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Exploring the role of circulating exosome-derived transfer RNA fragments in patients with ischemic stroke

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Abstract

Objectives: to investigate exosome-derived transfer RNAs (tRNAs) in the plasma of patients in the chronic phase of ischemic stroke (IS). tRFs may be disease biomarkers, especially for disorders linked to hypoxic and extracellular stress.

Material and methods: We assessed samples from ten patients who had ischemic stroke (IS), collected them six months after the ictus, and compared to samples obtained from ten controls. We used small RNA sequencing for tRNA identification and quantification.

Results: We found that the most abundant class of tRFs identified in the patients' samples were 3' fragments (37.7%). Most interestingly, we found increased levels of two tRFs in patients, namely Phe-^{GAA} (p = 0.0146) and Leu-^{TAG} (p = 0.0331). These two fragments originate from mitochondrial tRNAs and may be related to mechanisms leading to abnormal regulation of cellular energy, DNA transcription, and oxidative stress. We also identified decreased tRNA-Gly and tRNA-Val derivatives in patients. Furthermore, we found that the expression signature of tRNA Phe-^{GAA} differentiates patients from controls with an area under the receiver operating characteristics (ROC) curve (AUC) = 0.78.

Conclusions: Our results indicate differential expression of circulating exosome-derived tRFs in the chronic stages of IS. The tRFs with the greatest differences in expression identified in this study may be linked to the mechanisms leading to disease, most likely related to underlying risk factors that may persist after the acute event. Thus, we provide suggestive evidence that tRFs may assist in risk stratification for individuals with increased susceptibility to IS.

Introduction

Stroke is one of the leading causes of death and disability worldwide [1]. The timely and adequate treatment of patients with stroke determines the short- and long-term prognosis [2,3]. Most importantly, stroke treatment extends beyond the acute phase, and preventive measures addressing risk factors are more likely to decrease disease burden with a lower cost [4–7]. Currently, there is great interest in developing treatment strategies directed toward neuroprotection and the control of mechanisms leading to tissue damage [3]. In this context, many recent studies have investigated circulating molecules, especially non-coding RNAs (ncRNAs), as potential biomarkers and novel therapeutic targets [8–11]. ncRNAs are regulatory molecules involved in many processes relevant to stroke pathogenesis, such as angiogenesis, vascular homeostasis, and inflammation [12–15]. ncRNAs can be released from cells through passive apoptosis or active processes associated with lipoproteins or extracellular vesicles, such as exosomes [16]. These vesicles are responsible for intercellular communication and molecular delivery [17]. In addition, the release of exosomes is known to be induced under specific biological conditions [18,19], making them a potential source of disease biomarkers [20,21]. Among the many classes of ncRNAs that may be transported inside these vesicles, there are transfer RNAs (tRNAs). These are associated with the

translational process, representing 4–10% of all circulating RNA molecules, from which around 1% can be observed inside circulating vesicles [22–25].

It is known that tRNAs have an important role in regulating protein synthesis and gene expression [26]. More recently, tRNAs have been linked to the control of cell death and proliferation, genome stability, and oxidative stress [23,24,26]. Most interesting, there is an increase in tRNA cleavage in certain conditions, a phenomenon that generates tRNA fragments (tRFs) (Fig. 1) [23,26]. Furthermore, tRFs have been linked to the mechanisms leading to several diseases, including neurological disorders [28–34]. Furthermore, tRNAs have been proposed as potential biomarkers for early tissue damage in stroke [33]. Indeed, a few studies have shown increased tRF levels in the acute phase of stroke in neuronal cell cultures and patient samples [33–35]. Furthermore, the levels of tRFs increased significantly in the first 24 h after stroke onset and could be correlated to infarct size and hematoma volumes in patients [33]. Most interestingly, higher levels of tRFs seven days after the ictus were associated with poorer functional outcomes [33]. However, there is no information about the levels of tRNAs in the chronic phase of stroke, making it difficult to know whether these molecules are markers of acute tissue damage or perhaps associated with the mechanisms leading to the disease that persists beyond the ictus. Thus, we designed the current study to investigate exosome-derived tRNAs in the plasma of patients in the chronic phase of ischemic stroke (IS).

