Supplementary Information for manuscript:

Elevation in plasma tRNA fragments precede seizures in human epilepsy

Marion C. Hogg[1,2], Rana Raoof[1,3], Hany El Naggar[4], Naser Monsefi[1], Norman Delanty[2,4], Donncha F. O'Brien[5], Sebastian Bauer[6,7,8], Felix Rosenow[6,7,8], David C. Henshall[1,2] & Jochen H.M. Prehn[1,2*]

1. Department of Physiology and Medical Physics, Royal College of Surgeons In Ireland, St. Stephen's Green, Dublin D02 YN77, Ireland.

2. FutureNeuro Research Centre, Royal College of Surgeons In Ireland, St. Stephen's Green, Dublin D02 YN77, Ireland.

3. Department of Anatomy, Mosul Medical College, University of Mosul, Mosul, Iraq.

4. Department of Neurology, Beaumont Hospital, Beaumont, Dublin, Ireland.

5. Department of Neurosurgery, Beaumont Hospital, Dublin, Ireland.

6. Epilepsy Center Hessen, Department of Neurology, Baldingerstr, 35043, Marburg, Germany.

7. Epilepsy Center Frankfurt Rhine-Main, Neurocenter, Goethe-University, Schleusenweg 2-16, Haus 95, 60528, Frankfurt, Germany

8. LOEWE Center for Personalized Translational Epilepsy Research (CePTER), Frankfurt, Germany.

Supplementary Methods

TLE patients and healthy controls

A 10 ml blood sample (pre-seizure) was taken on admission. A post-seizure sample was collected 24 h after experiencing an electro-clinical seizure documented by video-EEG monitoring. The interval between pre-seizure blood sampling and seizure occurrence varied among patients (median 31 hours, range 00:11-205:46), as did the number and type of seizures experienced. 32 non-fasting male and female healthy control volunteers (MAR, n = 16; DUB, n = 16) were recruited.

Interictal activity analysis

Video-EEG recordings from a period of 18-24 hours upon arrival to the EMU were reviewed by a clinical neurologist and patients were classified into three groups based on the vEEG activity: rare, occasional, and frequent. tRNA fragment levels in pre-seizure samples were compared between groups.

Plasma preparation

Plasma was prepared within 1 h of collection by centrifuging at 1,300 x *g*, for 10 min, at 4°C, and stored at -80 °C. Haemolysis was assessed using a Nanodrop 2000, and samples with A_{414} >0.25 were excluded.

RNA Extraction

RNA was purified from plasma using the miRCURY RNA isolation kit for biofluids (Exiqon). A synthetic *C.elegans miRNA-39* spike-in RNA was added before purification. RNA purified from 200 ul plasma was eluted in 50 ul water.

Small RNA sequencing (RNA seq) and analysis

Small RNA seq (<50 nt) was performed on pooled plasma from 16 healthy controls and 16 focal epilepsy patients pre and post seizure samples. RNA libraries were generated using NEBNEXT library generation kit (New England Biolabs Inc.). Single ends reads were sequenced on the Illumina system by Exiqon Services, Denmark. RNA seq data has been submitted to the gene expression Omnibus (GSE114701). Adapter sequences were removed and reads with a quality score of <20 were removed. Reads were aligned using Tophat (v 2.0.14) and Bowtie (v 2.2.5.0), allowing 1 hit per read, to a custom tRNA database built from the GtRNAdb (gtrnadb.ucsc.edu). Intron locations were added for 32 tRNAs, and a "CCA" tail was manually added. Reads aligning to tRNAs were pooled based on their iso-acceptor type for the initial RNA seq analysis. This approach was taken due to the highly similar sequence of multiple tRNAs from the same iso-acceptor type. Subsequently Taqman assays were designed to recognise specific tRNA fragments from each iso-acceptor type that showed high abundance and high fold change. The genomic origin of the specific tRNA fragments cannot be absolutely defined due to the presence of multiple copies of identical tRNA genes in the genome. Reads are expressed as counts per million (CPM) to correct for differences in read depth. Mature tRNA structures were downloaded from GtRNAdb 2.0 (1) and tRNA fragment secondary structures were predicted using the Vienna RNAfold program (2).

Taqman assays

Custom Small RNA Taqman assays were designed to recognise tRNA fragments (ThermoScientific). The Taqman assay technology was developed to specifically amplify mature miRNAs without detecting the pre- or pri-miRNA that contains identical sequence, hence this technology was used here to amplify tRNA fragments without recognising the full length tRNA. The stem-loop primer used in the reverse transcription step of the Taqman assay inhibits binding to sequences with 3' extensions, such as full-length tRNAs. A similar protocol has been used to selectively quantify tRNA fragments previously (3). Primary hippocampal neuron samples were normalised to U6, human plasma samples were normalised to *C.elegans* miRNA-39 spike-in. 100 ng (cells or tissues) or 2 ul (biofluids) RNA was used per reverse transcription reaction. Quantification was performed on the Quantstudio 5 384-well PCR machine (ThermoFisher Scientific) and fold-change determined using the $2^{-\Delta\Delta Ct}$ method. Outliers +/- 2 standard deviations from the mean were excluded.

Primary hippocampal neuron culture and *in vitro* hyperexcitation model.

Primary mouse hippocampal neurons were dissected from E16-E18 C57Bl/6 embryos as described (4). On DIV 12 cells were incubated with 5 μM FLUO-4 for 45 minutes before media was replaced with experimental buffer (120 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM HEPES, 1.2 mM Na_2SO_4, 15 mM glucose, and 1.2 mM $CaCl_2$, 1mM $MgCl_2$). Cells were transferred to the heated stage of a LSM 5 Live microscope (Zeiss) and imaged to confirm spontaneous firing of neurons. Media was replaced with experimental buffer containing 0 or 1 mM MgCl2, and images were collected at 5Hz. Cells were incubated for 2 hours and total RNA

and media collected, importantly 2 hours in magnesium-free experimental buffer does not induce neuronal cell death (5).

