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FRANCESCO FISICARO

Circulating Biomarkers for Stroke in Cancer Patients: a Pilot Study

Ph.D. Coordinator: Prof. Stefania Stefani

Supervisor: Prof. Manuela Pennisi

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ABSTRACT

The association between stroke and cancer seems to be rising over time. It is known that patients with cancer are at a relatively higher risk to die because of a stroke. However, the precise reason for increased stroke risk in cancer patients is still debated, and many facets of this association remain uncertain, including the specific molecular mechanisms responsible for this severe complication. In the last years, the role of epigenetic alterations in stroke, in particular the modulation of microRNA (miRNA) expression, is gaining growing attention. In this research project, we hypothesized that cancer patients with stroke or at risk for stroke might show a specific expression pattern of miRNAs. For this purpose, we aimed to analyze blood samples obtained from patients with active solid cancer and stroke in order to identify any epigenetic circulating biomarker or causative factor that might predict or be associated with a higher risk of stroke. Briefly, a prospective cross-sectional study was performed. Four groups of adult patients were enrolled: Group 1 consisted of subjects with active cancer and acute ischemic stroke; Group 2 was represented by patients with acute ischemic stroke without evidence of cancer; Group 3 included patients with active solid cancer without stroke; Group 4 included healthy subjects. To obtain more reliable results on circulating miRNA alterations associated with ischemic stroke, liquid biopsy samples were analyzed by using a custom detection and amplification protocol based on the high-sensitive droplet digital PCR (ddPCR) system. The data generated were analyzed considering the patients' clinical and pathological features in order to identify novel effective epigenetic biomarkers associated with different clinical patterns. Overall, the results of the study allowed the identification of two miRNAs, hsa-miR-125b-5p and hsa-miR-199a-5p, involved in the regulation of multiple genes associated with key cellular and molecular pathways responsible for the alteration of the complement and coagulation cascades or the alteration of inflammatory and angiogenetic processes. These novel candidate biomarkers showed a good predictive value for stroke risk in cancer patients. Finding from this study might possibly lead to an improvement in the management and diagnostic strategies for the early identification of these patients, although further validation is needed.

SOMMARIO

L'associazione tra ictus e cancro sembra essere in aumento nel corso del tempo. È risaputo che pazienti con cancro presentano un rischio relativamente più alto di morire a causa di un ictus. Tuttavia, la ragione precisa per l'aumentato rischio di ictus in pazienti con cancro è ancora dibattuta e molti aspetti di questa associazione rimangono incerti, inclusi gli specifici meccanismi molecolari responsabili di questa severa complicanza. Negli ultimi anni, il ruolo di alterazioni epigenetiche nell'ictus, in particolare la modulazione dell'espressione di microRNA (miRNA), sta ricevendo una crescente attenzione. In questo progetto di ricerca, abbiamo ipotizzato che pazienti con cancro e ictus o a rischio di ictus potessero mostrare uno specifico aspetto di espressione di miRNA. Per questo motivo, abbiamo mirato ad analizzare campioni ematici ottenuti da pazienti con cancro solido attivo e ictus con lo scopo di identificare eventuali biomarcatori epigenetici circolanti o fattori causali che potessero predire o essere associati con un più alto rischio di ictus. In breve, è stato eseguito uno studio traversale prospettico. Sono stati reclutati quattro gruppi di pazienti adulti: il Gruppo 1 consisteva in soggetti con cancro attivo e ictus ischemico acuto; il Gruppo 2 era rappresentato da pazienti con ictus ischemico acuto senza evidenza di cancro; il Gruppo 3 includeva pazienti con cancro solido attivo senza ictus; il Gruppo 4 includeva soggetti sani. Per ottenere risultati più affidabili sulle alterazioni di miRNA circolanti associati a ictus ischemico, campioni di biopsia liquida sono stati analizzati usando un protocollo modificato di rilevazione e amplificazione basato su un sistema droplet digital PCR (ddPCR) ad alta sensibilità. I dati prodotti sono stati analizzati considerando le caratteristiche cliniche e patologiche dei pazienti per identificare nuovi efficaci biomarcatori epigenetici associati a differenti quadri clinici. Complessivamente, i risultati dello studio hanno permesso l'identificazione di due miRNA, hsa-miR-125b-5p e hsa-miR-199a-5p, coinvolti nella regolazione di molteplici geni associati a pathway cellulari e molecolari chiave responsabili dell'alterazione del complemento e della cascata coagulativa o dell'alterazione di processi infiammatori e angiogenici. Questi nuovi possibili biomarcatori hanno mostrato un buon valore predittivo per il rischio di ictus in pazienti con cancro. I risultati

di questo studio potrebbero condurre a un miglioramento nella gestione e nelle strategie diagnostiche per la identificazione precoce di questi pazienti, sebbene una ulteriore validazione sia necessaria.

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1 Introduction

1.1 Stroke in cancer patients

1.1.1 Epidemiology

Cancer and stroke are the second and the fifth leading cause of death in the United States, respectively [1]. The estimated lifetime incidence of malignant cancer is 40%, and currently, 13 million Americans are living with cancer [2]. Both diseases cause substantial disability and societal costs [3]. It has been suggested that cancer-related coagulation disorders, tumor embolism or adverse effects from oncological treatment could be a trigger for ischemic stroke [4–7]. About 10% of hospitalized ischemic stroke patients have a co-morbid cancer, and this association seems to rise over time. In fact, between 1997 and 2006 there was a significant decrease in the prevalence of stroke hospitalizations, but not among cancer patients [8]. A recent Dutch study found that in a cohort of stroke patients, the prevalence of cancer, most prominently cancer of the central nervous system, head and neck, lower respiratory, and urinary tract, was higher than in the general population [9]. Several previous studies have shown that ischemic stroke can be the first manifestation of cancer [10–14]. In addition, in a Norwegian cohort of 1,282 ischemic stroke patients with no history of cancer, approximately 4% of patients received a diagnosis of cancer after a stroke [15]. The median time from stroke onset to cancer diagnosis was 14.0 months. 41.8% of patients were diagnosed with cancer within 1 year and 23.6% within 6 months [15]. Overall, the risk of stroke among cancer patients is twice that of the general population and rises with longer follow-up time. The relative risk of stroke, compared to the general population, is highest in those with cancers of the brain and the upper gastrointestinal tract. Patients of any age diagnosed with brain tumors are at risk for stroke throughout life [16]. Indeed, patients with acute ischemic stroke in the setting of active cancer face a substantial short-term risk of recurrent ischemic stroke and other types of thromboembolism. In addition, most patients with cancer and stroke are elderly and male, although patients of any age, sex, or race/ ethnicity can be affected [17]. The most common types of

cancer among hospitalized stroke patients are prostate, breast, gastrointestinal, and colorectal [8].

1.1.2 Clinical manifestations

The clinical presentation of stroke in cancer patients is similar to that occurring in the general population, although ischemic strokes tend to be more severe at hospital admission in cancer than in non-cancer patients, and more often involve infarcts in multiple vascular territories of the brain. In-hospital mortality is also significantly higher [18]. Indeed, patients with gastrointestinal cancers (especially that of the pancreas, liver, and esophagus) are at a relatively high risk to die because of a stroke at any time after diagnosis [16]. Furthermore, cancer patients with ischemic stroke face high rates of recurrence. In a retrospective study conducted at a cancer center, 31% of active cancer patients were diagnosed with a recurrent thromboembolic event by 3 months, including 13% with recurrent ischemic stroke, which is nearly three-fold higher than typical recurrent stroke rates in non-cancer patients [17]. Cancer types classically associated with venous thromboembolism, such as pancreas, gastric, and lung, seem to have the highest risks of arterial thromboembolism [19]. Overall, about 50% of cancer-associated strokes are deemed cryptogenic after evaluation, compared to 30% of cryptogenic strokes in the general population [17]. Most cancer patients with ischemic stroke present with hemiparesis, speech disturbance, and/or visual field changes [5]. However, because of frequent embolic sources causing multifocal infarcts, encephalopathy is also common [20]. One common theory is that many cryptogenic strokes in cancer patients are from cardioembolic manifestations of cancer-mediated hypercoagulability, specifically nonbacterial thrombotic endocarditis, which comprises sterile, platelet-fibrin vegetations on cardiac valves [17].

1.1.3 Treatment

While cancer patients may be more likely to have contraindications to thrombolysis than non-cancer patients, active cancer by itself is not exclusionary for recanalization therapies [21]. Furthermore, although there have been no trials evaluating tissue plasminogen activator treatment in cancer patients with stroke,

cohort studies have suggested that selective treatment in patients who otherwise meet eligibility criteria is likely safe [22,23] and effective, as further confirmed by e recent meta-analysis [24]. Endovascular therapy is another treatment option for cancer patients with acute stroke but there are no trial data in this population. However, case series suggest that it might be beneficial for select patients with good premorbid functional status who develop large vessel occlusive strokes [25–28], which is also confirmed by a recent meta-analysis [29].

Owing to the lack of solid treatment data to guide clinicians, the optimal antithrombotic to prevent recurrent stroke in cancer patients is unclear [19]. Both anticoagulation and antiplatelet therapy have been proposed thus many neurologists use theoretical considerations and institutional practice patterns to determine which antithrombotic agent to prescribe [19].

1.1.4 Pathophysiology

The precise mechanisms for increased stroke risk in cancer patients are still debated, although it is likely to be multifactorial. A probable contributor is an acquired state of hypercoagulability, due to cancer itself. Supporting this hypothesis is the observation that the stroke risk associated with cancer is highest immediately after diagnosis, when cancer activity, and hence acquired hypercoagulability, is generally most intense and then attenuates over time as cancer activity decreases with anticancer treatments [30]. Additionally, variable risks by cancer type and stage, which are tumor-related factors directly linked to the presence and severity of cancer-mediated hypercoagulability, also implicate cancer's procoagulant effects as a driver of stroke risk in these patients [31]. Increased stroke risk might also be a consequence of anti-neoplastic treatments [19]. Recently, another theory postulated the involvement of the so-called "tumor embolus" in the pathogenesis of cancer-related stroke, paving the way for new studies on Circulating Tumor Cells (CTCs) potentially responsible for cerebral thromboembolism [32–34].

Like in the general population, large artery atherosclerosis and small vessel disease are common stroke mechanisms in cancer patients, accounting for one-quarter to one-third of all events [17]. Less common but noteworthy stroke

mechanisms in cancer patients include atrial fibrillation, septic embolism, intravascular coagulation, tumor embolism, intracranial vessel compression, hyperviscosity, and cerebral vein thrombosis [19]. Paradoxical embolism is another important factor to be considered in cancer patients with cryptogenic stroke, as about 25% of the general population has right-to-left shunts and up to 20% of cancer patients develop venous thromboembolism. In a study of 184 ischemic stroke patients, including 11 with comorbid cancer, right-to-left shunts were present in 55% of patients with cancer versus 15% of those without. [35].

Despite accumulating knowledge, many other aspects of the association between cancer and stroke remain uncertain. Consequently, the specific mechanisms responsible for stroke in cancer patients are still unclear [19].

Recently, Navi and coworkers have proved that cancer-related ischemic stroke has a distinct blood mRNA expression profile. In particular, these patients showed an upregulation of multiple genes and molecular pathways implicated in autophagy signaling, immunity/inflammation, and gene regulation, including interleukin (IL-1), interferon, relaxin, mammalian target of rapamycin signaling, SQSTMI1 (sequestosome-1), and CREB1 (cAMP response element binding protein-1) [36]. Some of these molecules could represent candidate biomarkers for an accurate prediction of stroke risk in cancer patients or for an early detection of cancer after a stroke; they might also be useful in clarifying the pathophysiology of stroke in cancer and, consequently, for the development of targeted therapy.

In this context, the role of epigenetics in stroke is gaining importance. Epigenetics is the study of molecular and cellular processes responsible for specifically modulating single gene expression and functional gene networks, including the molecular interactions between genes and the environment [37]. In particular, DNA methylation, the most well-characterized epigenetic mechanism, plays a critical role in the regulation of global and specific gene expression profiles and in the promotion of important cellular processes, such as the maintenance of genomic stability, X chromosome inactivation, and genomic imprinting [38]. DNA methyltransferases (DNMTs) mediate DNA methylation

by transferring methyl groups from S-adenosylmethionine to cytosine residues in various genomic regions [38]. Abnormal DNA methylation profiles have been associated with a broad spectrum of disorders, including stroke [39] and cancer [40,41]. The role of DNA methylation in cerebral ischemia is multifaceted, with genome-wide and gene-specific effects that influence the vulnerability of the CNS to injury, suggesting that the dynamic modulation of DNMT expression and the status of DNA methylation represent important mechanisms for preventing cell death in cerebral ischemia [38,39].

Non–protein-coding RNAs (ncRNAs) represent another class of epigenetic factors involved in both physiological and pathological processes. Among these, microRNAs (miRNAs) are a subclass of ncRNAs with 18-24 nt in length nucleotide regulatory ncRNAs that either repress translation or inhibit the stability and deployment of their target mRNAs. Transcripts encoding synaptic proteins comprise the largest group of predicted miRNA targets, suggesting seminal roles for miRNAs in activity-dependent synaptic plasticity and memory formation (38). miRNA expression has been found to be dysregulated in cancer (43) and neurodegenerative diseases (44). Emerging evidence suggests miRNAs may play an important role in explaining variation in stroke risk and recovery in both monkey models (45) and humans (46–48), yet there are still few longitudinal studies examining the association between whole blood miRNAs and stroke (49).

1.2 Key Features of microRNAs

As already mentioned, miRNAs are small non-coding single-strand RNA molecules of about 18-24 nt involved in several physiological and pathological processes during the entire life of the individuals. Of note, miRNAs are involved in the modulation of gene expression through the binding and regulation of messenger RNA (mRNA). miRNA-mRNA interaction occurs in specific gene regions with sequence homology. Specifically, in the case of a perfect match between miRNA and its targeted mRNA the gene target is totally degraded through the RISC protein complex (described below). In the case of a partial match between miRNA and the 3' untranslated regions (3'UTR) of mRNA the

RISC complex is able to transiently inhibit mRNA translation into protein [42]. Depending on the gene target affected, miRNAs are able to influence other epigenetic mechanisms, e.g. by regulating DNA methylation phenomena via the interaction with the mRNA coding for several methyltransferases (DNMTs) or other enzymes such as those responsible for histone modifications [43].