Methods

Cohort

We collected samples from ten patients with chronic IS, six to ten months after the ictus, and ten control subjects without stroke. We obtained written informed consent from all patients and controls. The research protocol and the informed consent form were approved by the Research Ethics Committee of the University of Campinas (UNICAMP), Campinas, SP, Brazil. All subjects were over 50 years old, age-matched, and were followed prospectively in the outpatient clinic of the UNICAMP hospital (Table 1). Stroke diagnoses and risk factors were determined following a detailed clinical and neuroimaging investigation performed by two neurologists, AS and WMA, who have training in neurovascular disorders. Patients were classified into IS subtypes, thrombotic or embolic, according to the TOAST classification [36]. In addition, stroke severity was evaluated by the attending neurologists using the National Institute of Health Scale Score (NIHSS) [37]. Control subjects were evaluated in the hypertension clinic. Based on clinical and magnetic resonance imaging (MRI) evaluation, they had not experienced a stroke or neurological damage.

Table 1
Cohort characterization according to age, sex, risk factors, and stroke classification[32].

		Patients with ischemic stroke	Controls
Age, years (mean ± standard deviation)		65.5 ± 7.7	64.5 ± 6.2
Sex	Female	5	8
	Male	5	2
Hypertension	Yes	4	10
	No	6	0
Diabetes mellitus	Yes	3	5
	No	7	5
Dyslipidemia	Yes	5	6
	No	5	4
Smoking	Yes	2	3
	No	4	7
	Ex	4	0
Alcoholism	Yes	2	0
	No	6	2
	Ex	1	0
	NA	1	8
Carotid Stenosis	Yes	3	0
	No	7	10
Stroke Classification	Thrombotic	5	-
	Embolic	5	-
NIHSS (mean)	Acute	12.2	-
	Chronic	4.2	-
Thrombolysis	Yes	1	-
	No	5	-
	NA	4	-

Analysis

We purified exosomes from plasma samples using the miRCURY Exosome Serum/Plasma kit (Qiagen, Inc.) and extracted total RNA using the miRNeasy Serum/Plasma Advanced (Qiagen, Inc.). Then, we performed small RNA sequencing with the QIAseq miRNA library kit (Qiagen, Inc.), following the manufacturer's recommended protocol. All samples were sequenced simultaneously on NextSeq 500 equipment (Illumina, Inc) with 75 single-end cycles.

The raw data were quality filtered and trimmed by fastx_toolkit, and adaptor sequences were removed using Cutadapt. Quality control was performed using FastQC to ensure high-quality data. Filtered reads were first mapped to rat tRNA sequences using Bowtie, allowing one mismatch. Next, we analyzed the data from patients and controls using DeSeq2, with the threshold p-value adjusted with the Bonferroni test [38,39]. We also constructed a scatterplot to observe the distribution of the fragment lengths and positions over the tRNA molecules. Finally, we evaluated the tRNA targets using the tRFTars prediction tool (http://trftars.cmuzhenninglab.org:3838/tar/) [40]. Subsequently, we selected the genes with a false discovery rate (FDR) < 0.05 to construct enriched molecular pathways using the Reactome Software [41]. Finally, we built receiver operating characteristic (ROC) curves using GraphPad software (San Diego, CA, USA).

Results

We identified 61 exosome-derived tRFs in patients and controls (Table 2), which corresponds to approximately 1% of the total exosomal RNA identified in the small RNA sequencing (Supplementary File, Supplementary Fig. 1). Overall, we observed an increased concentration of tRFs in controls (57.6%) compared with patients (42.4%). In addition, we found that 59% of the differentially expressed tRFs were downregulated in patients, while 41% were upregulated. The distribution of the different types of tRF fragments was 37.7% of 3' fragments, 18% of undetermined fragments (potentially 3' or 5' fragments), 11.5% of half tRFs, and 6.6% of 5' fragments (Supplementary File, Supplementary Fig. 2A and 2B). Most interesting, we found two tRFs significantly increased in patients, namely Phe-^{GAA} (p = 0.0146) and Leu-^{TAG} (p = 0.0331). Although these differences were subtle and did not survive the corrections for multiple comparisons (Table 2), they may still indicate biological relevant results, as depicted in Fig. 2.