Statistical analysis

Statistical analysis was performed in Graphpad Prism or SPSS. Data are fold change compared to control samples. Mouse hippocampal neuron experiments were analysed by two-tailed Student's t-test. Human plasma were not normally distributed therefore Kruskal-Wallis and Wilcoxon Signed Rank tests were used. For all analyses a p-value of less than 0.05 was considered significant. ROC analysis was performed in SPSS to determine the area under a curve (AUC) and Youdens J statistic was used to identify the optimal discriminatory tRNA level.

Surgically resected patient tissue

Focal epilepsy patients who were assessed to be suitable for surgical resection were recruited at the Department of Neurology, Beaumont Hospital, Dublin, Ireland. Informed consent was obtained for all patients and ethical approval was obtained from the Research Ethics Committee at the Royal College of Surgeons in Ireland (REC 13/75).

Fresh frozen tissue was mounted in OCT and sectioned on a cryostat at -22^{O}C. 12 um sections were either mounted on SuperFrost Plus slides (ThermoScientific) for histological analysis or collected in Eppendorf tubes for RNA extraction in Trizol (ThermoScientific). For histological analysis Nissl staining was performed. Briefly, slides were post-fixed in 4% pFa for 10 minutes and washed in PBS. Slides were stained in 0.1% cresyl violet acetate solution at 65^{o}C for 20 minutes. Slides were washed extensively in water and dipped in successive ethanol solutions, 2 dips each

of 70%, 80%, 90%, 95% (plus one drop glacial acetic acid), 100%. Slides were then incubated in Histoclear solution (National Diagnostics) for twice for 5 minutes and mounted in DPX solution.

Supplementary References

1. Chan PP, Lowe TM. GtRNAdb 2.0: an expanded database of transfer RNA genes identified in complete and draft genomes. Nucleic Acids Res. 2016;44(D1):D184-9.
2. Lorenz R, Bernhart SH, Honer Zu Siederdissen C, Tafer H, Flamm C, Stadler PF, et al. ViennaRNA Package 2.0. Algorithms Mol Biol. 2011;6:26.
3. Honda S, Loher P, Shigematsu M, Palazzo JP, Suzuki R, Imoto I, et al. Sex hormone-dependent tRNA halves enhance cell proliferation in breast and prostate cancers. Proc Natl Acad Sci U S A. 2015;112(29):E3816-25.
4. Jimenez-Mateos EM, Engel T, Merino-Serrais P, McKiernan RC, Tanaka K, Mouri G, et al. Silencing microRNA-134 produces neuroprotective and prolonged seizure-suppressive effects. Nat Med. 2012;18(7):1087-94.
5. Deshpande LS, Lou JK, Mian A, Blair RE, Sombati S, DeLorenzo RJ. In vitro status epilepticus but not spontaneous recurrent seizures cause cell death in cultured hippocampal neurons. Epilepsy Res. 2007;75(2-3):171-9.

	Group	Sex	Age	Diagnosis	AED	Group	Sex	Age
Dublin Cohort	TLE	F	18	TLE	LEV, LTG	C	F	25
	TLE	F	25	LEFT TLE	LEV, LTG	C	F	30
	TLE	F	28	RIGHT TLE	ESLI, LEV, ZNS	C	F	34
	TLE	F	45	LEFT TLE	LEV, CBZ	C	F	38
	TLE	F	64	RIGHT TLE	LEV, PHE	C	F	38
	TLE	F	79	TLE (CORTICAL DYSPLASIA)	ESLI , LEV, VAP	C	F	45
	TLE	M	18	LEFT TLE	OXC, CLOB	C	M	23
	TLE	M	25	LEFT TLE	LEV, LAC	C	M	24
	TLE	M	27	BILATERAL TLE	VAP, CLOB	C	M	25
	TLE	M	35	LEFT TLE	LEV, CLOB	C	M	31
	TLE	M	36	LEFT TLE	LEV, LTG	C	M	34
	TLE	M	47	LEFT TLE	LEV, LAC	C	M	37
	TLE	M	52	RIGHT TLE	ESLI, LAC, LEV	C	M	41
	TLE	M	61	BILATERAL TLE	LEV, PHY	C	M	46
	TLE	M	67	RIGHT TLE	LTG, CLOB	C	M	51
	TLE	M	74	TLE	TPN, LEV	C	M	52
Marburg Cohort	TLE	F	29	LEFT TLE	CBZ,LTG,TPM	C	F	25
	TLE	F	29	RIGHT temporo-parietal epilepsy	LTG, LEV	C	F	26
	TLE	F	30	LEFT TLE	CBZ, LEV	C	F	28
	TLE	F	33	RIGHT FTLE	LTG	C	F	33
	TLE	F	34	focal epilepsy	LCM, LTG	C	F	35
	TLE	F	37	RIGHT TLE	n/a	C	F	40
	TLE	F	49	LEFT focal epilepsy	CBZ	C	F	45
	TLE	M	18	RIGHT TLE	LEV	C	F	46
	TLE	M	23	mesial LEFT TLE	LEV	C	M	25
	TLE	M	34	RIGHT TLE	VPA, CBZ	C	M	33
	TLE	M	35	focal epilepsy	LEV, OXC, ZNS	C	M	34
	TLE	M	46	RIGHT TLE	OXC, LEV	C	M	35
	TLE	M	52	LEFT TLE	OXC, LEV, LCM	C	M	35
	TLE	M	52	focal epilepsy	LEV, ZNS	C	M	48
	TLE	M	57	LEFT TLE or FLE	TPM	C	M	55
	TLE	M	62	RIGHT TLE	LEV, PGB, Clonazepam	C	M	65

Table 1: Patient demographics. 32 epilepsy patients and age-matched healthy controls were recruited at two independent epilepsy monitoring units in Dublin and Marburg. Age, sex, diagnosis, and current AEDs are indicated for patients, and age and sex are indicated for healthy controls.

	Control	Pre-seizure	Post-seizure
Input	30336921	48665926	32808642
Mapped	227578	524797	272138
Mapped (%)	0.80	1.10	0.80

Table 2: RNA Seq reads aligned to tRNAs. Total reads and reads aligned to tRNAs for Control, Pre-seizure and post-seizure pooled RNA seq samples. The mean Phred score was >28 for all sequences indicating they were of very high quality.