In addition, miRNAs themselves are actively regulated by DNA methylation and epigenetic mechanisms. In this context, it has been demonstrated that some miRNAs located in introns can be transcribed from promoters present in CpG regions regulated by DNA methylation; therefore, in case of hyper-methylation these miRNAs are silenced, otherwise, they are up-regulated in case of CpG hypomethylation [44].

At present, more than 2,600 human miRNAs have been identified. For the majority of these miRNAs the precise mRNA targets were identified by using both computational and experimental approaches [45]. Such studies revealed how a single miRNA is able to bind and modulate several targeted mRNAs, thus influencing multiple molecular pathways. Therefore, the study of the role of miRNAs in both physiological and pathological processes is extremely complex.

Different studies have already demonstrated how numerous miRNAs are dysregulated in several pathologies, including cancer, neurodegenerative disorders and other acute and chronic diseases [46,47]. Due to the strong association between the dysregulation of specific miRNAs and the presence of pathological conditions, different authors have proposed the use of miRNAs as predictive biomarkers for both the diagnosis and prognosis of human diseases [46,47]. In addition, it was also demonstrated how miRNAs and other epigenetics alterations (including DNA methylation and histone modifications) represent early events predisposing to the development of disease, further corroborating the diagnostic potential of miRNAs [48].

The following paragraphs will briefly describe the biogenesis and maturations of miRNAs and their involvement in the development of tumors and in the pathogenesis of stroke.

1.2.1 Biogenesis and Maturation of microRNAs

The biogenesis of miRNAs is characterized by a multi-step process that starts with a primary transcript RNA (pri-miRNA) that is processed into a mature single-strand miRNA through different protein complexes. The coding sequences of miRNAs are usually interspersed in the non-coding regions of the human DNA; however, some miRNAs are coded by intron or exon regions. The transcription of miRNAs is operated by RNA polymerase II which produces a pri-miRNA of about 200 bp in length. Subsequently, the pri-miRNA is processed in the nucleus by nucleoprotein complex called Drosha that produces a stem-loop structure of ∼70–100 bp defined precursor miRNA (pre-miRNA). The cleavage operated by Drosha is facilitated by the DGCR8 protein [49]. The pre-miRNAs obtained by Drosha are subsequently transferred into the cytoplasm via an exportin-5-RanGTP-dependent mechanism [50]. In the cytoplasm, pre-miRNAs are further processed by Dicer, with the help of transactivating response RNA-binding protein (TRBP) and argonaut 2 AGO2 proteins which produce a double-strand molecule of mature miRNA [51]. Finally, the thermodynamically more unstable strand detaches from the double-stranded molecule thus obtaining two mature single-strand miRNA molecules.

The released 18–24 nt mature miRNAs are bound by a complex termed miRNA-associated RNA-induced silencing complex (miRISC) [52]. miRNAs can direct the RISC to downregulate gene expression by using two different post-transcriptional mechanisms: mRNA cleavage or translational repression (Figure 1). As stated above, a single miRNA can regulate multiple target genes, while a single gene can be targeted by multiple miRNAs, suggesting that the miRNAome and mRNAome interaction is a complicated network that should be analyzed by using computational and high-throughput technologies.

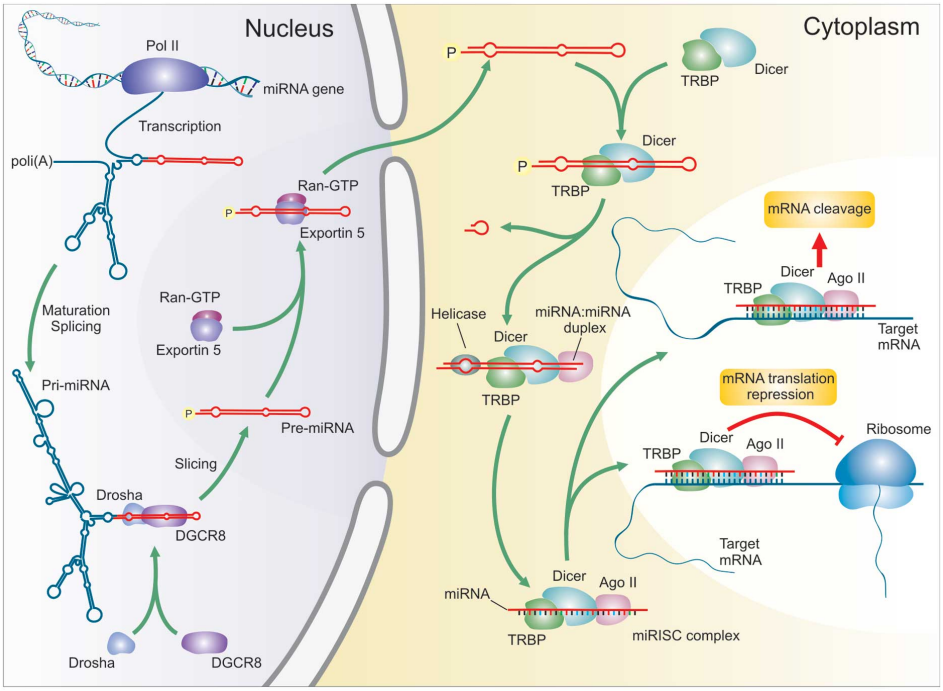

Figure 1. Biogenesis and maturation of human microRNAs.

1.2.2 Clinical Role of microRNAs in Cancer

Although miRNAs play important roles in healthy individuals, they have also been implicated in a wide range of diseases including cardiovascular, neurological, diabetes and obesity. Consequently, miRNAs are being studied in the clinical setting as diagnostic and prognostic biomarkers [53]. Altered expression profiles of miRNAs have been observed in specific tumors, demonstrating how these molecules may also be involved in cancer development. In the case of tumors, experimental approaches have indicated that some miRNAs act as tumor suppressors and others as oncogenes [54]; for this reason, they have important roles in cancer development, disease progression and prognosis.

The analysis of miRNAs expression levels may be helpful in different pathologies for the prognostic evaluation of the patient, to predict the effectiveness of the treatment and follow-up the patients, to predict the

therapeutic response to treatments, and to study the patients' susceptibility to other complications or a more aggressive phenotype [55–57].

These properties of miRNAs have been already validated for different tumors, in which miRNAs allow to distinguish tumor cells from normal and can be used also for the prediction of patients' prognosis [58–60]. Furthermore, miRNA expression patterns have been shown to have relevance for the biological and clinical behavior of human B-cell chronic lymphocytic leukemia and solid tumors, including oral cancer [61]. Significant results were also obtained in the prediction of therapeutic efficacy during cancer treatments, suggesting how the analysis of specific miRNAs can predict the response to therapy and suggest to the clinicians the best therapeutic schedule administered to the patient thus proposing novel personalized treatment approaches [62,63].

More in general, alterations in the expression levels of specific miRNAs have been observed and characterized in different tumors; in particular, many computational and experimental studies revealed that several miRNAs are differentially expressed in tumors as to the matched normal tissues [58–60]. In addition, it was also observed as miRNAs can modulate both tumor suppressors or oncogenes thus playing a diametrically opposite function. Indeed, miRNAs can be differentiated into tumor suppressor miRNAs able to bind and inhibit oncogenic factors and tumor-promoting miRNAs directed against mRNAs with tumor suppressor function [64].

Such results were mainly obtained by using microarray or NGS platforms for the analysis of both tissue and liquid biopsy samples of cancer patients and healthy donors. However, due to the huge number of human miRNAs and their multiple mRNA targets conflicting results about the precise miRNAs involved in the development and progression of tumors were generated. Therefore, the studies aimed at identifying the role of miRNAs in cancer need to be further validated by functional experiments performed on in vitro and in vivo models and by using validation techniques including RT-qPCR or droplet digital PCR (ddPCR).

More recently, different studies have been performed by using both computational and experimental approaches to identify a panel of miRNAs to be

used for the early diagnosis of specific tumors or to define the prognosis of cancer patients. The results of such studies revealed a set of miRNAs that are often dysregulated in multiple cancer. Among these, there are the miRNAs hsa-miR-21-5p and hsa-miR-125b-5p known to be over-expressed and down-regulated, respectively in almost all solid tumors [65]. Of note, these two miRNAs are also dysregulated in different pathological conditions including cardiovascular and brain disorders [66,67].

By searching the involvement of miRNAs in cancer it is possible to find thousands of studies reporting miRNA signatures or panels predictive for the presence of specific tumors. However, the majority of these studies are only theoretical and mainly related to the analysis of already existing databases collecting miRNA expression data of different tumors by using several and different platforms. Therefore, the data generated by the analysis of these sources of data may generate confusing results as no validation experiments or effective normalization of data across platforms are performed.

As regards the involvement of miRNAs in the most frequent tumors, different studies have highlighted key miRNAs associated with breast cancer, colorectal cancer, oral cancer, uveal melanoma etc. [60,68–70].

Regarding breast cancer, it was demonstrated that the down-regulation of hsa-miR-125b-5p is associated with a worse prognosis [71,72]. Other miRNAs strongly involved in breast cancer are miR-18a, miR-22, miR-181, miR-206, and miR-221/222 due to their regulating role towards the expression of the estrogen receptor-α in breast cancer cells [73].

Other miRNAs often associated with the development of breast cancer are the miRNAs of the let-7 family which are often down-regulated in breast cancer and associated with a worse prognosis [74].

Studies on breast cancer cell lines also revealed the involvement of miR-7-5p in the regulation of apoptotic processes. This miRNA together with other miRNAs including miR-15a and miR-16, are able to regulate the apoptotic processes through the regulation of BMI1, a protein involved in the downregulation of the

anti-apoptotic protein BCL2 and in the upregulation of pro-apoptotic proteins [75].

Several other upregulated and down-regulated miRNAs including miR-27a, miR-32, miR-205-3p, miR-221/222, miR-1271, as well as miR-17-5p, miR-134, miR-139-5p, miR-200b, miR-214, miR-218, miR-543, miR-1301-3p, and miR-4458 are able to influence breast cancer cell apoptosis highlighting how epigenetic mechanisms strongly influence the survival potential of cancer cells [75].

As regards colorectal cancer (CRC), different studies have tried to identify miRNAs able to regulate the key molecular pathways altered in this tumor including the EGFR pathway, the RAS signaling pathway, the Wnt/Beta-catenin pathway, etc. Wnt signaling pathway is the most altered in CRC, with mutations in APC/β-catenin genes (found in over 75% of CRC cases). All these pathways influence the tumorigenesis of colon cells and are affected by the expression of selected miRNAs. Among Wnt-interacting miRNAs, miR-135a and miR-135b represent two of the most studied miRNAs in CRC. These two miRNAs are over-expressed in CRC and mediated the down-regulation of APC levels which thus has a reduced control activity on Wnt pathway [76]. Other known miRNAs are those belonging to the miR-34 family (mir-34a/b/c) which are considered tumor suppressor miRNAs as they can effectively bind and down-regulate different genes of the Wnt pathway, like WNT1, WNT3, LRP6, β-catenin and LEF1 [77]. The regulation of miR-34 family seems also influenced by TP53 which acts as a positive transcription factor for the transcription of these miRNAs able to inhibit the Wnt pathway.

Other miRNAs are able to directly target and influence the expression levels ok key oncogenes such as KRAS and BRAF, often mutated in CRC. Among these, the miR-31 is considered a tumor-promoting miRNAs as it indirectly enhances RASA1 [78]. Finally, as regards BRAF, it is recognized by the miR-378 [79]. On the other hand, activating mutations of BRAF seem to be associated with the over-expression of miR-31 and a more aggressive tumor phenotype [80,81].

Other miRNAs have been identified for oral cancer as both diagnostic and prognostic biomarkers [69]. Among these, the experimental validation of hsa-miR-196a-5p, hsa-miR-503-5p, hsa-miR-133a-3p and hsa-miR-375-3p confirmed the high predictive value of computational analyses for the identification and selection of novel candidate biomarkers as well as the stability of miRNAs in different biological fluids including plasma, serum and saliva [82].

Of great importance is also the identification of miRNAs that can be used as therapeutic targets or drugs. Some researchers have used miRNAs as target therapy, with antagomir chemically modified oligonucleotides that competitively bind miRNAs, inhibiting their expression [83]. An example of the application of this therapy has been studied in hepatic metabolic diseases, where miR-122 is selectively inhibited by using antagomir. The results obtained showed an evident decrease in plasma cholesterol levels, both in obese and normal mice with reduced toxicity [84]. However, the use of antagomir as target therapy still has limitations today. Their mechanisms of action should be further studied and understood, in order to identify more precisely all the gene targets and their respective and suitable drug therapy [85].

Overall, several studies have been conducted on this topic generating conflicting data about the tumor suppressor or tumor-promoting role of some miRNAs like miR-139 [68]. Therefore, further studies are needed to clearly identify miRNAs involved in cancer development and as potential indicators of tumor complications, including stroke.