Table 2
Differential expression of exosome-derived tRNA fragments (tRFs) in patients with ischemic stroke compared with controls.

tRFs	baseMean	log2FC	p	p-adj
Phe-^{GAA}	60.13	0.62	0.0146	0.8908
Leu-^{TAG}	50.12	0.67	0.0331	0.9816
Gly-^{GCC}	2437.29	-0.86	0.0600	0.9816
Ser-^{AGA}	91.07	-0.40	0.0644	0.9816
Glu-^{CTC}	3065.82	-0.53	0.1176	0.9936
nmt-tRNA-Ser-^{TGA}	1.06	-1.75	0.1249	0.9936
Tyr-^{ATA}	4.51	-0.96	0.1316	0.9936
Trp-^{CCA}	87.23	0.52	0.1527	0.9936
SeCe-^{TCA}	17.11	-0.52	0.1895	0.9936
Cys-^{GCA}	94.78	0.62	0.2064	0.9936
Pro-^{AGG}	33.55	-0.31	0.2263	0.9936
Leu-^{AAG}	76.00	0.33	0.2271	0.9936
Asp-^{GTC}	312.51	-0.22	0.2360	0.9936
Pro-^{CGG}	33.89	-0.30	0.2886	0.9936
Val-^{CAC}	1011.23	-0.42	0.3119	0.9936
Val-^{AAC}	928.28	-0.43	0.3145	0.9936
Val-^{TAC}	39.46	-0.32	0.3227	0.9936
Tyr-^{GTA}	30.85	0.29	0.3302	0.9936
Gln-^{TTG}	33.56	0.34	0.3525	0.9936
Leu-^{CAA}	68.87	-0.16	0.3882	0.9936
Pro-^{TGG}	62.51	0.25	0.4192	0.9936
Asn-^{GTT}	60.40	0.25	0.4778	0.9936
Cys-^{ACA}	2.87	0.59	0.4964	0.9936

tRFs	baseMean	log2FC	p	p-adj
Ser-^{TGA}	156.17	0.13	0.5075	0.9936
Ile-^{GAT}	2.29	-0.38	0.5332	0.9936
Gly-^{TCC}	38.48	-0.13	0.5464	0.9936
Met-^{CAT}	19.13	0.27	0.5477	0.9936
nmt-tRNA-Leu-^{TAA}	0.41	0.93	0.5692	0.9936
Arg-^{TCT}	67.11	-0.13	0.5702	0.9936
Sup-^{CTA}	0.62	-0.58	0.6207	0.9936
Glu-^{TTC}	584.30	-0.14	0.6603	0.9936
Sup-^{TTA}	4.98	-0.23	0.6964	0.9936
Arg-^{TCG}	25.96	-0.10	0.7245	0.9936
Ser-^{GCT}	137.94	0.07	0.7374	0.9936
Asn-^{ATT}	0.78	-0.34	0.7382	0.9936
Arg-^{CCT}	23.92	-0.11	0.7449	0.9936
Thr-^{AGT}	8.93	-0.11	0.7483	0.9936
nmt-tRNA-Pro-^{TGG}	0.18	-0.80	0.7633	0.9936
Thr-^{TGT}	16.89	-0.10	0.7639	0.9936
Lys-^{CTT}	290.25	0.08	0.7657	0.9936
Leu-^{TAA}	43.76	-0.08	0.7703	0.9936
Ala-^{CGC}	108.38	0.06	0.8095	0.9936
nmt-tRNA-Gln-^{TTG}	2.26	-0.15	0.8120	0.9936
iMet-^{CAT}	67.76	-0.07	0.8132	0.9936
Ser-^{CGA}	148.99	0.05	0.8251	0.9936
Gly-^{CCC}	657.89	-0.06	0.8309	0.9936
Ser-^{ACT}	2.85	0.13	0.8334	0.9936