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A)
Human_GluCTC      TCCCTGGTGGTCTAGTGGTtAGGATTCGGCGCTCTCACCGCCGCGGCCCGGGTTCGATTC        60
Mouse_GluCTC      TCCCTGGTGGTCTAGTGGTtAGGATTCGGCGCTCTCACCGCCGCGGCCCGGGTTCGATTC        60
5'GluCTC          TCCCTGGTGGTCTAGTGGTTAGGATT----------------------------------        26
                  *************************
Human_GluCTC      CCGGTCAGGGAA        72
Mouse_GluCTC      CCGGTCAGGGAA        72
5'GluCTC          ------------        26

B)
Mouse_AlaTGC      GGGGATGTAGCTCAGTGGTAGAGCGCATGCTTAGCATGCATGAGGtCCTGGGTTCGATCC        60
Human_AlaTGC      GGGGATGTAGCTCAGTGGTAGAGCGCATGCTTAGCATGCATGAGGtCCCGGGTTCGATCC        60
5'AlaTGC          GGGGATGTAGCTCAGTGGTAGAGC------------------------------------        24
                  ************************
Mouse_AlaTGC      CCAGCATCTCCA        72
Human_AlaTGC      CCAGCATCTCCA        72
5'AlaTGC          ------------        24

C)
Human_GlyGCC      GCATGGGTGGTTCAGTGGTAGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTTCGATTCC        60
Mouse_GlyGCC      GCATGGGTGGTTCAGTGGTAGAATTCTCGCCTGCCACGCGGGAGGCCCGGGTTCGATTCC        60
5'GlyGCC          GCATGGGTGGTTCAGTGGTAGAATT-------------------------------------        25
                  *************************
Human_GlyGCC      CGGCCCATGCA         71
Mouse_GlyGCC      CGGCCCATGCA         71
5'GlyGCC          -----------         25
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Figure 1: Alignment of human and mouse tRNA sequences and tRNA fragments identified in this study. tRNAs are highly conserved indicating assays designed to human tRNA fragments would also detect tRNA fragments in mouse samples.

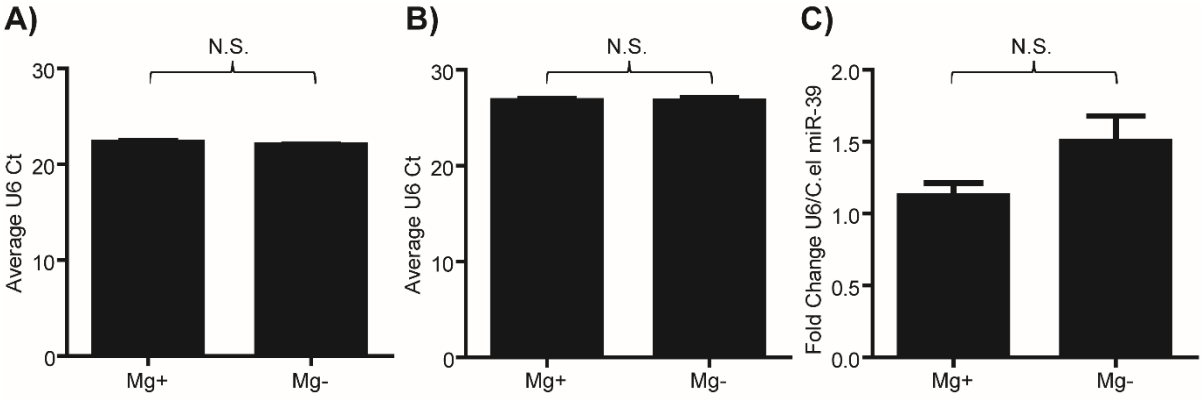

Figure 2: U6 levels are constant across mouse hippocampal neuron experiments. A) Intracellular and B) Extracellular average U6 Ct values from primary mouse hippocampal neurons cultured in the presence (Mg+) or absence (Mg-) of Magnesium show no significant difference in levels. C) Extracellular U6 normalised to *C.elegans* miRNA 39 spike-in also shows no significant difference between Mg+ and Mg- cultures.

	5'GlyGCC	5'AlaTGC	5'GluCTC
AUC	0.816	0.916	0.802
p-value	0.000027	1.86E-08	0.000069
Youdens	1.36	1.33	2.13
Sensitivity	0.67	0.97	0.59
Specificity	0.93	0.87	0.9

Table 3: Summary of ROC analysis from Figure 3.