1.2.3 microRNAs and Stroke

Besides their involvement in cancer development and progression, human miRNAs have extensively been associated with the pathogenesis and clinical features of different neurological and neurodegenerative conditions including neuronal differentiation, neurogenesis, neural cell specification, neurodevelopmental function, neurodegenerative disorders (Alzheimer's and Parkinson's diseases), etc. [86]. Even in stroke some preliminary studies have identified distinct expression patterns of miRNAs associated with the modulation of cellular and molecular processes associated with an increased risk

of ischemic stroke including the pathway of the complement and coagulation cascade, platelet activation and the pathway responsible for the formation atherosclerotic plaques [87]. More in detail, these studies allowed the identification of key miRNAs involved in specific predisposing mechanisms. In this context, it was demonstrated that hsa-miR-21-5p and hsa-miR-126-5p are able to modulate several genes involved in atherosclerosis. Similarly, Goedeke and colleagues demonstrated that the dysregulation of two miRNAs, hsa-miR-33 and hsa-miR-135b-5p are associated with hyperlipidemia [88]. Other miRNAs, including the hsa-miR-155-5p and the hsa-miR-222 and hsa-miR-210-5p are respectively associated with hypertension and plaque rupture suggesting how an imbalance of these miRNAs may be responsible for an increased risk of ischemic stroke in presence of other risk factors [89]. Besides these specific miRNAs associated with key pathological conditions correlated with stroke there are a plethora of accessory miRNAs involved in the modulation of inflammatory processes which may play a concomitant pathogenetic role in patients with stroke [90].

On the other hand, stroke itself is responsible for profound cellular, molecular and epigenetic alterations. In this context, it was demonstrated that transient focal ischemia induces an alteration in levels of microRNAs predicted to target proteins known to mediate inflammation, transcription, neuroprotection, receptor function, and ionic homeostasis in the brain as demonstrated in rat models [91].

As mentioned in the above chapters, the biogenesis and regulation of miRNAs are finely modulated by several environmental factors and proteins. Indeed, it was demonstrated that transient ischemia influences the mRNA levels of proteins involved in the biogenesis of miRNAs, including Drosha, Dicer and other cofactors (Pasha and Exportin 5) [91,92]. It was also observed that during brain ischemia there is an acute modulation of the expression levels of miR-145 as a rescue mechanism to reduce the damages induced by ischemia. Indeed, miR-145 is able to selectively inhibit the mRNA coding for the enzyme superoxide dismutase-2 (SOD2), known to be involved in the reduction of damages induced by hypoxia and reactive oxygen species [91]. In particular, Dharap and

colleagues observed that the expression levels of miR-145 were significantly modulated in the postischemic brain of rats from 3 h to 3 days after transient MCAO.

In post-ischemic stroke, it was also observed that several miRNAs able to modulate genes involved in the regulation of brain microenvironments are dysregulated. In particular, Deng and colleagues observed that miR-21 is significantly up-regulated after stroke. Of note, miR-21 is one of the key miRNAs involved in human pathologies, especially in cancer, due to its ability to alter different cellular and molecular pathways (e.g. the MAPK pathway). The group of Deng revealed that, in post-ischemic brain, miR-21 is up-regulated to limit the brain damages induced by the Matrix metalloprotease-9 (MMP9) responsible for the post-ischemic blood-brain barrier (BBB) disruption and the formation of lesions after cerebral ischemia [93].

These and other studies have partially clarified the role of miRNAs in postischemic events; however, the role of miRNAs in inducing stroke is still debated and only a few data have been generated on this topic. Expression profiling experiments performed on ischemic rat brains revealed significant changes in the expression levels of several microRNAs whose dysregulation was concomitantly observed also in blood samples suggesting that liquid biopsy samples could be analyzed to predict ischemic stroke in patients at risk for this pathology (e.g. cancer patients) [94]. The results obtained in rats were partially confirmed in humans. Tan and colleagues observed that the expression levels of miRNAs involved in endothelial cell and vascular functions as well as in erythropoiesis, angiogenesis, neural function, and hypoxia were significantly altered in peripheral blood samples obtained in ischemic stroke patients. Such alterations of miRNA expression were persistent after months from the stroke episode suggesting that in the case of indolent stroke events the expression levels of these miRNAs could be altered and predictive for more severe episodes [95].

From a mechanistic point of view, in vitro and animal studies have demonstrated that miRNAs are able to modulate the expression levels of insulin-like growth factor 1 (IGF-1) known to play protective roles before and during ischemic

stroke. In detail, it was demonstrated that IGF-1 is negatively modulated by the miR-1 and miR-let-7. In rat models of endothelin (ET)-1 induced middle cerebral artery occlusion, it was demonstrated that the inhibition of both miR-1 and miR-let-7 results in an increased expression of IGF-1 which mitigates the detrimental effects of stroke by reducing estrogen neurotoxicity and infarct volume [96,97]. In similar stroke models, it was also demonstrated that the overexpression of miR-223 induces a significant down-regulation of a subunit of glutamate receptor by targeting the 3'-UTR region of both GluR2 and NR2B. The same miRNA is also able to inhibit NMDA-induced calcium influx in hippocampal neurons by protecting the brain from neuronal cell death following transient global ischemia and excitotoxic injuries [98]. Another pathogenetic mechanism mediated by miRNAs in ischemic brain is the alteration of neural progenitor cells of the subventricular zone. Indeed, it was demonstrated that miR-124a inhibits the proliferation of ischemic neural progenitor cells and also plays a role in the neuronal differentiation of the progenitor cells.

Overall, all these studies highlighted that miRNAs are actively involved in both pre- and post-ischemic processes. At present, the majority of the studies are performed on rat cells or in vivo animal models, while studies on human neurons and clinical evaluation in patients with stroke or patients at risk for this pathology are very few and often inconclusive. Therefore, there is an urgent need to clarify the role of miRNAs in stroke in order to provide new insights into the epigenetic and molecular mechanisms underlying stroke-inducing and stroke-induced damages.

2 Aim of the study

It has been clearly established that cancer increases the risk of stroke, although the precise mechanisms of this association have not been clarified yet. There is currently no resource to assist clinicians, including primary care physicians, oncologists, neurologists, neurosurgeons, and cardiologists, in identifying cancer patients at the highest risk of stroke.

On this basis, the purpose of this project was to identify potential causative factors of stroke in patients with cancer. Findings from this study might help neurologists to more effectively prevent and treat stroke in individuals with cancer, while oncologists would be able to promptly identify and properly manage stroke-related cancer.

The significance of the present project relies on the possibility of identifying diagnostic and prognostic biomarkers for the prediction of stroke in cancer patients at risk. On the bases of the recent findings on epigenetics in stroke, we hypothesized that patients with cancer at risk for stroke might present a specific expression pattern of miRNAs. Therefore, we wanted to analyze liquid biopsy samples obtained from patients with active solid cancer and stroke in order to identify specific epigenetic biomarkers with clinical values for patients at risk. The identification of such biomarkers could clarify the molecular mechanisms responsible for the development of stroke in cancer patients suggesting new therapeutic targets and strategies to be implemented for the clinical management.

3 Methods

3.1 Study design

This was a prospective cross-sectional pilot study assessing the bilateral mechanisms underlying ischemic stroke and cancer. Signed informed consent was provided by all individuals before participation in the study, which was approved by the Ethics Committee of the "Azienda Ospedaliera Universitaria Policlinico Gaspare Rodolico-San Marco" of Catania (approval code 154/2020/PO/prot. n. 48055) and performed according to the 1964 Declaration of Helsinki and its subsequent amendments.

Four groups of consecutive adult patients were enrolled, according to inclusion and exclusion criteria (see below). The lack of any previous study did not allow the estimation of optimal sample size; however, as many patients as possible were recruited. Group 1 consists of subjects with active cancer and acute ischemic stroke. Group 2 is represented by patients with acute ischemic stroke, without any evidence of cancer. Patients in Groups 1 and 2 were enrolled at 96 hours +/- 24 hours from the stroke onset. Group 3 includes patients with active solid cancer but without stroke. Group 4 consists of healthy donors with no past cancers and no evidence of cancer and stroke. The inclusion of these groups allowed us to confirm that any differences found between stroke patients with and without cancer are not merely incidental, regardless of thrombotic status. Demographic features, co-morbidities, and stroke severity (for Groups 1 and 2 only) were recorded on admission using a standardized structured form. All participants underwent a battery of hematological biomarker testing.

The circulating biomarkers were evaluated by analyzing serum samples collected at 96 hours (+/-24 hours) after stroke (Groups 1 and 2). For each patient or healthy donor, two blood draws were obtained. For stroke and cancer patients routine blood tests were performed to evaluate the main clinical-pathological features of patients. As regards to the exploratory analysis, epigenetic biomarkers were analyzed in liquid biopsy samples by using the ddPCR System. Finally, the data generated by ddPCR investigations were analyzed considering the clinical-pathological features of patients enrolled within Groups in order to

establish the diagnostic and prognostic values of the selected biomarkers as well as their sensitivity, specificity and the positive predictive value.

3.2 Participants

Group 1 and 2 enrollments occurred at the Cerebrovascular Disease Unit of the "Azienda Ospedaliera Universitaria Policlinico - San Marco" of Catania, whereas the Oncology Unit of the "Azienda Ospedaliera Cannizzaro" of Catania recruited participants of the Group 3. Healthy donors were already available at the biobank established at the Experimental Oncology Laboratory of the University of Catania. In the same laboratory all the molecular analyses were performed.

Subjects were enrolled according to the following criteria.

Inclusion criteria:

- 18 years of age or older, of both sexes;
- Evidence of active solid cancer (Groups 1 and 3 only);
- Imaging-confirmed evidence of acute ischemic stroke (Groups 1 and 2 only);
- Available for blood draw at 96 hours (+/- 24 hours) from the pre-morbid time (Groups 1 and 2 only) or within 2 weeks from enrollment (Group 3 only).

Exclusion criteria:

- Primary or secondary brain tumor;
- Any hematological cancer;
- Treatment with intravenous or intraarterial thrombolysis or mechanical thrombectomy;
- Platelets < 50,000/mm3;
- Hemodialysis within the last 14 days;
- Pregnancy;
- Infection within 14 days according to the Infectious Diseases Society of America (IDSA) criteria;

- Any other acute or chronic neurological or neuropsychiatric disorder;
- Any acute or chronic critical medical illness;
- Any previous thrombotic or coagulation pathology.

As already stated, healthy donors were already recruited within previous case-control studies.

3.3 Computational Selection of Stroke-Associated and Cancer-Associated microRNAs

Epigenetic biomarkers were first selected by analyzing bioinformatics data obtained from Gene Expression Omnibus DataSets (GEO DataSets) database. In particular, an advanced research was performed on GEO DataSets for the selection of microarray miRNA expression profiling datasets related to stroke by using the following search terms “((stroke) AND "non coding rna profiling by array"[DataSet Type]) AND "homo sapiens"[Organism]".

Through this advanced research different datasets were obtained. However, for our study, only the datasets which met the following inclusion and exclusion criteria were selected.

Inclusion Criteria:

- Datasets containing miRNA expression levels of stroke and non-stroke patients;
- Datasets with miRNA expression levels from both tumor and normal tissue samples;
- Datasets containing miRNA expression data at least of 10 samples (either stroke or controls).

Exclusion Criteria:

- Datasets containing only samples from stroke patients;
- Datasets with miRNAs expression levels obtained from animal samples or cell lines;

- Datasets containing miRNAs not correctly annotated or with obsolete annotation (without the specific sequence of each miRNA contained in the platform).

After dataset selection, differential analyses were performed to identify the differentially expressed miRNAs in stroke compared to non-stroke patients. For each of the selected datasets, the data matrices already normalized by the GEO2R software available on GEO DataSets were downloaded. Differential analyses were also performed using the GEO2R tool. After differential analyses, each miRNA was annotated according to the nomenclature reported by the latest version of the miRBase database (V. 22) (http://www.mirbase.org/). The differential expression levels of miRNAs observed in stroke compared to non-stroke patients were expressed as base 2 logarithm of the fold change (log2FC). To strengthen the analysis, only differentially expressed miRNAs with a p-value $p<0.05$ were considered statistically significant.

For the identification of the miRNAs involved in stroke, the lists of miRNAs differentially expressed in each dataset were combined using a software for the generation of Venn diagrams (Venn Diagrams of the Bioinformatics & Evolutionary Genomics (http://bioinformatics.psb.ugent.be/webtools/Venn/)). In this way, it was possible to identify the miRNAs with concordant expression levels among datasets and potentially involved in stroke.

After the merge of miRNA lists, only those highly upregulated or downregulated and with concordant expression levels at least in two of the selected datasets were considered as potential biomarkers. The log2FC value for each miRNA was reported.

For the selection of cancer-associated miRNAs a different approach was adopted. Since stroke patients with cancer were affected by different tumors, a pan-cancer analysis was performed to identify the miRNAs most commonly altered in multiple tumors. For this purpose, the miRNA expression data deposited on The Cancer Genome Atlas Pan-Cancer database (TCGA PANCAN) were analyzed. The data were downloaded by using the UCSC Xena Browser (https://xenabrowser.net) of the University of California Santa Cruz. For the

analysis of miRNAs differentially expressed in tumor samples versus normal controls the data reported on the "miRNA mature strand expression - Batch effects normalized miRNA data" dataset was considered. Of note, this dataset contains the miRNA expression data of a total of 10,188 samples obtained from different tumors (No. 9,534) and matched normal samples (No. 654) thus representing the more reliable cohort of cancer patients currently available on bioinformatics platforms.

The data downloaded from the TCGA database were manually analyzed by performing differential analyses on Microsoft's Excel. No normalization was needed as the TCGA data are already normalized. To strengthen the analysis and to select only representative miRNAs, a p-value of $p<0.01$ and a Log2FC value of $\pm$ 1.5 were adopted for the selection of significantly up-regulated and significantly down-regulated miRNAs.

Finally, to further corroborate the pathogenetic role of computationally selected miRNAs in stroke, a pathway prediction analysis was performed by using the computational tool DIANA-miRPath. This software allowed the identification of the molecular pathways and related genes altered by the selected miRNAs. In particular, this analysis was performed by analyzing the miRNA interactions obtained from the database DIANA-TarBase v7.0. The detected interactions, whether validated or predicted, can be then combined with fusion algorithms and meta-analysis thanks to the DIANA-mirPath tool.

After the *in silico* selection of miRNAs, the diagnostic and prognostic significance of the putative biomarkers were validated on the liquid biopsy samples obtained from the patients enrolled in the different groups and from healthy donors.