tRFs	baseMean	log2FC	p	p-adj
Arg-^{CCG}	24.62	0.06	0.8663	0.9936
Lys-^{TTT}	120.17	0.04	0.8727	0.9936
Pro-^{GGG}	0.11	-0.44	0.8846	0.9936
Ala-^{AGC}	102.54	0.02	0.8908	0.9936
Ala-^{TGC}	139.91	0.03	0.9112	0.9936
Ile-^{AAT}	39.58	0.03	0.9129	0.9936
Arg-^{ACG}	25.56	-0.03	0.9273	0.9936
SeC-^{TCA}	0.29	-0.16	0.9295	0.9936
His-^{GTG}	110.25	-0.02	0.9338	0.9936
Ile-^{TAT}	2.86	-0.04	0.9607	0.9936
nmt-tRNA-Gln-^{CTG}	0.03	-0.13	0.9661	0.9936
Thr-^{CGT}	35.71	0.01	0.9692	0.9936
Leu-^{CAG}	150.69	0.01	0.9774	0.9936
Gln-^{CTG}	70.27	0.00	0.9943	0.9943

Next, we performed a pathway analysis based on the target genes of the tRFs Phe-^{GAA} and Leu-^{TAG}. These were tRFs showing differential expression based on the nominal p-value < 0.05). We also included tRFs Glu-^{CTC}, Glu-^{TTC}, Gly-^{CCC}, Gly-^{GCC}, Val-^{AAC}, Val-^{CAC} due to their high sequencing depth, over 500 reads. We observed pathways associated with DNA translation and gene expression regulation, programmed cell death, the cell cycle, and inflammatory processes (Supplementary File, Supplementary Table 1)[41]. Furthermore, we found enrichment of some pathways, such as *TRAIL signaling*— correlating with the tRNAs Leu-^{TAG}, Glu-^{CTC}, Glu-^{TTC}, and Val-^{AAC}/Val-^{CAC}—and *TP53 Regulates Transcription of Caspase Activators and Caspases* [41] – correlating with the tRNAs Glu-^{CTC}, Glu-^{TTC}, Gly-^{CCC}, and Gly-^{GCC} (Fig. 3; Supplementary File, Supplementary Table 1). Based on these tRFs, we performed a prediction analysis to evaluate the ability of these fragments to differentiate the patient and control groups. The greatest area under the receiver operating characteristics (ROC) curve (area under the curve - AUC) = 0.78 was obtained for Phe-^{GAA} (Supplementary File, Supplementary Fig. 3).

Discussion

Stroke is a multifactorial disease characterized by obstruction of the blood flow to the brain and can be classified into two large groups, ischemic (IS) or hemorrhagic (HS) [1]. In addition, the TOAST classification categorizes IS into five subtypes according to etiology [36]. Considering the different stages of the disease, IS can be divided into three main phases: acute (at the beginning of symptoms), sub-acute (a transition stage for deficit delimitation), and chronic (associated with the establishment and recovery of deficits) [3]. Although there have been several studies in the field, stroke diagnosis still relies predominantly on clinical and neuroimaging evaluations [42]. Thus, there is great interest in identifying molecular biomarkers, including ncRNAs, that could assist in the diagnosis and predict the prognosis of patients with IS. Furthermore, these molecules could provide insights into the mechanisms leading to IS and serve as targets for novel therapeutic and preventive measures [9,43–46].

tRNAs are novel molecules associated with several regulatory processes, such as gene expression and ncRNA biogenesis [24]. It has been observed that under specific biological conditions—for example, during stress or hypoxia—cleavage on the anticodon loop of mature tRNA molecules by the endonuclease Angiogenin may occur, creating 29–50 nucleotides molecules known as half tRFs [23,27]. Depending on the loop end, half tRFs can be classified as 5' or 3' half tRFs [23] (Fig. 1). Another cleavage may occur by Dicer and RNases at the mature tRNA ends, generating fragments of 14–30 nucleotides called 5' or 3' tRFs [23]. Thus, a disease linked to stress and hypoxic conditions could increase the number of tRNA derivatives, which in turn may serve as biomarkers [29,34,47,48]. Furthermore, it has been shown that some of these tRFs are associated with the Argonaute (AGO) protein complex and, similarly to microRNAs, may lead to suppression of target genes [49]. However, differently from microRNAs, due to their transcription-independent biogenesis, tRFs can respond quickly to changes in extracellular conditions [50,51]. tRNA internalization in exosomes is important to increase stability, protecting these molecules from RNase activity [51,52].