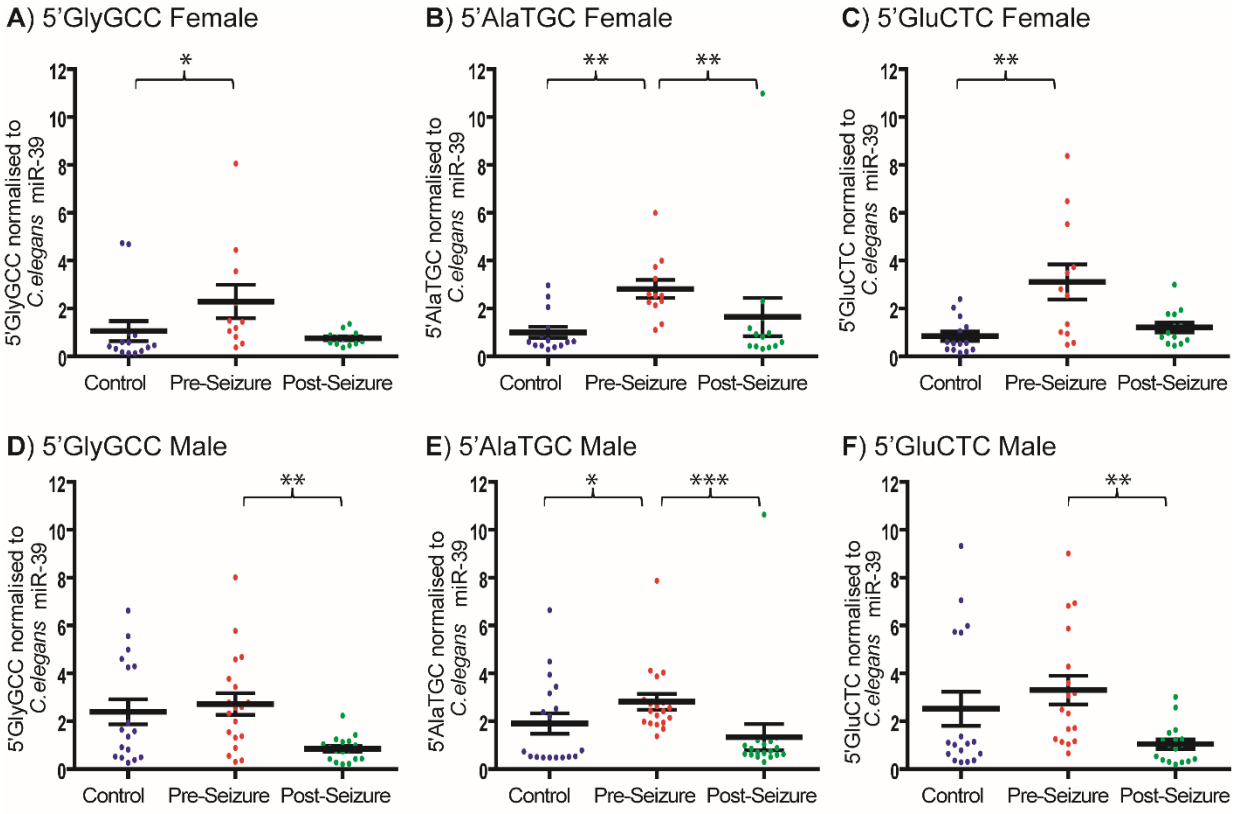

Figure 3: Analysis of tRNA fragment levels in males and females. Separating the controls and patients analysed in the main manuscript Figure 3 by sex revealed that 5'AlaTGC is significantly elevated in pre-seizure males and females and significantly decreases post seizure in both groups. 5'GlyGCC and 5'GluCTC were significantly deceased following seizures in males. Data were analysed using Kruskal-Wallis test where A) 5'GlyGCC in females p=0.0129, B) 5'AlaTGC in females p=0.0006, C) 5'GluCTC in females p=0.0074, D) 5'GlyGCC in males p=0.0033, E) 5'AlaTGC in males p=0.0004, and F) 5'GluCTC in males p=0.0036. Controls include 14 female and 18 male, epilepsy patients include 13 females and 19 males.

A)

Ranked by Fold Change	Isoacceptor type	Control CPM	Pre-Seizure CPM	Post-Seizure CPM	Fold Change (Pre/Con)
34	ValAAC	345.12	248.49	229.45	0.72
51	ProAGG	121.83	50.75	43.56	0.42

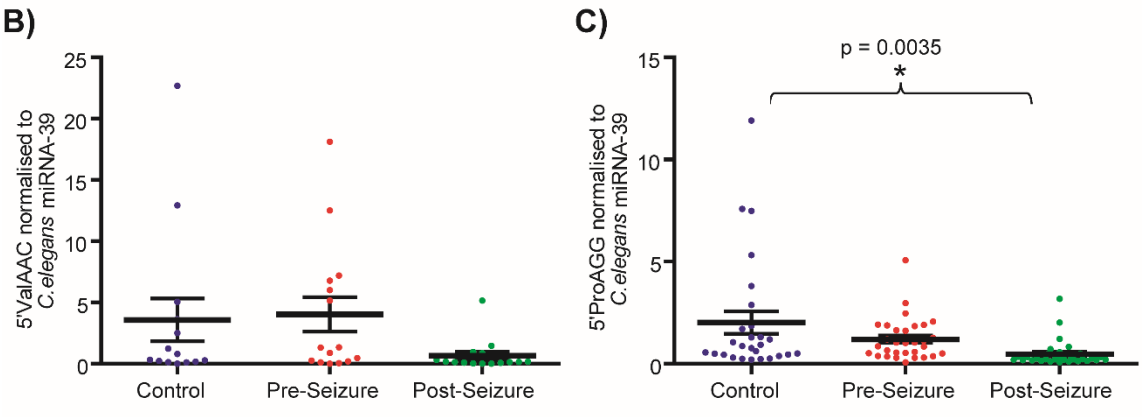

Figure 4: Analysis of tRNA fragments that are not elevated in pre-seizure samples compared to controls. Two tRNA fragments were chosen for further analysis to highlight that not all tRNA fragments are elevated in pre-seizure samples. A) RNA seq analysis of reads aligning to tRNAs ranked by fold change between pre-seizure and control samples indicated #34 ValAAC and #51 ProAGG were higher in controls than pre-seizure samples. Custom Taqman assays were developed to analyse levels of B) 5'ValAAC and C) 5'ProAGG levels in 32 pre and post seizure samples and 32 healthy controls. 5'ValAAC was not detectable in some samples (n= 14-15 samples per group).