3.4 RNA Extraction and microRNA Reverse Transcription

As previously mentioned, miRNAs can be easily detected in different biological fluids, therefore, they can be used as non-invasive biomarkers for the diagnosis of different diseases thanks to their stability. On these bases, together with the Experimental Oncology Laboratory of the University of Catania, a novel

effective isolation and amplification protocol for the analysis of miRNAs was developed in this study. Of note, the protocol can be applied for the analysis of different liquid biopsy samples, including saliva, serum, plasma, CSF and urine. By using the protocol described in detail below, the circulating miRNAs were extracted from the serum samples obtained from the patients enrolled in the study.

Briefly, the extraction of circulating miRNAs was performed by using the miRNeasy Serum/Plasma kit (Qiagen, Cat. No. 217184) by modifying and optimizing the commercial protocol in some steps to obtain a higher yield of circulating miRNAs which could have been underrepresented.

First, serum samples were centrifuged at 2.000g x 10 minutes at room temperature to pellet down protein aggregates and debris. Subsequently, 200 µL of samples were used as described in the Qiagen miRNeasy Serum/Plasma protocol. Before the extraction protocol, a known concentration of an exogenous synthetic oligonucleotide, UniSP4, was added as exogenous control useful to perform the absolute quantification of extracted miRNAs (Cat. No 219610, Qiagen, Hilden, Germany). In addition, to optimize miRNAs extraction and purification, molecular biology-grade chloroform was used (Serva cat. n. 39553.01).

After RNA extraction, 2 µL of total RNA, miRNAs included, were selectively reverse transcribed into cDNA using the miRCURY LNA RT Kit (Qiagen – Cat. N. 339340). In particular, the miRCURY LNA RT technology was adopted to ensure the efficient polyadenylation of miRNAs and the subsequent reverse transcription into cDNA in a single reaction step.

3.5 ddPCR microRNA Quantification

All the validation analyses were performed at the Experimental Oncology Laboratory of the Department of Biomedical and Biotechnological Sciences of the University of Catania. In particular, for the detection of the miRNAs expression levels the following droplet digital PCR-based method (ddPCR) was developed.

The new method adopted for the quantification of miRNA was based on a custom Qiagen-BioRad hybrid protocol using specific primers for the selected miRNAs as well as for the exogenous normalizer UniSp4 spike-in control (miRCURY LNA miRNA PCR Assays x200, Qiagen, Cat. No. 339306).

The use of ddPCR was justified by the use of low-quality samples such as liquid biopsy samples with a low amount of miRNAs. The adoption of this protocol ensures a higher sensitivity and specificity compared to other protocols based on real-time PCR or microarray platforms as through ddPCR it is possible to divide the sample into thousands of droplets, in each of which a single reaction runs. The nano-partition of samples is obtained through a water-oil emulsion, which permits to generate thousands of droplets, with a maximum of 20,000 droplets. Each droplet contains, theoretically, specific primers and dye, Taq polymerase, the amplification buffer and target DNA, which is amplified through a classic PCR amplification protocol. After droplet amplification, each generated droplet is read using a droplet reader, which is capable to evaluate the positive through a CCD camera.

For the ddPCR analyses (Figure 2), each cDNA sample was diluted from pure to 1:10 in sterile H_2O depending on the absolute abundance of each miRNA. Before use, the cDNA and all the ddPCR reagents were thawed on ice.

For this study, a ddPCR reaction mix was prepared as follows:

- 11 µL of 2x QX200TM ddPCRTM EvaGreen® Supermix (Cat. N. 1864034 – Bio-Rad, Hercules, California, USA);
- µL of miRCURY LNA miRNA PCR Assays (x200), i.e. specific primers for the three selected miRNAs and for the Unisp4 spike-in control (Qiagen - Cat. N. 339306);
- 6.9 µL of RNase and DNase free-water;
- µL of cDNA (diluted from pure to 1:10) in order to obtain a final volume of 22 µL.

Thus, a final volume of 22 µL. Subsequently, 20 µL of the ddPCR reaction mix was used to generate 20,000 droplets with the QX200 droplet generator (Bio-Rad, Hercules, California, USA) through specific cartridges with wells for

amplification mixture and droplet generation oil-linked each other with microchannels and a vacuum system to generate the droplets. After generating the droplets each containing a single copy of target cDNA, the whole reaction was charged into a 96-well plate sealed with an aluminum foil and amplified using a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, California, USA) following the thermal conditions reported below:

- 95°C for 5 minutes, in order to activate the polymerase;
- 94°C for 30 seconds to guarantee the denaturation, 60°C for 1 minute to facilitate the annealing: these steps constitute the amplification cycle, which is repeated 40 times;
- minutes at 4°C and subsequently 98°C for 10 minutes in order to stabilize the droplets;
- 4°C infinite hold.

A ramp rate of 1.6 °C/s was used between each step/cycle of the amplification protocol. After amplification, negative and positive signals were read in the QX200 Droplet Reader (Bio-Rad, Hercules, California, USA) using a capillary tube where every single droplet flow and a laser excitation source plus a detector to collect the fluorescent signals of positive droplets (Figure 2). All experiments were performed in triplicate.

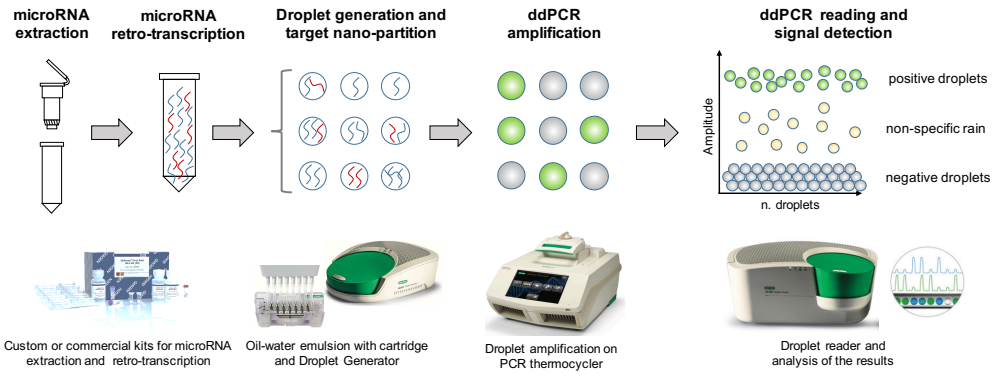

Figure 2. Schematic representation of the ddPCR workflow for the analysis of miRNAs.

Finally, the ddPCR results were analyzed by using the QuantaSoft software, which allows the statistical analysis of the obtained data by applying specific correction factors, including the Poisson distribution, used for the calculation of the copies of circulating miRNAs based on the number of droplets.

3.6 Further Bioinformatics Analyses

In order to better clarify the involvement of the predicted dysregulated miRNAs in the pathogenesis of cancer-related stroke, several computational approaches were used. First, the targeted genes shared among the experimentally validated miRNAs were identified using the miRWalk bioinformatics tool (http://mirwalk.umm.uni-heidelberg.de) as already described [82]. Briefly, this tool is able to identify the genes targeted by the selected miRNAs through the analysis of the interaction data contained in the latest version of TargetScan, MirDB and miRTarBase as well as from in-house generated data.

Subsequently, the protein-protein interaction (PPI) network, clinical phenotype and molecular pathways were evaluated using, respectively, STRING v11.0 and GO PANTHER v15.0 software [99,100].

3.7 Statistical Analyses

Differential analyses were performed for each selected dataset by using the GEO2R software already available in GEO DataSets. The absolute quantification of miRNA was performed by using the QuantaSoft software (Bio-Rad). D'Agostino-Pearson normality test was used to assess the distribution of miRNA n. copies/µL and for the data obtained from TCGA PANCAN Database. Mann-Whitney non-parametric test was used to evaluate the statistical difference between two groups; Kruskal-Wallis non-parametric test (and the post-hoc Dunn's multiple comparison test) was used to assess the statistical differences existing among the expression levels of three or more groups. The sensitivity and specificity of the three selected miRNAs were assessed by performing the receiver operating characteristics curve (ROC). All analyses were performed in triplicate and the data were analyzed using Prism version 8 (GraphPad software, La Jolla, CA, USA). $p<0.05$ was considered statistically significant.

4 Results

4.1 Differential Analyses and Identification of Stroke-Associated and Cancer-Associated microRNAs

The advanced search on GEO DataSets database performed to select miRNA expression microarray dataset related to stroke patients allowed the identification of a total of 22 microarray miRNA expression datasets related to humans. However, by applying the inclusion and exclusion criteria described in the "Methods" section, only five datasets were included in the present study for the differential analyses. Table 1 reports the main features of the selected datasets (Table 1).

Table 1. Microarray miRNA expression datasets related to stroke and included in the study.

Series Accession	No. of Controls	No. of Stroke	Total Number	Type of Sample	Platform	Ref.
GSE117064	1612	173	1785	Serum Samples	3D-Gene Human miRNA V21_1.0.0	[101]
GSE95204	15	15	30	Pooled Plasma Samples	Exiqon miRCURY LNA microRNA array V.7	N/A
GSE86291	4	7	11	Plasma Samples	Agilent-046064 Unrestricted_Human_miRNA_V19.0	[102]
GSE55937	24	24	48	Paxgene Venous Blood Samples	Affymetrix Multispecies miRNA-3 Array	[103]
GSE60319	117	82	199	Pooled Serum Samples	Exiqon miRCURY LNA microRNA array V.7	N/A

All the five selected datasets contained miRNA expression data of both stroke and non-stroke patients and were selected to identify the differentially expressed miRNAs associated with the onset of the disease. Overall, the five selected datasets contained the miRNA expression data of a total of 2,073 samples of which 1,772 were normal samples and 301 were serum, plasma or blood samples obtained from ischemic stroke patients.

After the selection of datasets, the differential analyses within stroke and normal samples of each dataset were performed by using GEO2R. For each dataset was obtained a list of miRNAs up-regulated or down-regulated with a p-value of $p<0.05$ and a Log2FC value of ± 1.5. Figure 3 shows the Volcano Plot graphs obtained for each dataset by performing GEO2R differential analyses (Figure 3).

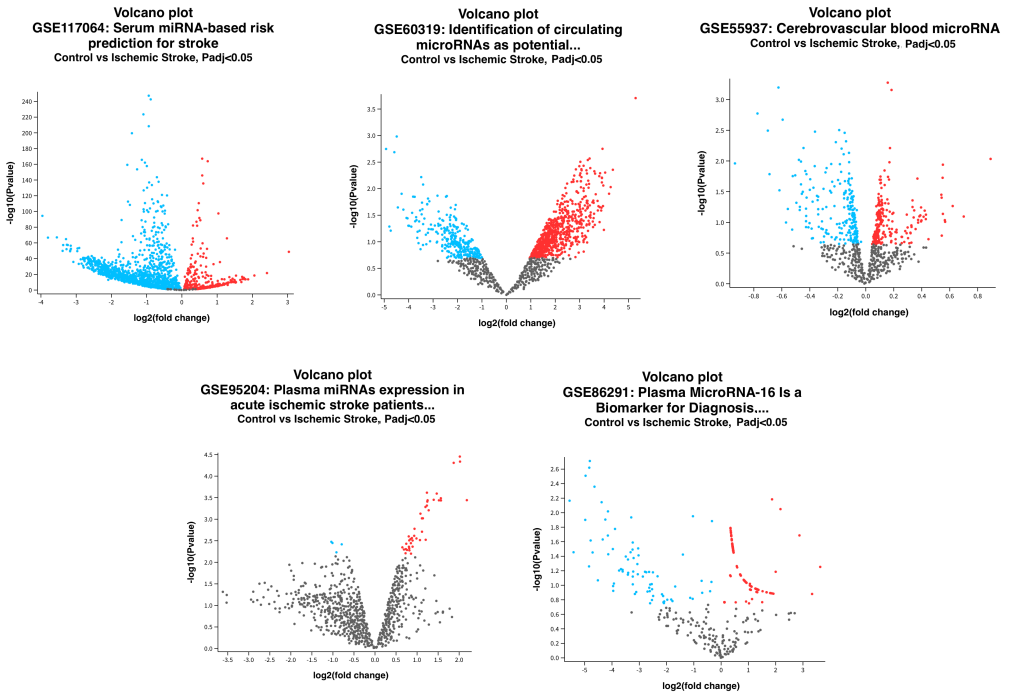

Figure 3. Significantly dysregulated miRNAs in the selected datasets.

As shown in Figure 3, the two datasets with the highest number of dysregulated miRNAs were the GSE117064 and GSE60319 containing also the highest number of samples, i.e. 1,785 and 199 total samples, respectively.

The lists of significantly dysregulated miRNAs obtained from the selected datasets were then merged in order to identify those miRNAs significantly up-regulated or down-regulated and reported into two or more datasets with concordant expression levels. By merging the obtained lists, a total of 52 miRNAs were shared at least between two or more datasets. However, some of these miRNAs showed opposite expression levels in the datasets where they were found, thus such non-concordant miRNAs were not considered as miRNAs associated with ischemic stroke (data not shown). After the removal of biased miRNAs a list of 21 miRNAs was obtained (Table 2).

Table 2. List of significantly dysregulated miRNAs associated with ischemic stroke.