Over the past few years, there has been significant interest in investigating a possible relationship between changes in tRNA and the mechanisms of disease [30,33,34,53,54]. Indeed, it is known that the human brain is sensitive to defects in tRNAs, and many neurological diseases may be linked to these modifications [55–59]. For example, reducing tRNA-^{ARG} can cause protein misfolding in brain cells, inducing neurodegenerative changes in mice [60]. Moreover, researchers showed that a reduction in mature tRNAs due to mutations in cleavage and polyadenylation factor I subunit 1 (CLP1) caused neuromotor disabilities and increased oxidative stress *in vivo* and *in vitro* models [61]. Furthermore, an *in vitro* study found increased levels of half tRFs after cell stress induced by oxygen deprivation, which was reversed after treatment with neuroprotective agents, leading to the normalization of tRF levels [62]. In addition, 5' tRFs could be increased in tissues by post-transcriptional modifications of RNA molecules. They may affect protein translational rates and stress pathways, such as apoptosis, inducing a reduction in the size of hippocampal and cortical neurons [55]. Furthermore, disruptions in tRNA biosynthesis may change neuronal homeostasis, leading to neuronal damage and abnormal neurological development due to modification of protein synthesis, tRNA splicing, and post-transcriptional mutations [48]. In Parkinson's

disease, researchers identified a set of tRFs that could be potential biomarkers for the presence of dementia in patients when compared with control subjects [32]. In amyotrophic lateral sclerosis, 5' tRF-^{ALA} associated with the presence of the C9ORF72 variant contributes to motor neuron death and the progression of neurodegeneration [31].

A few studies investigating tRNAs have been performed in cell culture, animal models, and biological samples from patients with IS. However, these studies have been limited to the acute phase of the disease [33–35, 63]. The researchers generally observed higher tRNA expression in patients with acute stroke than controls, indicating increased tRNA cleavage during hypoxia and stress conditions [33–35, 63]. Li and collaborators [63] also identified an increase in 5' half tRFs with a similar cleavage pattern 24 h after ischemic onset in animal models of IS[63], indicating that tRF biogenesis is strictly regulated during ischemic conditions. Nguyen et al. [34] observed a set of tRNAs (Tyr-^{GTA}, Thr-^{CGT}, and Val-^{CAC}) that may act as diagnostic biomarkers in the acute stage and could distinguish among stroke subtypes: IS, HS, and stroke mimics. In addition, Ishida et al. [33] evaluated patients in the acute and subacute stages of IS and HS. They observed an increase in tRNAs in patients upon hospital admission. The levels of these molecules seven at seven days after the stroke ictus correlated with the patient's poor functional outcome 30 days after the acute event [33]. Recently, Winek et al. [50] observed an increase in tRFs in the acute phase of IS accompanied by a decrease in microRNA levels, a change that may affect the neuroimmune response in IS by regulating the gene expression of CD14 + monocytes.

Unlike these previous reports, we performed our study in the chronic phase of IS. We aimed to investigate whether these tRFs are only markers of acute tissue damage or may be associated with persistent biological changes that could be observed beyond the ictus. Our results revealed an overall decrease in tRFs in the plasma of patients in the chronic stage of IS compared with controls, indicating that after the initial increase in tRFs due to acute tissue damage, there is a decrease in tRFs levels over time. Furthermore, we detected increased 3' tRFs in chronic IS, a finding different from the increased 5' tRFs reported in acute IS [63], suggesting a different cleavage pattern of tRNAs during stroke recovery.

Furthermore, we identified specific differentially expressed tRFs in patients with chronic IS compared with controls, namely Phe-^{GAA} ($p = 0.0146$) and Leu-^{TAG} ($p = 0.0331$), both upregulated. We also found that the plasma concentration of tRNA Phe-^{GAA} could differentiate patients from controls with 78% accuracy. These fragments are derived from mitochondrial tRNAs and have been proposed as potential biomarkers of cancer and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) [53,64–67]. In patients with leukemia, the tRFs Phe-^{GAA} and Leu-^{TAG} are increased [53,65], and the overexpression of these molecules seems to be correlated with a poorer prognosis [53,65,68,69], probably due to induction of angiogenesis and macrophage migration. Interestingly, these two mechanisms may be relevant for stroke recovery [70–72]. Furthermore, according to tRFTars and Reactome pathway analysis, Phe-^{GAA} and Leu-^{TAG} regulate many genes associated with DNA replication, the cell cycle, and inflammation (Supplementary File, Supplementary Table 1) [40,41].