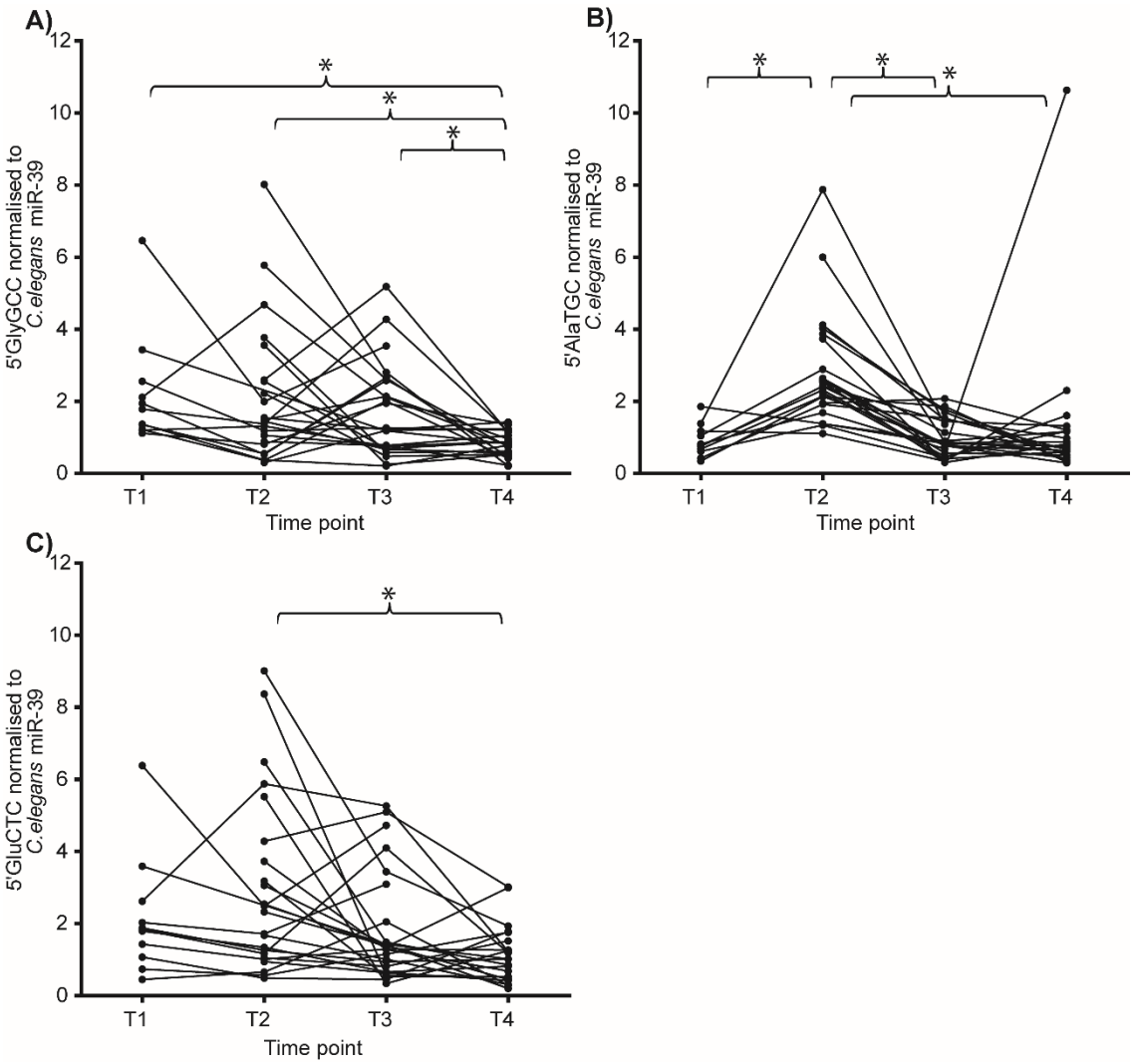

Figure 5: Analysis of tRNA fragments across 4 time points in epilepsy patients. Plasma samples collected at intervening time points were available for 24/32 focal epilepsy patients and tRNA fragment levels were analysed. T1 = on arrival to the EMU, T2 = 24 hours after T1 if no seizure occurred, T3 = 1 hour after seizure, and T4 = 24 hours after seizure. Figure 3 in the main text displays data from T2 and T4 time points for all 32 patients. One or more seizures occurred between T2 and T3 time points. All tRNA fragment levels were significantly higher in pre-seizure samples compared to post seizure samples. Data was analysed by Kruskal-Wallis test where * indicates $p < 0.05$.

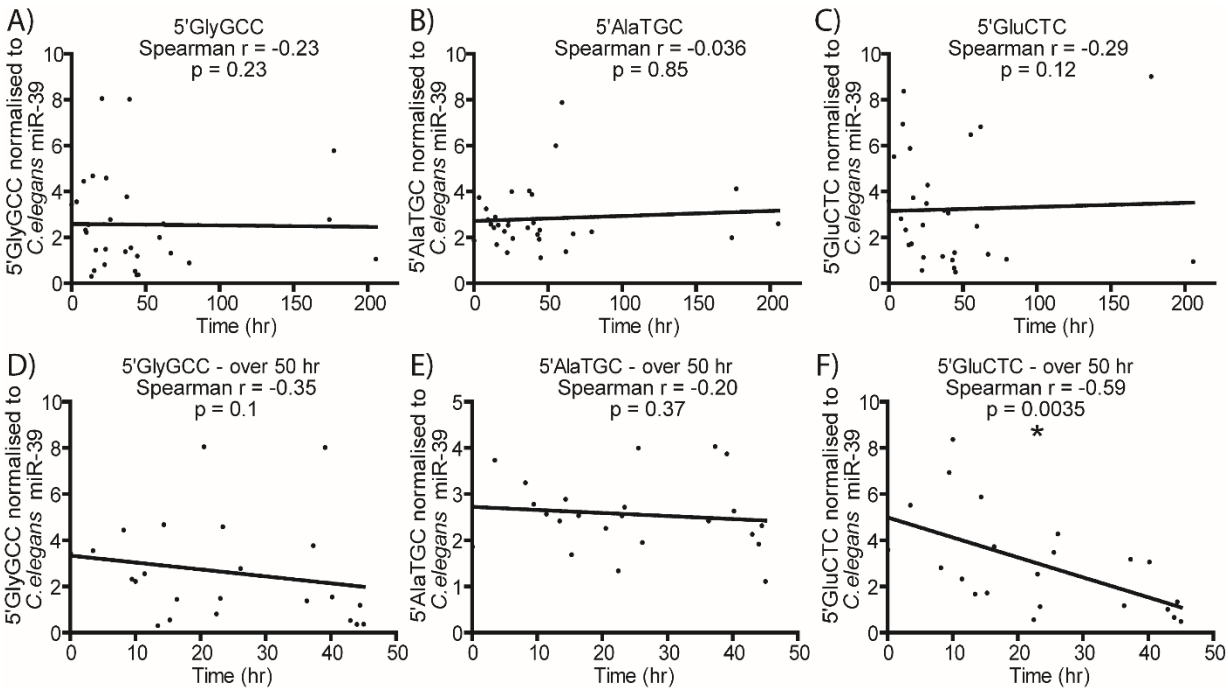

Figure 6: Correlation of time interval between pre-seizure blood collection and onset of seizure and tRNA fragment level. A-C) Correlation analysis for all samples, and D-F) Correlation for samples collected within under 50 hours before seizure onset. Pre-seizure samples collection to seizure onset time interval (hours) is plotted on the x axis and tRNA fragment level normalised to *C.elegans* spike-in on the y axis. Correlation was assessed with Spearman's r-value showing no significant correlation between time interval and plasma tRNA fragments levels when analysing all samples; however, when restricting samples to under 50 hours a significant correlation is observed with 5'GluCTC (panel F) and 5'GlyGCC and 5'AlaTGC show a similar trend.