ID miRNA	Selected Datasets				
	GSE117064	**GSE55937**	**GSE60319**	**GSE95204**	**GSE86291**
hsa-miR-325	-1.622968	#N/D	#N/D	-1.72726	#N/D
hsa-miR-3680-5p	-2.132791	-0.1159172	#N/D	#N/D	#N/D
hsa-miR-487b-3p	-2.173912	-0.4500085	#N/D	#N/D	#N/D
hsa-miR-4699-5p	-2.116649	-0.1042418	#N/D	#N/D	#N/D
hsa-miR-4326	-2.008259	-0.086989	#N/D	#N/D	#N/D
hsa-miR-657	-2.103296	-0.1233589	#N/D	#N/D	#N/D
hsa-miR-4794	-1.705221	-0.0957129	#N/D	#N/D	#N/D
hsa-miR-551a	-1.504001	-0.1485051	#N/D	#N/D	#N/D
hsa-miR-550a-3p	-2.124432	-0.3970621	#N/D	#N/D	#N/D
hsa-miR-1271-5p	-1.846671	-0.6174458	#N/D	#N/D	#N/D
hsa-miR-1272	-2.317538	-0.2472692	#N/D	#N/D	#N/D
hsa-miR-3124-3p	-1.568819	-0.1431867	#N/D	#N/D	#N/D
hsa-miR-21-5p	-3.265176	#N/D	-2.7883779	#N/D	#N/D
hsa-miR-199a-5p	-2.12514	#N/D	-4.0462008	#N/D	#N/D
hsa-miR-3591-3p	-2.670594	#N/D	-3.4848425	#N/D	#N/D
hsa-miR-3148	-1.757252	#N/D	-3.5702612	#N/D	#N/D
hsa-miR-503-5p	-2.058424	#N/D	-4.0117492	#N/D	#N/D
hsa-miR-1263	#N/D	-0.4227158	#N/D	-1.993044	#N/D
hsa-miR-140-3p	#N/D	-0.2804625	#N/D	#N/D	-4.81174
hsa-miR-4739	#N/D	#N/D	-2.1054684	#N/D	-3.24582
hsa-miR-125b-5p	#N/D	0.1136982	3.3839589	#N/D	#N/D

As shown in Table 2, the majority of miRNAs were shared between the GSE117064 and the GSE55937 datasets. More importantly, different miRNAs were also shared between the GSE117064 and the GSE60319 (a total of five miRNAs) with very similar Log2FC values. This data can be explained considering that both datasets contain miRNA expression data obtained from serum samples. Noteworthy, although statistically significant, the GSE55937 dataset using the Affymetrix Multispecies miRNA-3 Array platform showed the lowest Log2FC value. This data can be explained considering that the dataset contains miRNA expression data obtained from whole blood, therefore the slight differential expression of miRNAs in patients with stroke compared to healthy donors may be due to a bias introduced by the intracellular miRNA content of PBMCs.

Overall, the results obtained from the analysis of GEO DataSets miRNA microarray expression datasets allowed the identification of a panel of miRNAs whose dysregulation is associated with ischemic stroke and that could be used to predict the risk of stroke in patients at risk for this disease. Among the 21 miRNAs identified, the miRNAs hsa-miR-125b-5p, hsa-miR-21-5p, hsa-miR-503-5p and hsa-miR-199a-5p were those with the highest dysregulation levels.

Besides the analyses performed on miRNA expression data related to patients with ischemic stroke, a further computation analysis was performed on the miRNA expression data collected by The Cancer Genome Atlas consortium. As the cancer patients with stroke recruited in the present study were affected by different types of tumors, a pan-cancer analysis was performed to identify the key miRNAs dysregulated in cancer. For this purpose, the data related to 10,188 tumor and normal samples contained in “miRNA mature strand expression - Batch effects normalized miRNA data” dataset were downloaded.

miRNAs contained in the data matrix were annotated according to the latest nomenclature and differential analyses were performed as previously described [69,70].

After differential analyses, a list of 28 dysregulated miRNAs in all tumors (both liquid and solid tumors) was identified (Table 3).

Table 3. List of dysregulated miRNAs in cancer (TCGA PANCAN database).

ID miRNA	Mean Expression		p-value	Log2FC
	Tumor	Normal		
Up-regulated				
hsa-miR-210-3p	8.693	6.441	4.01E-120	2.252
hsa-miR-183-5p	12.329	10.173	5.70E-145	2.155
hsa-miR-182-5p	13.539	11.755	8.07E-113	1.785
hsa-miR-96-5p	3.947	2.196	9.18E-187	1.751
hsa-miR-21-5p	17.131	15.670	3.51E-113	1.461
hsa-miR-199a-5p	12.217	10.774	2.81E-165	1.443
hsa-miR-196a-5p	5.128	3.724	7.98E-27	1.404
hsa-miR-130b-3p	3.892	2.509	5.42E-139	1.384
hsa-miR-503-5p	3.824	2.510	2.42E-105	1.314
hsa-miR-92b-3p	6.867	5.573	4.54E-91	1.294
hsa-miR-196b-5p	7.374	6.092	9.82E-25	1.283
Down-regulated				
hsa-miR-195-5p	4.729	5.984	5.46E-117	-1.254
hsa-miR-140-3p	1.230	2.572	1.23E-47	-1.342
hsa-miR-99a-5p	8.850	10.244	5.08E-111	-1.394
hsa-miR-144-5p	5.722	7.117	9.08E-49	-1.394
hsa-miR-30a-5p	13.928	15.403	2.03E-82	-1.474
hsa-miR-125b-3p	15.448	16.929	2.07E-98	-1.481
hsa-miR-451a	7.915	9.397	2.19E-54	-1.482
hsa-miR-145-3p	4.650	6.136	4.36E-150	-1.486
hsa-miR-145-5p	10.175	11.661	4.21E-113	-1.486
hsa-miR-30c-2-3p	5.314	6.844	1.10E-102	-1.530
hsa-miR-486-5p	6.335	7.878	2.28E-57	-1.543
hsa-miR-133a-3p	3.115	4.863	7.07E-64	-1.749
hsa-miR-30a-3p	12.166	13.964	1.60E-126	-1.799
hsa-miR-139-5p	5.322	7.156	5.04E-165	-1.834
hsa-miR-1	3.470	5.309	2.89E-66	-1.839
hsa-miR-139-3p	3.370	5.318	3.26E-173	-1.948
hsa-miR-204-5p	3.276	5.403	2.03E-77	-2.127

Of these significantly dysregulated miRNAs in cancer, 11 were strongly up-regulated while 17 were strongly down-regulated. As will be widely discussed in the Discussion section, almost all of the selected miRNAs are known to be altered in multiple cancer and to be involved in the regulation of both oncogenes

and tumor suppressor genes as well as key cellular and molecular pathways including cancer cell proliferation, loss of apoptosis, tumor migration and infiltration and alteration of the tumor microenvironment.

More importantly, by comparing the list of miRNAs significantly altered in stroke patients with that of miRNAs dysregulated in cancer patients it is possible to note that five miRNAs were shared, i.e. hsa-miR-125b-5p, hsa-miR-21-5p, hsa-miR-503-5p, hsa-miR-199a-5p and hsa-miR-140-3p. Therefore, to focus the validation analyses only on miRNAs potentially involved in cancer-mediated stroke we selected these five miRNAs as novel potential predictive biomarkers of stroke risk in cancer patients.

To further correlate the dysregulation of these five selected miRNAs with cellular and molecular processes potentially associated with the occurrence of stroke in cancer patients, further computational investigations were performed.

First, a pathway prediction analysis was performed to evaluate the overall pathophysiological roles of the five selected miRNAs. For this purpose, the DIANA-mirPath pathway prediction software was used. Notably, this software reports of all the predicted 3′-UTR gene targets of selected miRNAs according to experimentally validated miRNA interactions derived from DIANA-TarBase v7.0 or microT-CDS algorithms. These interactions (predicted and/or experimentally validated) were subsequently combined with sophisticated merging and meta-analysis algorithms by DIANA-mirPath and giving as a result the genes and pathways targeted by a specific miRNA and the statistical significance of this interaction. Therefore, by using DIANA-mirPath two independent prediction analyses were performed. In the first analysis, the five selected miRNAs were imputed in the search tool of DIANA-mirPath to identify all the genes and pathways modulated by the selected miRNAs. While, in the second approach, a reverse pathway analysis was performed by searching two KEGG pathways notoriously altered and involved in the occurrence of stroke, i.e. the “platelet activation (hsa04611)” and the “complement and coagulation cascades (hsa04610)” pathways.

Through the first approach, it was observed that the five miRNAs were able to alter a total of 57 different cellular and molecular pathways and a total of 1,165 genes. Some of these genes were targeted by two or more miRNAs and were altered within different pathways (Appendix A, Supplementary Table 1).

Noteworthy, among the most altered pathways there were different cancer-related pathways and different signal transduction pathways highlighting how the selected miRNAs were able to modulate a plethora of cellular processes. The most targeted pathways were the Hippo signaling pathway (hsa04390), the Proteoglycans in cancer (hsa05205), the Cell cycle (hsa04110), the p53 signaling pathway (hsa04115), the Focal adhesion (hsa04510) and the Bladder cancer (hsa05219) pathways, all targeted by four of the five selected miRNAs. Other significantly dysregulated pathways with numerous genes affected by the selected miRNAs were the Pathways in cancer (hsa05200) (74 modulated genes) and the MAPK signaling pathway (hsa04010) (49 modulated genes) (Appendix A, Supplementary Table 1). All these data are also evident in the heatmap generated by the DIANA-mirPath tool (Figure 4).

Figure 4. Heatmap of the interaction between the selected miRNAs and different cellular and molecular pathways according to DIANA-miRpath predictions.

As shown in Figure 4, hsa-miR-125b-5p and hsa-miR-21-5p were those with the highest interaction levels with the 57 KEGG pathways. Regarding hsa-miR-125b-5p, it is possible to note that this miRNA has a strong interaction with two key pathways responsible for an increased risk of stroke, the Fatty acid biosynthesis (hsa00061) and the Fatty acid metabolism (hsa01212) pathways (Figure 4). This analysis showed also that hsa-miR-125b-5p and hsa-miR-21-5p act in the same miRNA cluster, while the other three miRNAs belong to a separate cluster (Appendix A, Supplementary Figure 1). Similarly, the 57 modulated pathways were also analyzed through a dendrogram revealing multiple subclusters among the pathways identified (Appendix A, Supplementary Figure 2).

To obtain more reliable data about the involvement of these five miRNAs in the development of stroke, the second DIANA-mirPath analysis was carried out by searching the miRNA interactors with two stroke-related pathways, the Platelet activation (hsa04611) and the Complement and coagulation cascades (hsa04610) pathways.

This second analysis revealed that both pathways are widely modulated by miRNAs. The Platelet activation (hsa04611) pathway is actively modulated by a total of 652 miRNAs as predicted by DIANA-mirPath and TarBase v.7, while the Complement and coagulation cascades pathway (hsa04610) is modulated by a total of 282 miRNAs (data not shown). Among these miRNAs, all the five selected miRNAs were able to interact with the Platelet activation (hsa04611) pathway, while four of the selected miRNAs, with the exclusion of hsa-miR-503-5p, were able to modulate the Complement and coagulation cascades (hsa04610) pathway by interacting with multiple genes (Table 4).

Table 4. Genes targeted by the five selected miRNAs in "Platelet activation" and "Complement and coagulation cascades pathways".

miRNA ID	Platelet Activation		Complement and coagulation cascades	
	No. Genes	Gene ID	No. Genes	Gene ID
hsa-miR-21-5p	13	ACTB, ARHGEF12, AKT2, ARHGAP35, RASGRP1, F2R, FGB, PIK3R1, GNAQ, MAPK1, TLN1, COL5A2	3	F2R, PLAT, FGB
hsa-miR-125b-5p	7	ARHGEF12, PPP1CC, MAPK14, PLA2G4F, RASGRP1, AKT1, MYLK	2	CD59, SERPINE1
hsa-miR-199a-5p	2	COL5A1, COL1A1	2	CD59, PLAU
hsa-miR-140-3p	7	PRKCI, RHOA, COL5A1, PRKG1, MYL12A, PPP1CB, ADCY6	1	SERPINE1
hsa-miR-503-5p	4	ACTB, GNAQ, AKT3, TLM1	/	/

This further analysis confirmed that the five miRNAs selectively modulate two of the most important pathways in stroke pathogenesis by interacting with multiple genes (Appendix A, Supplementary Figures 3 and 4). More in detail, within the "Platelet activation" pathway the genes most frequently altered were those belonging to the collagen family (COL5A2, COL5A1, COL1A1) and genes belonging to the MAPK and PI3K/Akt signal transduction pathways (MAPK1, MAPK14, AKT1, AKT3, PIK3R1, etc.). While the genes most frequently targeted within the "Complement and coagulation cascades" pathway were CD59 and SERPINE1, both genes actively involved in the alterations of complement activation and in the inhibition of plasminogen activation, respectively, as will be discussed in the Discussion section.

Overall, all these computational investigations allowed us to identify and select five miRNAs strongly associated with both cancer and stroke that could be used as predictive biomarkers in cancer patients at risk for brain ischemic events. Therefore, these miRNAs were selected for the validation analyses performed on liquid biopsy samples obtained from healthy donors, patients with cancer, patients with stroke and patients affected by both pathological conditions.

4.2 Analysis of the Circulating Expression Levels of microRNAs in clinical samples

Through computational analyses, the miRNAs hsa-miR-21-5p, hsa-miR-125b-5p, hsa-miR-503-5p, hsa-miR-199a-5p and hsa-miR-140-3p were selected as potentially involved in the pathogenetic mechanisms responsible for cancer-related stroke when dysregulated. In order to validate the computational results obtained through such bioinformatics integrated approaches a pilot case series of 11 patients with cancer and stroke, 15 patients with stroke, 28 patients with cancer and 10 healthy donors were included in the study. For all the participants in the study, written informed consent was obtained or was already available from previous studies (patients with cancer and healthy donors).

Due to the different sources of the patients and controls recruited in this study, clinical-pathological data were collected in a heterogeneous manner. Below are reported the main clinical-pathological and socio-demographic features of patients with stroke and patients with cancer and stroke (Table 5); the clinical characteristics of the cancer patients and healthy donors are reported in a separate table (Table 6).

Table 5. Clinical-pathological characteristics of stroke and cancer with stroke patients.