Of note, a mutation in mtRNA-Phe was identified in a patient with MELAS and carotid artery stenosis [67]. In that report, the authors speculate that tRNA-Phe mutations may affect the vessel architecture and predispose individuals to develop embolic strokes [67]. Furthermore, mutations in tRNA-Leu have been reported in patients with cancer [65,73], MELAS [73,74], and diabetes [75]. In these patients, the presence of mutations was associated with poor survival rates and recurrent stroke episodes. Mutations of mitochondrial tRNAs are usually described as inhibiting mitochondrial DNA translation, which affects the regulation of homeostasis, cellular energy through the respiratory chain, and production of reactive oxygen species [73,76].

Although not reaching statistical significance, other interesting tRFs identified as downregulated in our patients are tRNA-Gly and tRNA-Val derivates (Gly-^{CTC}, Gly-^{CCC}, Gly-^{GCC}, Val-^{AAC}, and Val-^{CAC}). These tRNAs have been observed, as differentially expressed, in animal models of acute IS and are associated with inhibiting angiogenesis and cell proliferation [63]. Val-^{CAC} was identified as upregulated in patients with acute IS and intracerebral hemorrhage, and its concentration was able to differentiate between the two different stroke subtypes [34].

Our study's strengths are that all patients were followed prospectively by the same group of neurologists and were subjected to the same thorough clinical and imaging investigation. In addition, all individuals in the control group had a brain MRI that excluded the possibility of an asymptomatic stroke event. However, our study also has limitations, such as the fact that we could not classify 26.2% of the tRFs sequenced. This limitation has been commonly reported in the literature due to technical issues in RNA sequencing experiments and annotations [80,81]. In addition, we did not study methylation and post-translational changes that may occur in tRNAs and may impact tRNA function, structure, and stability [22,27].

In conclusion, we identified a different expression signature of exosome-derived tRFs in patients with chronic IS compared with controls. Of note, we found increased levels of Phe-^{GAA} and Leu-^{TAG}, which are linked to mechanisms such as regulation of cellular energy, transcription, and oxidative stress. In particular, the levels of Phe-^{GAA} discriminate patients with IS from controls. Based on our results, we hypothesize that the differential levels of circulating exosome-derived tRFs in chronic IS may be linked to the underlying risk factors for stroke that persist after the acute event. Thus, our findings warrant additional studies to explore the potential role of tRFs as biomarkers and therapeutic targets for secondary prevention of IS, assisting in risk stratification of individuals with increased susceptibility for IS.

Declarations

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Author contributions

Amanda Donatti, conceptualization, methodology; **Yan Yan**, conceptualization, methodology; **Morten T Venø**, formal analysis; **Fabiana Oliveira**, investigation; **Alessandro Souza**, clinical investigation; **Wagner M Avelar**, clinical investigation; **Wilson Nadruz**, clinical investigation; **Jørgen Kjems**, supervisor; **Iscia Lopes-Cendes**, supervision.