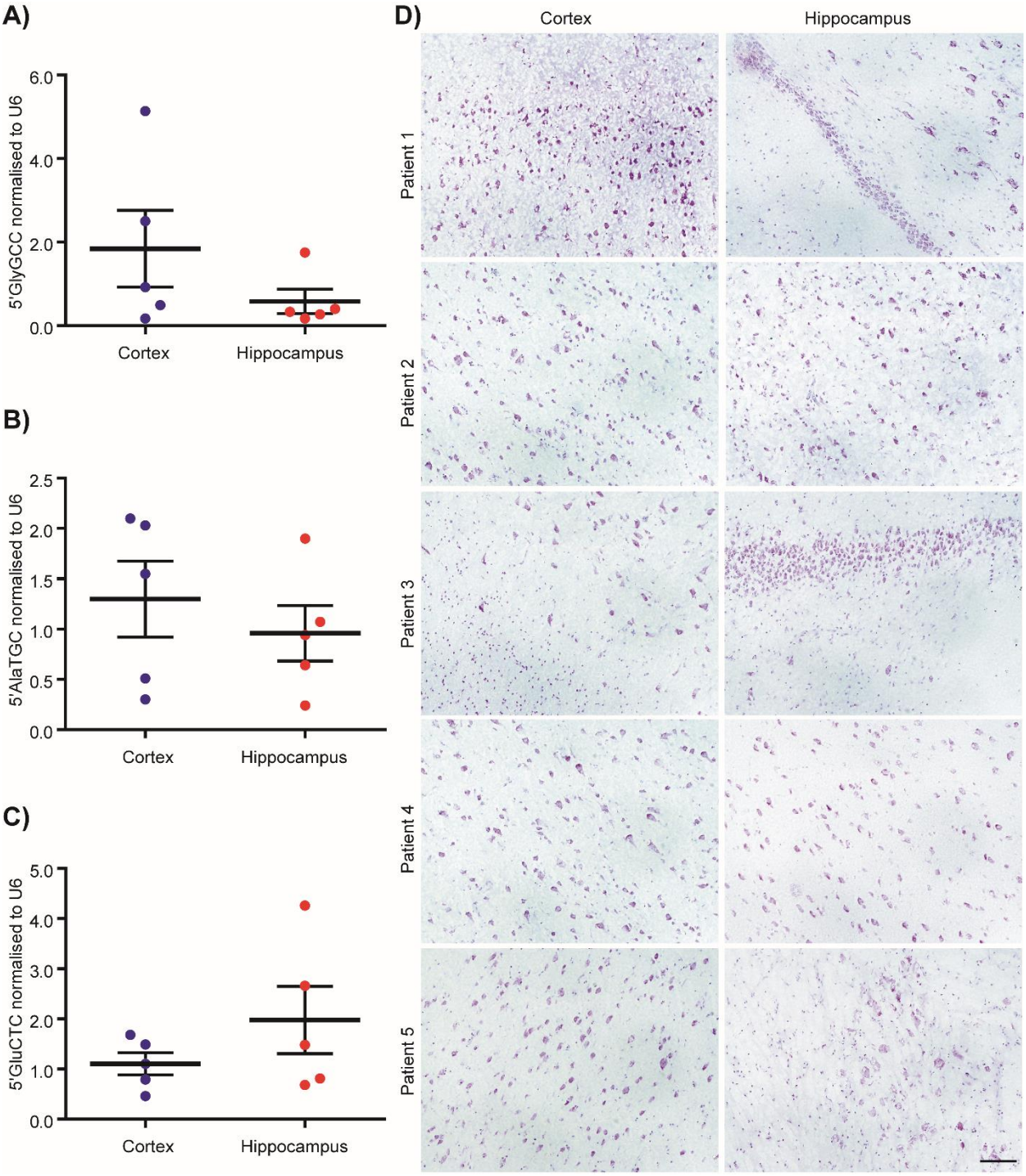

Figure 7: tRNA fragments are detectable in surgically resected focal epilepsy patient brain tissue. A) 5'GlyGCC, B) 5'AlaTGC, and C) 5'GluCTC were quantified in surgically resected cortical and hippocampal tissue from five of the focal epilepsy patients in the Dublin cohort of the study. tRNA fragments were detected in all regions analysed but no significant difference in levels could be detected. D) Histological analysis of Nissl stained neighbouring sections revealed no gross structural changes were apparent in these tissue samples. Scale bar 100 µm.

A)

#	MRI Result
1	Positive (HS)
2	Positive (PNH)
3	Positive (Hamartia)
4	Positive (Resection of dermoid cyst)
5	Negative
6	Positive (Resection of cavernoma)
7	Positive (Cortical Dysplasia)
8	Positive (Cavernoma)
9	Positive (Low-grade tumor)
10	Negative
11	Negative
12	Positive (AVM)
13	Negative
14	Unclear (MR lesion detectable, but lesion is not epileptogenic)
15	Positive (Residual HS after partial hippocampal resection)
16	Positive (Partial selective amygdalo-hippocampectomy right (2001), partial infarction of MCA (2001))
17	Negative
18	Positive (Previous left lateral temporal hippocampectomy)
19	Negative
20	Positive (Right MTS)
21	Unclear (Findings suspicious of right MTS)
22	Positive (Cortical Dysplasia)
23	Positive (Left MTS)
24	Unclear (Multicystic nodular mass in the left hippocampus)
25	Negative
26	Positive (HS)
27	Positive (Left hippocampal atrophy with increased signal)
28	Positive (Extensive signal abnormality and volume loss in left hippocampus)
29	Negative
30	Positive (Cortical Dysplasia)
31	Positive (Right hippocampal atrophy)
32	Negative