	Cancer Patients with Stroke (N. 11)		Stroke Patients (N. 15)		p-value*
	N.	%	N.	%	
Sex					0.141
Male	5	45.5	12	80	
Female	6	54.5	3	20	
Age					0.845
<45	0		1	6.7	
45-69	2	18.2	6	40.0	
>70	9	81.8	9	60.0	
Mean	74.91 y/o		70.50 y/o		
Stroke Severity (NIHSS)	(N. 8)		(N. 12)		0.091
1-4	3	37.5	8	66.7	
5-15	5	62.5	4	33.3	
Hypertension	(N.10)		(N.13)		0.636
Yes	8	80.0	12	92.3	
No	2	20.0	1	7.7	
Hypercholesterolemia	(N.10)		(N. 12)		0.410
Yes	2	20.0	5	41.7	
No	8	80.0	7	58.3	
Diabetes	(N.10)		(N. 12)		0.634
Yes	3	30.0	6	50.0	
No	7	70.0	6	50.0	
Atrial fibrillation	(N.10)		(N.13)		0.661
Yes	3	30.0	3	23.1	
No	7	70.0	10	76.9	
CRP (mg/L)					0.466
Mean Value	49.033	/	24.536	/	
ESR (mm/h)					0.756
Mean Value	20.857	/	23.25	/	
Homocysteine (umol/L)					0.726
Mean Value	16.918	/	20.1	/	
Fibrinogen (mg/dL)					0.482
Mean Value	361.778	/	411.461	/	
D-dimer (ug/L)					0.116
Mean Value	518.667	/	312.4	/	
Antithrombin (%)					0.674
Mean Value	100.556	/	95.636	/	
Cancer Type					
Breast	3	27.3			
Colon	3	27.3		NA	
Prostate	2	18.2			
Other	3	27.3			

*p-value calculated with Fisher's Test and Student's t-test.

Table 6. Clinical-pathological characteristics of cancer patients and controls.

	Cancer Patients (N. 28)		Normal Controls (N. 10)		p-value*
	N.	%	N.	%	
Sex					0.749
Male	17	60.7	7	70	
Female	11	39.3	3	30	
Age					0.268
<45	3	10.7	0	0	
45-59	7	25.0	6	60	
>60	18	64.3	4	40	
Mean	68.23 y/o		70.21		
Smoke					0.700
Yes	10	35.7	3	30	
No	11	39.3	5	50	
Ex smoker	7	25.0	2	20	
Alcohol					
Yes	19	67.9	1	10	0.859
No	9	32.1	9	90	
Cancer Type					
Breast	9	32.1	NA		
Colorectal	19	67.9			
Tumor Stage					
T1	3	10.7			
T2	12	42.9	NA		
T3	7	25.0			
T4	6	21.4			
Node					
Positive	10	35.7	NA		
Negative	18	64.3			
Recurrence					
Yes	5	17.9	NA		
No	23	82.1			

*p-value calculated with Fisher's Test

Although the low number of patients and controls recruited, no statistical differences among clinical-pathological features exist in the different groups of patients as demonstrated by Fisher's test and Student's t-test.

In order to validate the potential involvement of hsa-miR-21-5p, hsa-miR-125b-5p, hsa-miR-503-5p, hsa-miR-199a-5p and hsa-miR-140-3p in cancer-related stroke as predicted by the bioinformatics analyses performed both on cancer patients (TCGA PANCAN analysis) and stroke patients (GEO DataSets analysis), the expression levels of the five selected miRNAs were analyzed on serum samples obtained from the case series described above and healthy controls. For this purpose, the absolute quantification of the circulating levels of the miRNAs hsa-miR-21-5p, hsa-miR-125b-5p, hsa-miR-503-5p, hsa-miR-199a-5p and hsa-miR-140-3p were investigated by using the high-sensitive ddPCR technology and a custom protocol developed to detect even slight variation in the expression levels of the selected miRNAs. Although performed in a preliminary cohort of samples, statistically significant results were obtained.

The ddPCR analysis performed on miRNAs obtained from serum samples allowed the generation of good-quality amplification signals for all the selected miRNAs and the UniSp4 control except for the miRNA miR-140-3p, probably due to a technical issue related to the generation of the commercial primers for this miRNA (Figure 5).

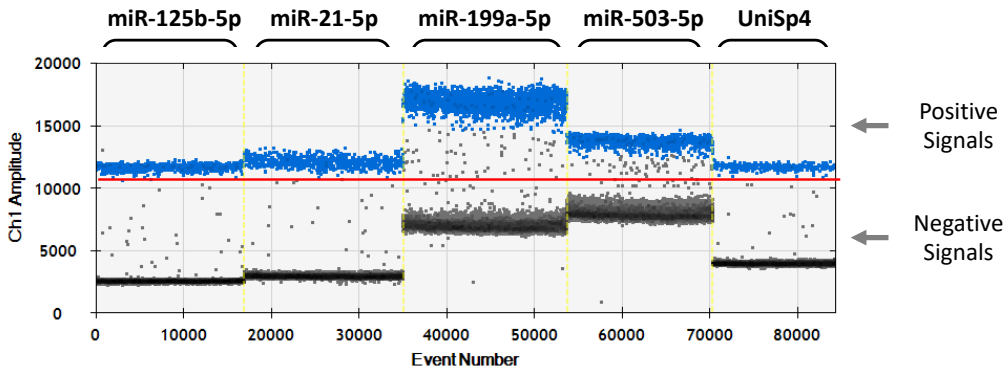

Figure 5. Positive ddPCR signals obtained for four of the five selected miRNAs and for the artificial control UniSp4.

To limit the variability due to the recruitment of samples in different studies, we chose to analyze differences in miRNA expression levels first in patients with

cancer, stroke and stroke&cancer. Subsequently, we performed a second analysis by comparing the expression levels of miRNAs in healthy donors, patients with stroke and patients with stroke&cancer.

Before analyzing the miRNA expression levels a normality test was performed revealing a non-gaussian distribution of the molecular data obtained from serum samples.

Regarding the analysis performed in the first group of patients (cancer vs stroke&cancer vs stroke), the results obtained through ddPCR revealed substantial variations of all the selected miRNAs in the different groups analyzed (Figure 6). More in detail, significant variations of miRNAs expression levels were observed for the miRNAs hsa-miR-125b-5p ($p<0.0001$) and hsa-miR-199a-5p ($p=0.117$). Regarding hsa-miR-125b-5p, the expression levels of this miRNA were significantly reduced in patients with stroke&cancer compared to patients with cancer ($p<0.01$) while a more evident reduction was observed in patients with stroke vs patients with cancer ($p<0.0001$). Concerning the data obtained for the miRNA hsa-miR-199a-5p, significant variations were observed only between cancer patients and patients with stroke&cancer ($p<0.05$), while only a non-significant decrement was observed between cancer patients vs patients with stroke. On the contrary, no significant variations were observed for the miRNAs hsa-miR-21-5p and hsa-miR-503-5p (Figure 6).

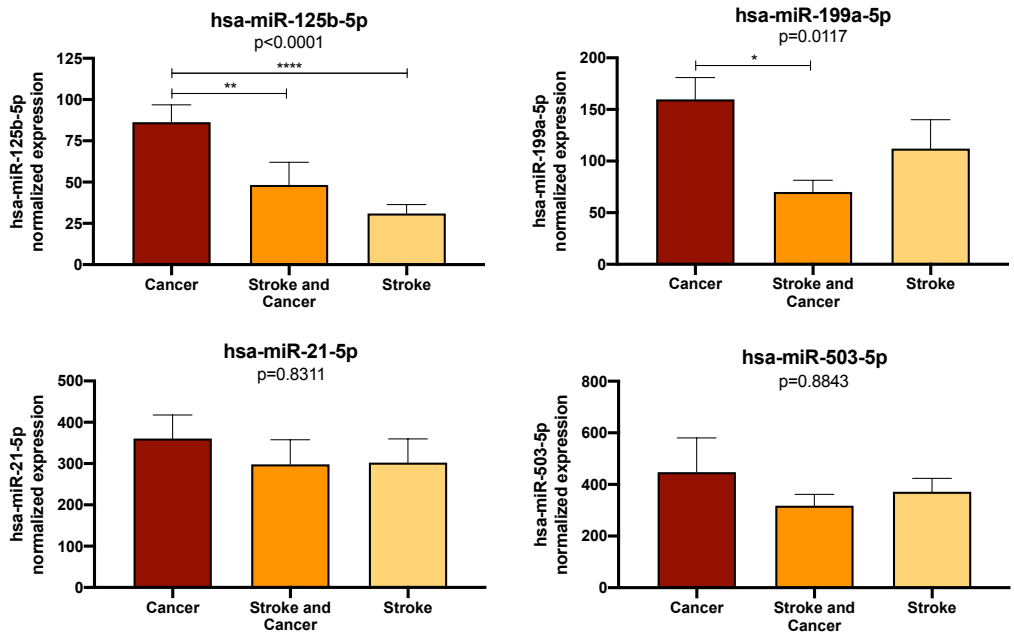

Figure 6. Statistical differences of the serum expression levels of the four selected miRNAs among cancer patients, patients with stroke and patients with cancer and stroke. The values are expressed as mean ± SEM. Kruskal-Wallis and Dunn's multiple comparison tests: * $p<0.05$; ** $p<0.01$; **** $p<0.0001$.

Similarly, by comparing the expression levels of the selected miRNAs in the group of healthy donors and in patients with stroke&cancer and stroke alone significant variations were observed (Figure 7).

In detail, these further analyses revealed that only the miRNA hsa-miR-199a-5p showed significant variation of the expression levels among groups ($p=0.0046$). Indeed, for this miRNA it was observed a more evident significant reduction of its expression levels between control and stroke&cancer patients ($p<0.01$) while a weaker, but significant, reduction was observed between control samples and samples obtained from patients with stroke ($p<0.05$). For the other three miRNAs, hsa-miR-125b-5p, hsa-miR-21-5p and hsa-miR-503-5p, no significant variations of miRNAs expression levels were observed. Only the miRNA hsa-miR-125b-5p showed trend of increment in case of stroke (both in patients with or without cancer), however, no significant data were obtained possibly due to the low number of samples included in each group (Figure 7).

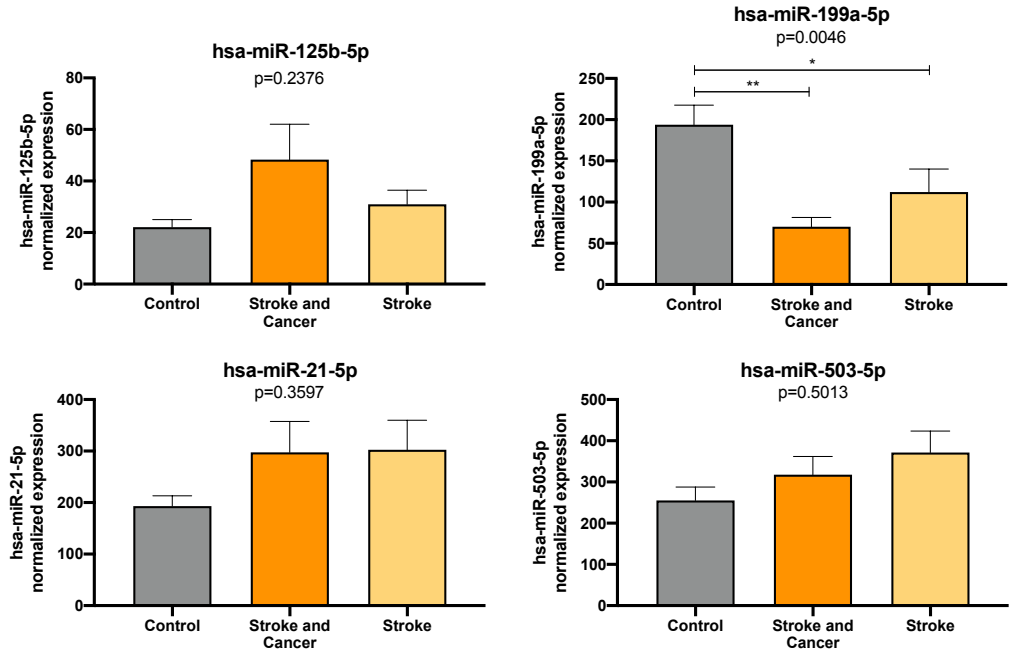

Figure 7. Statistical differences of the serum expression levels of the four selected miRNAs among healthy donors, patients with stroke and patients with cancer and stroke. The values are expressed as mean ± SEM. Kruskal-Wallis and Dunn's multiple comparison tests: * $p<0.05$; ** $p<0.01$.

Overall, the data obtained by analyzing the circulating levels of miRNAs in all the patients and controls recruited in this study revealed a potential involvement of the miRNAs hsa-miR-125b-5p and hsa-miR-199a-5p in the pathogenesis of cancer-related stroke. Of note, the data here obtained need to be further validated in a wider cohort of patients to further corroborate the preliminary findings observed.

As shown in Tables 2 and 3, opposite trends of expression were observed for some miRNAs. For example, the miRNA hsa-miR-21-5p is notoriously over-expressed in cancer patients compared to normal individuals, while in patients with stroke its expression levels are down-regulated. Similarly, hsa-miR-199a-5p and hsa-miR-503-5p are over-expressed in cancer and down-regulated in stroke, while the hsa-miR-125b-5p is known to be down-regulated in cancer as it acts as a tumor suppressor miRNA, while it is over-expressed in stroke. These data, together with those observed from the ddPCR results, suggest the existence of a balance between miRNA down-regulation and up-regulation responsible for

different clinical manifestations (e.g. increased risk of stroke or more aggressive tumor phenotype).

In order to better clarify the role of the selected miRNAs in cancer-related stroke, the expression levels of the miRNAs were evaluated only in patients with stroke&cancer and in patients with stroke to avoid potential bias introduced by the other two groups (only cancer and healthy donors) recruited in other studies. In addition, the expression levels of miRNAs were also evaluated considering specific clinical pathological features of patients with stroke (either cancer or not) including hypertension, hypercholesterolemia, diabetes, atrial fibrillation and other hematological parameters. Due to the low number of patients included in the study and some missing data such analyses were cumulative, considering stroke&cancer and stroke patients in the same group.