Data accessibility statement

Data available in article supplementary material

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Figures

Figure 1

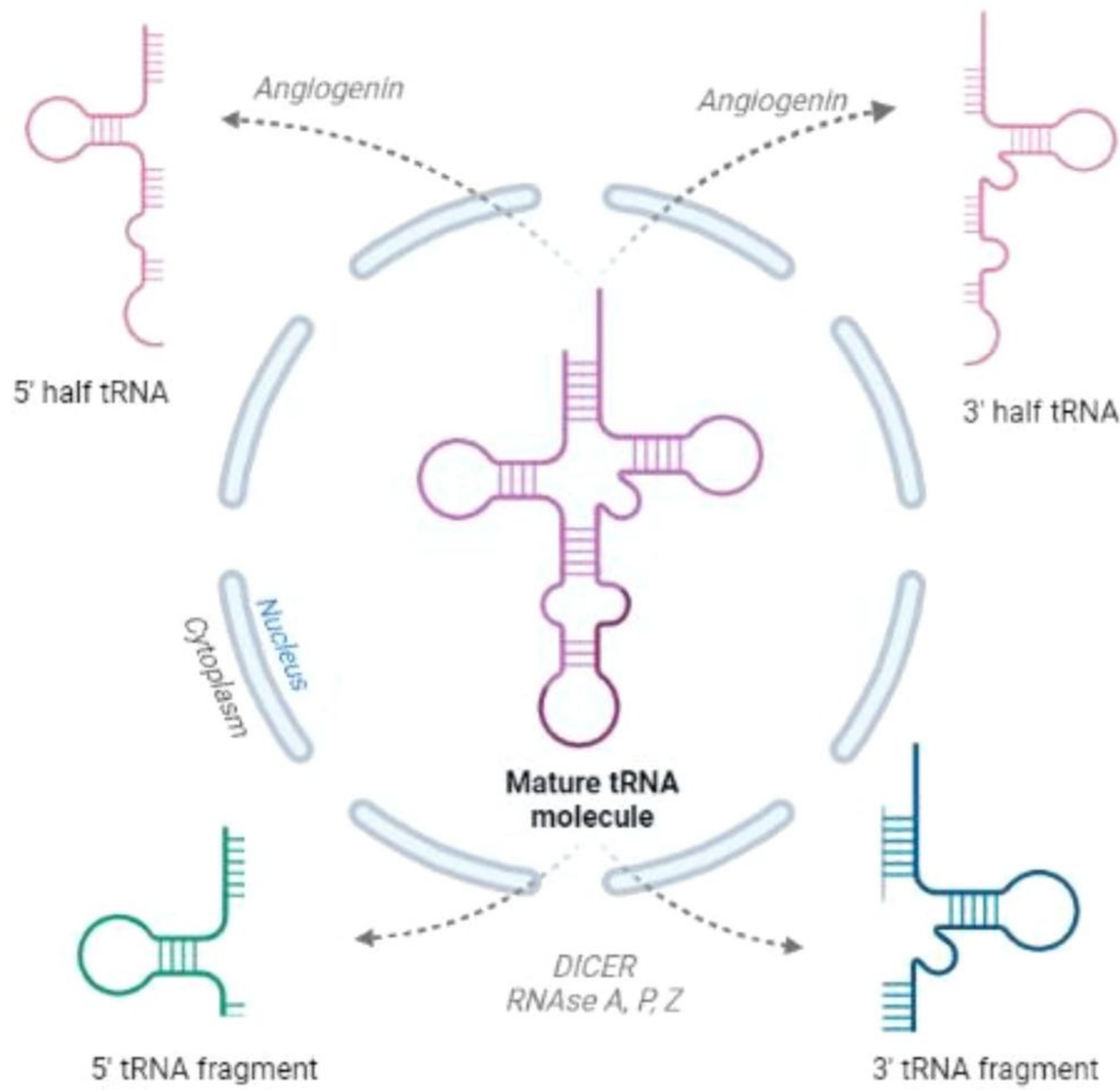

Figure 1

tRNA fragment (tRF) biogenesis from a mature tRNA molecule [23,26,27]. Created with BioRender.com