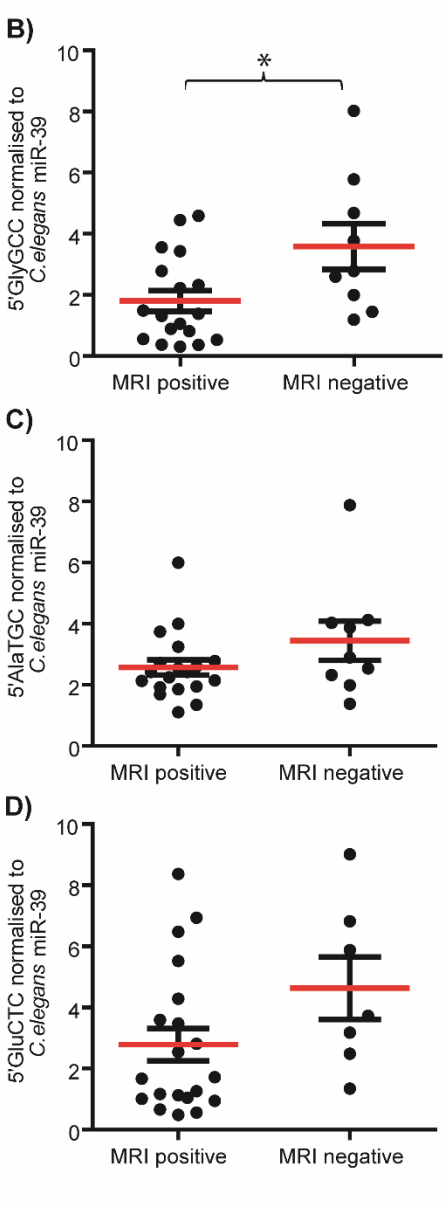

Figure 8: Analysis of structural abnormalities detected by MRI and tRNA fragment levels in pre-seizure samples. A) Summary of MRI findings, patients were classed as MRI positive or MRI negative, and 3 patients whose results were unclear/indeterminate were excluded from the analysis. Pre-seizure levels of B) 5'GlyGCC, C) 5'AlaTGC, and D) 5'GluCTC were increased in patients with no sign of structural abnormalities compared to those with lesions detected by MRI, with 5'GlyGCC levels significantly different as analysed by Mann-Whitney U test, $p = 0.02$. This data suggests that elevated tRNA fragment levels are not due to underlying tissue damage or scar formation which can be detected by MRI. Abbreviations: HS: Hippocampal Sclerosis, PNH: Periventricular Nodular Heterotopia, AVM: Arterio-Venous Malformation, MCA: Middle Cerebral Artery, MTS: Mesial Temporal Sclerosis.

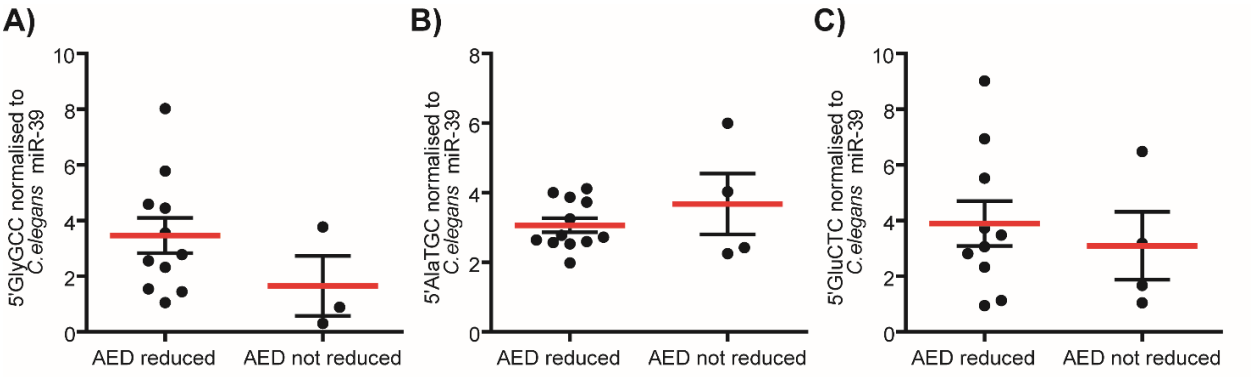

Figure 9: tRNA fragment levels in patients that did not have AEDs reduced and experienced seizures. Four focal epilepsy patients from the Dublin cohort did not have their AED medication reduced upon admittance to the EMU and experienced electro-clinical seizures. Comparing pre-seizure tRNA fragment levels between patients with and without AED reduction, we found no significant difference (Mann-Whitney U test).

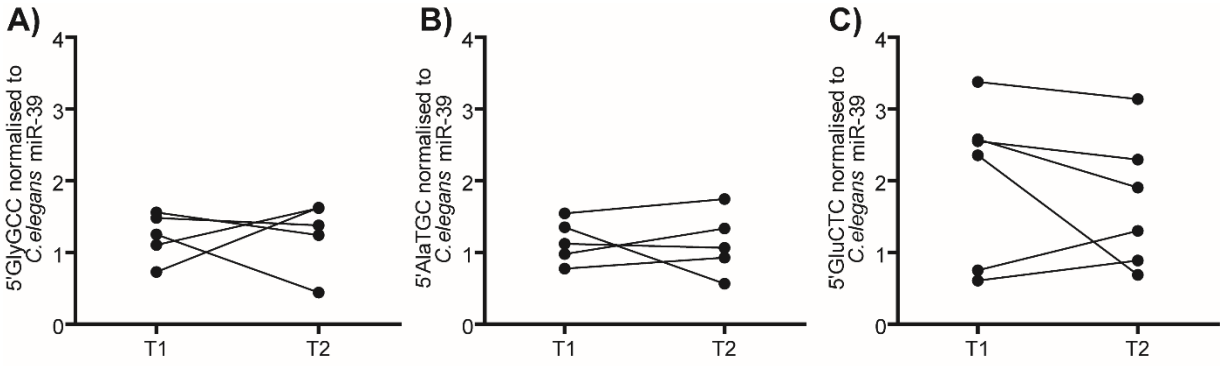

Figure 10: tRNA fragment levels in focal epilepsy patients with reduced AEDs that did not experience seizures. No signifcant changes in tRNA fragments A) 5'GlyGCC, B) 5'AlaTGC, or C) 5'GluCTC were detected in plasma samples collected 24 hours apart in patients whose AED medications were reduced but did not go on to expereience seizures. No significant difference in tRNA fragment levels was detected.