First, the expression levels of miRNAs between stroke&cancer and stroke patients were evaluated. The results revealed no statistical differences for the four selected miRNAs possibly due to the low number of samples analyzed. Overall, the levels of the miRNAs hsa-miR-21-5p and hsa-miR-503-5p were substantially similar in both groups, while non-significant decrement and a non-significant increment were observed for hsa-miR-125b-5p and hsa-miR-199a-5p, respectively (Figure 8).

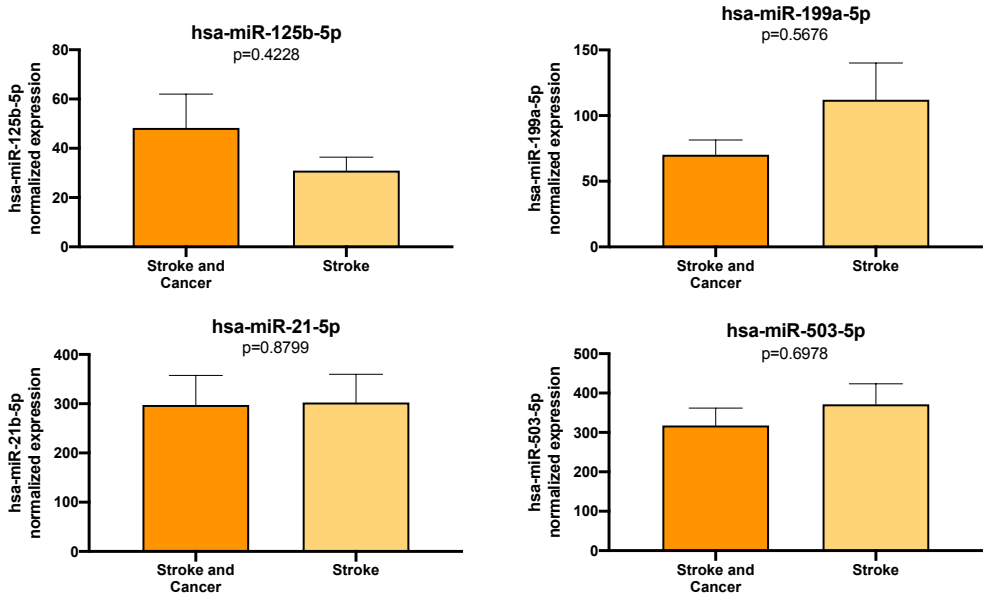

Figure 8. Statistical differences of the serum expression levels of the four selected miRNAs among patients with stroke and patients with cancer and stroke. The values are expressed as mean ± SEM. Mann-Whitney test.

Subsequently, the role of these miRNAs was evaluated considering the clinical-pathological features of stroke patients in order to establish if they can be associated with other risk factors to predict the risk of stroke.

First, the expression levels of miRNAs were evaluated according to the severity of stroke by using the National Institutes of Health Stroke Scale (NIHSS) score. All the patients were stratified into two groups as follows: NIHSS 1-4 (minor stroke); NIHSS 5-15 (moderate stroke) [104]. The results observed by analyzing patients according to their NIHSS score did not reveal any statistical differences among groups. Inconclusive results were obtained for the miRNAs hsa-miR-125b-5p and the hsa-miR-199a-5p which showed very variable expression levels, while for the miRNAs hsa-miR-21-5p and hsa-miR-503-5p a trend of increment was observed in more severe stroke, however, even these data were not statistically significant (Figure 9).

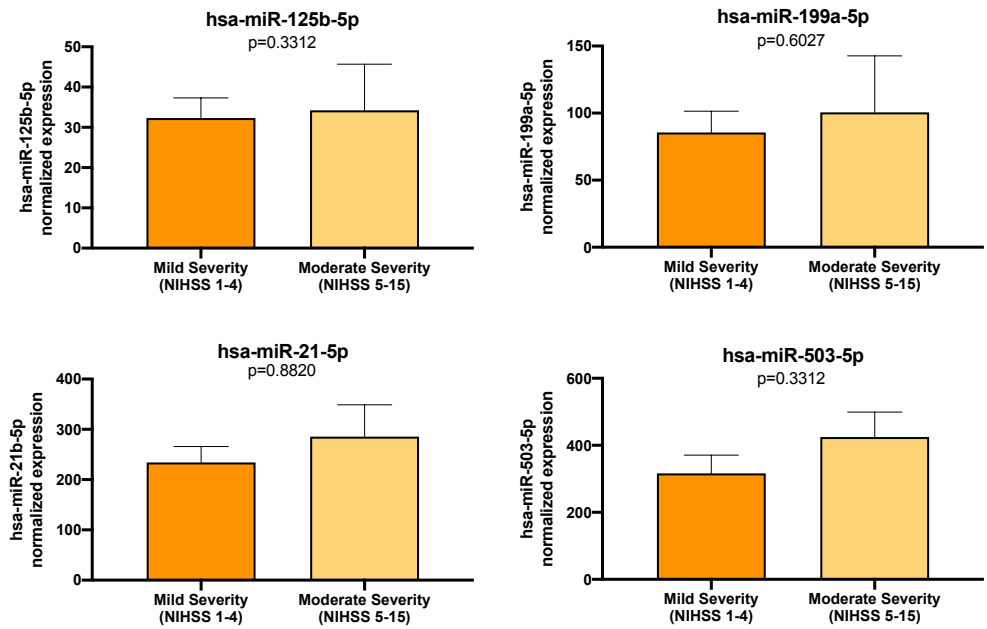

Figure 9. Statistical differences of the serum expression levels of the four selected miRNAs among patients with stroke and patients with cancer and stroke considering stroke severity (NIHSS). The values are expressed as mean ± SEM. Kruskal-Wallis and Dunn's multiple comparison tests.

No significant results were obtained by also analyzing miRNAs expression levels according to the presence or not of hypertension, hypercholesterolemia, diabetes, atrial fibrillation as well as hematological parameters including CRP, ESR, homocysteine, D-dimer, etc. (data not shown), therefore, further association with the selected miRNAs and patients' clinical features will be investigated in a wider cohort of patients.

Finally, the expression levels of the selected miRNAs were evaluated in the group of patients with cancer and stroke in order to establish if specific tumor types are more sensitive to the dysregulation of miRNAs. Among the cases recruited in this study, three have breast cancer, three were affected by colon cancer and two patients were affected by prostate cancer and the remaining three patients have different tumors (pancreatic cancer, cholangiocarcinoma and urothelial carcinoma).

These analyses did not show any significant data due to the limited number of samples available. Overall, patients suffering from colon cancer or pancreatic

cancer with stroke seem to have higher expression levels of all miRNAs analyzed. Breast cancer patients were those with the lower expression levels of miRNAs except for the miRNA hsa-miR-503-5p which showed higher but non-significant expression levels (Figure 10).

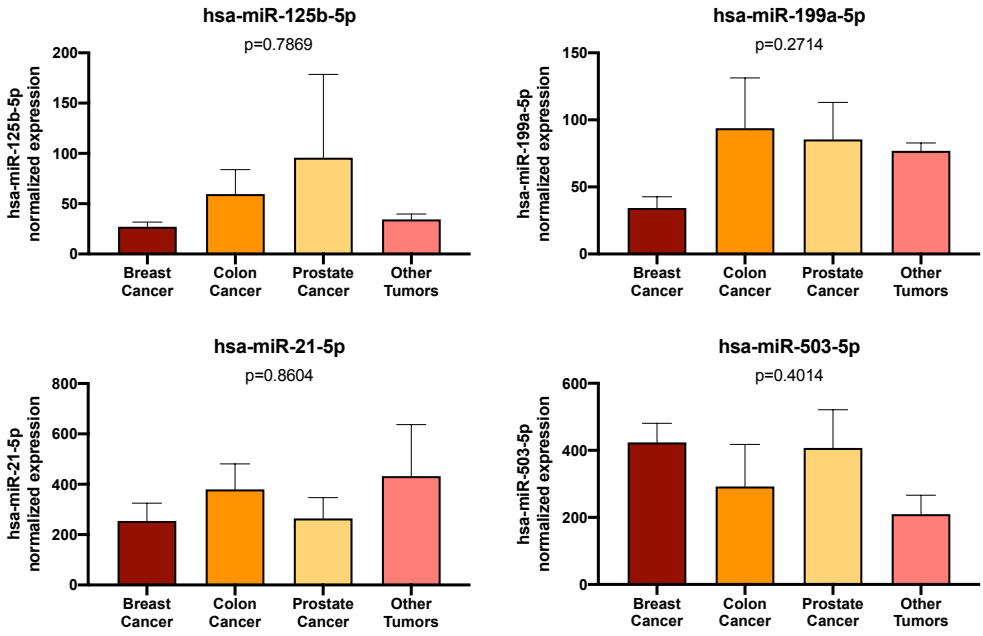

Figure 10. Statistical differences of the serum expression levels of the four selected miRNAs among patients with cancer and stroke considering the types of cancer. The values are expressed as mean ± SEM. Kruskal-Wallis and Dunn's multiple comparison tests.

Again, these results suggest the need of increasing the number of patients recruited in the study in order to obtain more reliable data. However, due to the overall not so common concomitant presentation of active cancer and stroke, longer studies are usually needed in this field. In addition, the COVID-19 pandemic has significantly slowed-down the recruitment of patients further complicating the collection of samples for the study.

As the primary endpoint of the study was to evaluate the diagnostic potential of miRNAs in predicting the risk of stroke in cancer patients, ROC curve analyses were finally performed for the two significantly dysregulated miRNAs, i.e. hsa-miR-125b-5p and hsa-miR-199a-5p. ROC analyses performed between the

expression levels of miRNAs observed in cancer patients compared with those observed in stroke&cancer patients revealed that both hsa-miR-125b-5p and hsa-miR-199a-5p have a good diagnostic potential in predicting the occurrence of stroke in cancer patients (Figure 11). In detail, the ROC analysis performed for the hsa-miR-125b-5p revealed an AUC value of 0.8244 with sensitivity and specificity rates of 82.3% and 75.0%, respectively (95% CI 0.6446 – 1.000, p=0.0013). The same analysis performed on the expression levels of hsa-miR-199a-5p revealed an AUC value of 0.7887 with sensitivity and specificity rates of 83.3% and 67.9%, respectively (95% CI 0.6434 – 0.9340, p=0.0042) (Figure 11).

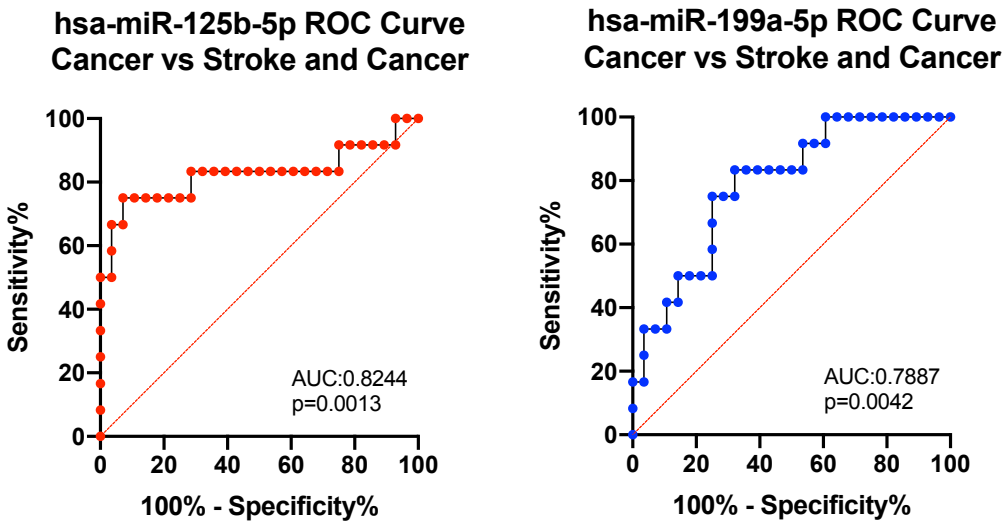

Figure 11. ROC analyses performed for the two significantly dysregulated miRNAs hsa-miR-125b-5p and hsa-miR-199a-5p in patients with cancer and patients with stroke and cancer.

Overall, the preliminary data obtained in this pilot study support the notion that both hsa-miR-125b-5p and hsa-miR-199a-5p may be used as novel biomarkers in cancer patients to predict the risk of complications like stroke.

4.3 Bioinformatics analysis of the functional roles of the two validated microRNAs in cancer and stroke

To further corroborate the involvement of the two validated miRNAs in the development of cancer-related stroke, additional in-depth computational analyses were performed. First, all the genes targeted by hsa-miR-125b-5p and hsa-miR-199a-5p were identified by using the bioinformatics tool miRWalk. By using this software, only genes with very high interaction levels with the two validated miRNAs were selected. Through this analysis it was possible to identify a very large number of genes targeted by both hsa-miR-125b-5p and hsa-miR-199a-5p (4,402 and 3,975 genes, respectively). By merging the lists of the genes targeted by these two miRNAs it was possible to observe a total of 390 genes concomitantly targeted by hsa-miR-125b-5p and hsa-miR-199a-5p (Figure 12) (Appendix A, Supplementary Table 2).