Figure 2

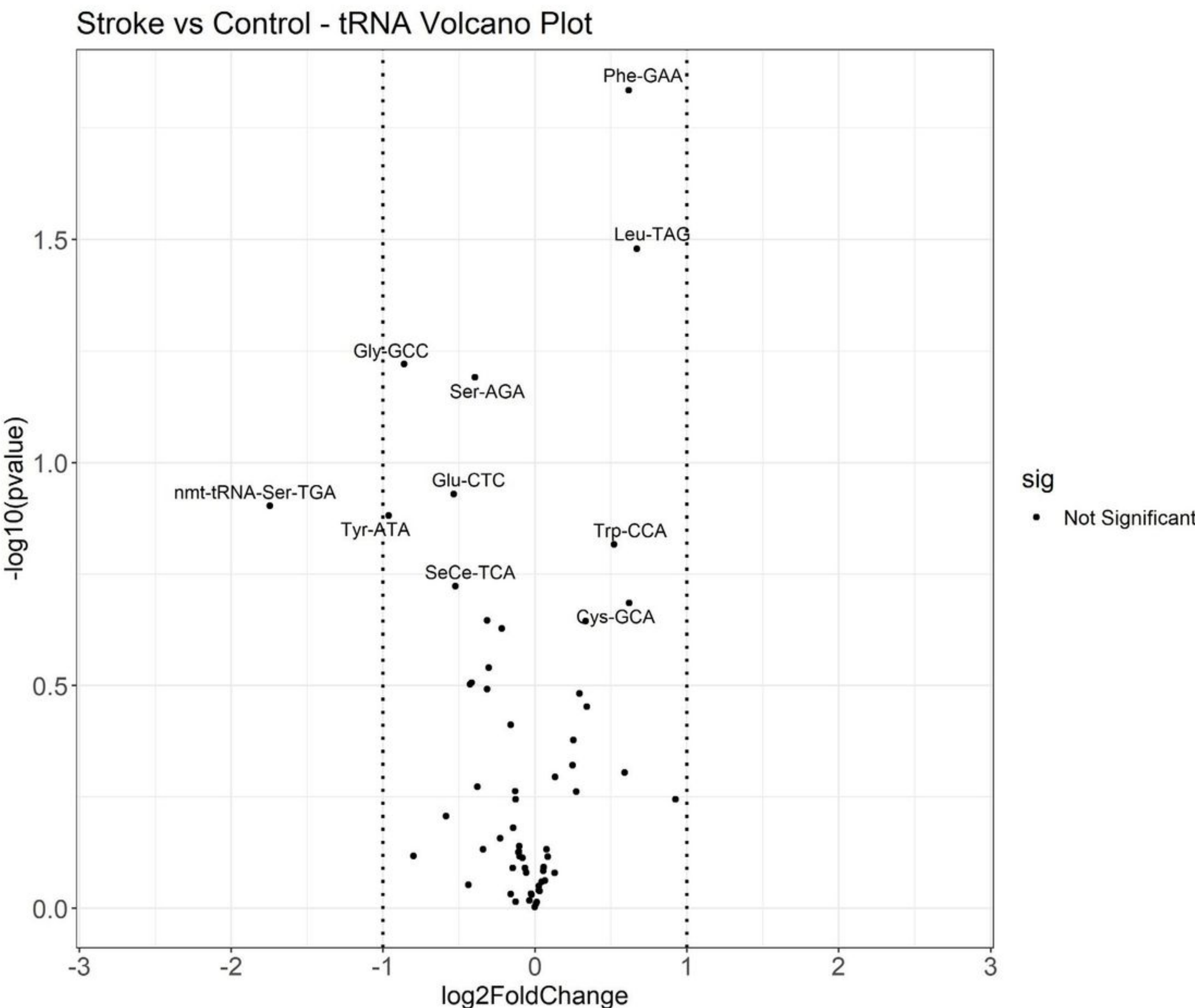

Figure 2

Graphical representation of tRFs distribution obtained by exosomal small RNA sequencing of plasma samples obtained from patients with chronic ischemic stroke and controls.

Figure 3

Molecular pathways associated with tRF expression

TRAIL signaling
TP53 Regulates Transcription of Caspase Activators and Caspases
RUNX1 regulates transcription of genes involved in WNT signaling
Transcriptional Regulation by E2F6
EGR2 and SOX10-mediated initiation of Schwann cell myelination
Interleukin-2 signaling
TP53 Regulates Transcription of Death Receptors and Ligands
Erythropoietin activates STAT5
STAT5 Activation
DNA Replication
Interleukin-9 signaling
Interleukin-21 signaling
DNA Replication Pre-Initiation
FasL/ CD95L signaling
TNFs bind their physiological receptors
CDC42 GTPase cycle
Lipophagy
RHOJ GTPase cycle
Recognition and association of DNA glycosylase with site containing an affected purine
SUMOylation of chromatin organization proteins
Transcriptional regulation by small RNAs

Pathways

2 4 6 8 10

tRFs

Figure 3

Distribution of molecular pathways according to the presence of two or more tRNA fragment (tRF) target genes. Each bar reflects the number of tRFs associated with the pathways.

Supplementary Files

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