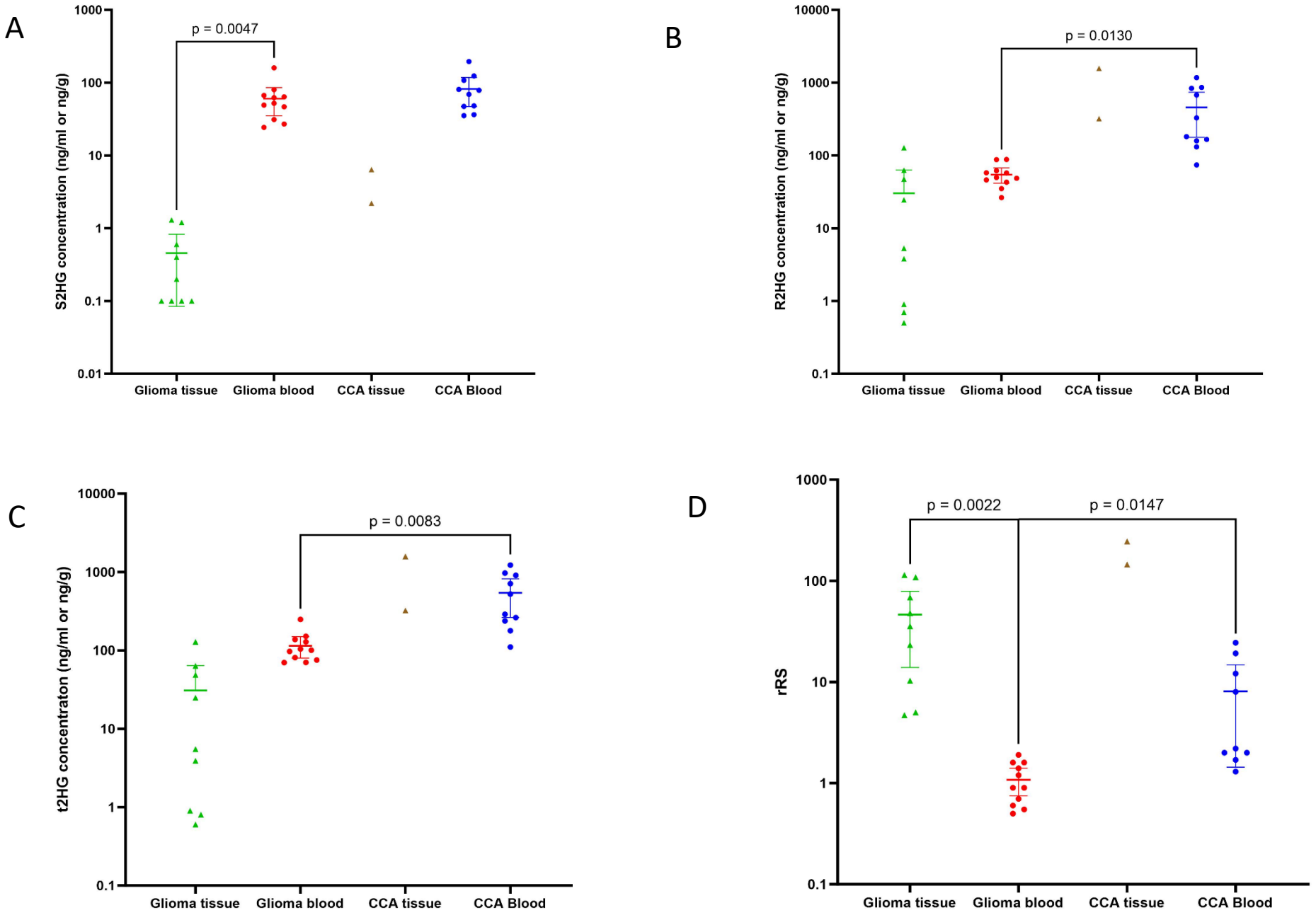

Figure 3