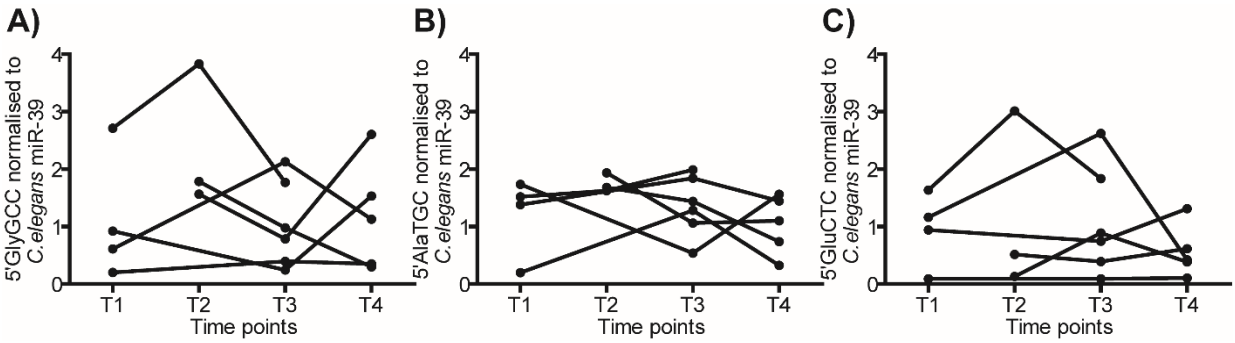

Figure 11: tRNA fragment levels in patients with psychogenic non-epileptic seizures (PNES). Six patients admitted to the EMU were subsequently diagnosed with PNES, analysis of tRNA fragment levels at timepoints T1-T4 indicated no significant difference in tRNA fragment levels were detected, and the "seizure-like" event occurred between time points T2 and T3.

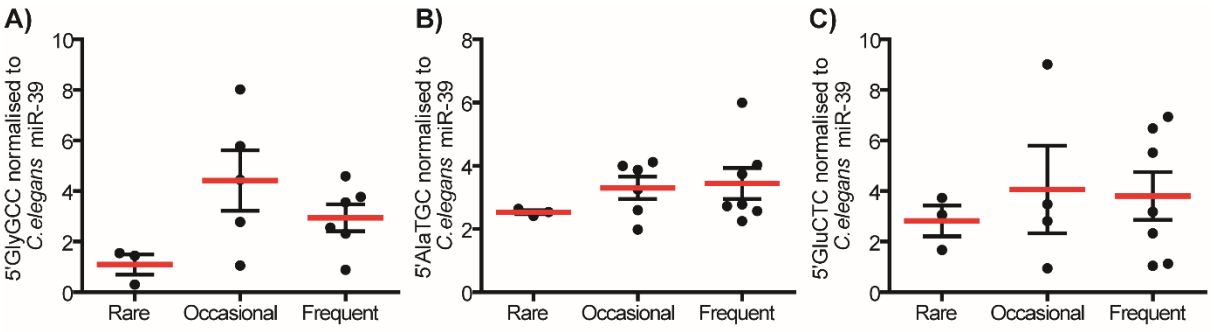

Figure 12: Analysis of pre-seizure tRNA fragments levels in relation to interictal activity. Video-EEG recordings from a period of 18-24 hours prior to seizure onset were reviewed by a Clinical Neurologist and patients were classified into 3 groups: Rare, Occasional, and Frequent interictal spiking activity. There was no significant difference in levels of A) 5'GlyGCC, B) 5'AlaTGC, or C) 5'GluCTC however all showed higher levels in patients experiencing occasional or frequent interictal activity.

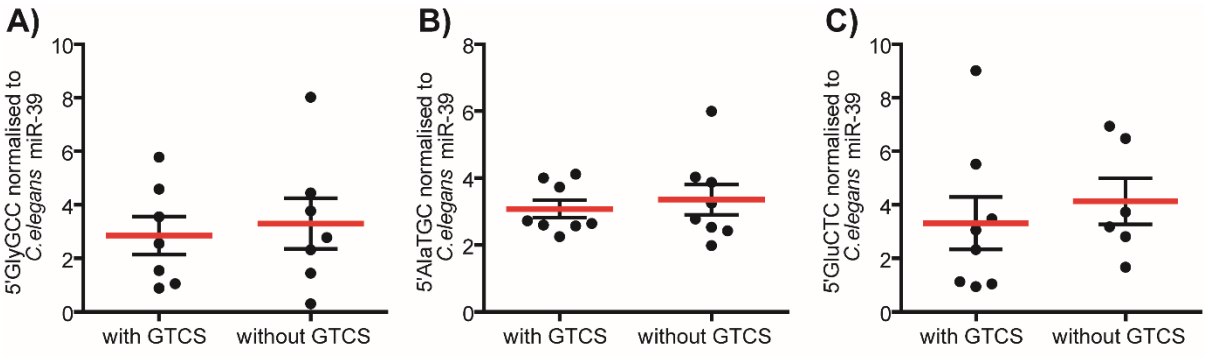

Figure 13: Analysis of pre-seizure tRNA fragment levels according to type of seizure experienced. All patients from the Dublin cohort (n = 16) experienced Complex Partial Seizures (CPS); however, some patients progressed on to generalized tonic-clonic seizures (GTCS). Comparison of tRNA fragment levels in patients with and without GTCS revealed no significant difference indicating pre-seizure plasma tRNA fragment levels cannot discriminate seizure type.