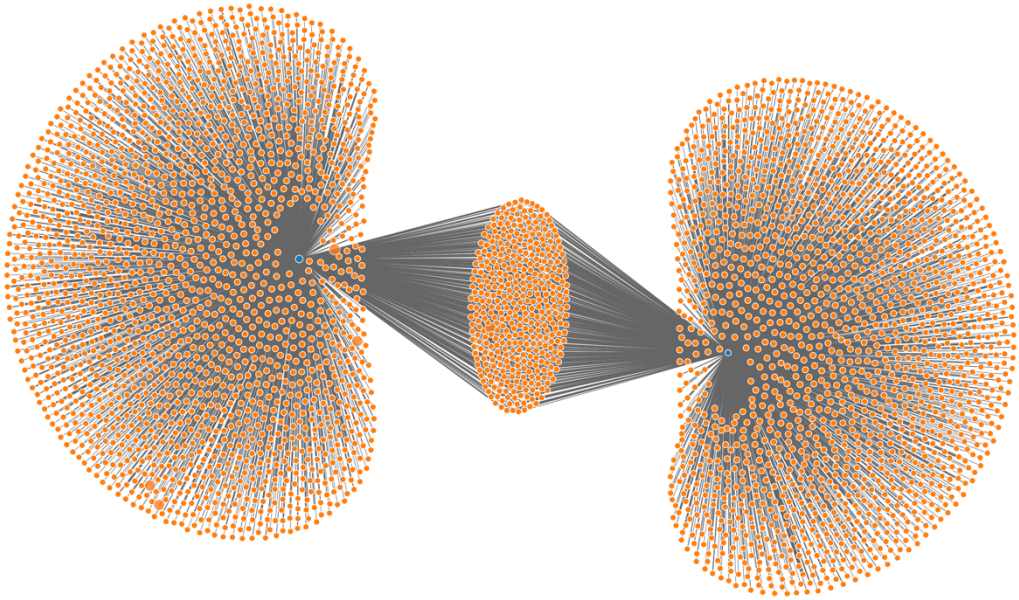

Figure 12. Schematic representation of the interaction network of hsa-miR-125b-5p and hsa-miR-199a-5p according to miRWalk analysis. hsa-miR-125b-5p network on the left and hsa-miR-199a-5p network on the right.

Overall, the miRWalk analysis revealed that the selected miRNAs were able to interact with different genes involved in multiple cellular and molecular

processes. Such interactions were strong as the binding between miRNAs and the 3'UTR region of targeted genes involved >10 nt.

To further establish the functional role of the 390 genes targeted by hsa-miR-125b-5p and hsa-miR-199a-5p a Protein-Protein interaction (PPI) analysis was performed through STRING. Due to the numerous genes identified, only protein-protein interactions with high confidence levels (0.900) were selected and displayed. As shown in Figure 13, STRING analysis revealed a very complex protein-protein interaction network involving 73 different genes that were interconnected to each other (Figure 13).

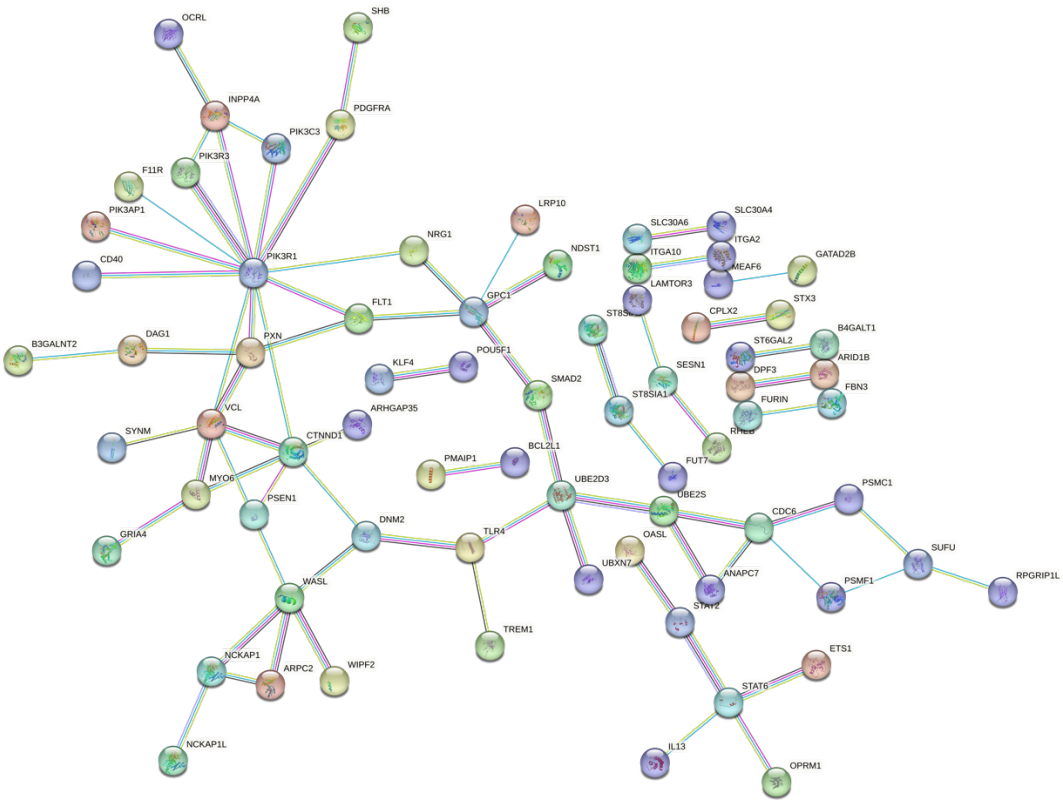

Figure 13. STRING protein-protein interaction network of 73 genes out of 390 targeted by both hsa-miR-125b-5p and hsa-miR-199a-5p.

In this intricate PPI network, key hub genes were UBE2D3, STAT6, GPC1, PIK3C3, etc., known to be associated with different pathological conditions, including cerebrovascular disorders.

More interestingly, by analyzing the functional role of these genes in the context of clinical phenotypes it was observed that these genes play fundamental regulating roles in both cardiovascular and neurological disorders. In detail, the modulation of these genes mediated by both hsa-miR-125b-5p and hsa-miR-199a-5p is associated with depressive symptoms, Alzheimer's disease, cognitive processes (intelligence, mental process, cognition), mental or behavioral disorders and cardiovascular diseases (Table 7). These data suggest that these miRNAs and their targeted genes may be used as biomarkers for the measurement of all these pathological conditions. In particular, the majority of these genes may serve as biomarkers for cardiovascular diseases and cardiovascular functions (Table 7).

Table 7. Phenotype enrichment of miRNA-targeted genes according to STRING (Monarch data)

Phenotype ID	Description	Count in Network	Strength	False Discovery Rate
EFO:0007006	Depressive symptom measurement	17 of 230	0.57	0.0123
EFO:0006514	Alzheimers disease biomarker measurement	24 of 416	0.46	0.0122
EFO:0004337	Intelligence	38 of 740	0.41	0.00049
EFO:0004323	Mental process	60 of 1342	0.35	3.49E-05
EFO:0003925	Cognition	59 of 1328	0.35	3.49E-05
EFO:0006848	Mental or behavioural disorder biomarker	69 of 1634	0.33	3.49E-05
EFO:0005278	Cardiovascular disease biomarker measurement	73 of 2232	0.22	0.0208
EFO:0004298	Cardiovascular measurement	77 of 2382	0.21	0.0198

Finally, a gene ontology analysis was performed through GO Panther database. In particular, the GO Panther analysis was performed to identify the main molecular pathways where the genes targeted by the validated miRNAs were involved (no investigations were performed on other gene ontology classes including cellular components, molecular function and biological processes).

Overall, GO Panther analysis revealed that the genes targeted by the validated miRNAs were involved in 67 different pathways (data not shown). In Figure 14 are reported the pathways containing at least five of the 390 genes identified by miRWalk considered the most represented and modulated pathways by the two selected miRNAs (Figure 14).

The three most altered pathways were the Integrin signaling, the Wnt signaling and the Gonadotropin-releasing hormone receptor pathways. More importantly, among the most targeted pathways there were key cellular and molecular pathways known to be involved in the pathogenesis of stroke including Angiogenesis, PDGF signaling pathway, VEGF signaling pathway, Inflammation and chemokine/cytokine signaling pathways, Endothelin signaling pathway, etc. (Figure 14).

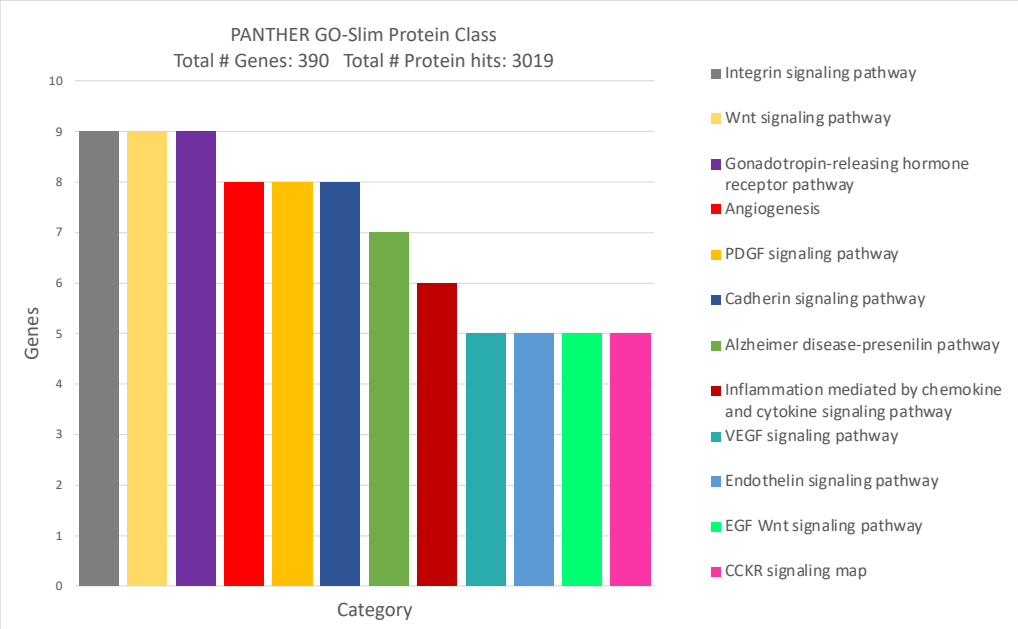

Figure 14. The GO PANTHER analysis revealed the cellular and molecular pathways of the 390 genes targeted by hsa-miR-125b-5p and hsa-miR-199a-5p.

These last data further suggest the involvement of the two selected and validated miRNAs in the pathogenesis of cancer-related stroke.

Of note, all these bioinformatics results need to be validated through both functional in vitro experiments and clinical evaluations. Therefore, future perspectives of the present study will be the analysis of a panel of circulating protein biomarkers associated with inflammation, angiogenesis, platelet activation and complement and coagulation cascades in order to confirm the regulatory role of hsa-miR-125b-5p and hsa-miR-199a-5p towards these further potential biomarkers predictive for stroke in cancer patients at risk for this pathology.

5 Discussion

Stroke is one of the deadliest acute diseases worldwide causing serious socio-demographic and socio-economic burdens due to the high disability and mortality rates [105].

Currently, there are no strategies for the early diagnosis of ischemic stroke, therefore, only therapeutic approaches are available, including thrombolysis and thrombectomy; however, the administration of lysing agents (e.g. tissue plasminogen) or the removal of the blood clot are often ineffective due to hypoxic brain damages, reperfusion injuries or hemorrhagic events [106].

Therefore, there is an urgent need to develop effective prevention strategies as well as novel diagnostic approaches for the early identification of hematological imbalances potentially associated with the onset of stroke.

Regarding the preventive strategies, primary prevention is aimed at reducing the risk of stroke in the general population avoiding some well-recognized risk factors like cigarette smoking. Secondary prevention has a different aim as is directed to patients at risk for this pathology by using anticoagulants or drugs that inhibit platelet activation and the formation of blood clots. This result can be achieved either by using antiplatelet activation factors or through the modulation of processes responsible for platelet aggregation [107].

About screening strategies or preventive diagnostic approaches, at present, there is a lack of clinical and molecular biomarkers for the diagnosis and prevention of stroke. Despite some studies trying to identify circulating serum biomarkers for the diagnosis of stroke, no conclusive results were obtained due to the multifactorial pathogenesis of this condition and the hidden underlying cellular and molecular mechanisms [108,109].

To overcome the current limitation related to the early diagnosis of stroke, in recent years, different studies have investigated the potential predictive role of ncRNAs to predict the risk of stroke. Beside the diagnostic potential of ncRNAs, the pathogenetic mechanisms driven by these epigenetic factors were also investigated [110,111].

In animal models of ischemic stroke, it was widely demonstrated that ncRNAs, including miRNAs, play fundamental roles in the modulation of the coagulation cascade responsible for cerebral ischemia.

As widely described in the Introduction section, miRNAs are small non-coding RNA molecules able to inhibit or modulate the expression levels of genes at post-transcriptional level. Although no certain results have been obtained, some preliminary studies demonstrated that miRNAs are actively involved in the multifactorial process responsible for ischemic stroke through the modulation of genes involved both in physiological and pathological processes [112,113].

Among the processes associated with an increased risk of stroke and regulated by miRNAs there are neuroinflammation, apoptosis, atherosclerosis, and angiogenesis, all mechanisms that are altered before and after ischemic stroke [90,114–116]. Other studies have demonstrated how miRNA and other ncRNAs (e.g. lncRNAs) can be selectively expressed in brain tissue or vascular endothelium regulating different physiological and pathological neurological processes in pre- and post-ischemic events [117–119]. Overall, all these studies suggest a pivotal role of miRNAs in the regulation of different processes related to stroke.

Besides the direct role of miRNA in the pathogenesis of stroke, a growing body of evidence has suggested that other pathological conditions may increase the risk of stroke, including cancer [120]. The main mechanisms involved with cancer-related stroke seem to be associated with direct effects mediated by tumors (e.g. arterial or venous neoplastic invasion, tumor emboli, metastatic circulating cells, blood vessel compression, etc.) or by the anticancer pharmacological treatment and radiation therapies which are associated with teratogen coagulopathies [121]. More recently miRNAs have also emerged as potential mediators of cancer-related stroke thus serving both as diagnostic biomarkers or therapeutic targets; however, the results of these preliminary studies were inconsistent and have not reached yet the general consensus by the scientific community.

To establish if miRNAs might serve as diagnostic and predictive biomarkers for cancer-related stroke we performed a computational and experimental study aimed at identifying miRNAs associated with the development of stroke in cancer patients.

In particular, a preliminary integrated computational study was performed by analyzing all the existing microarray miRNA expression datasets related to stroke and deposited on GEO DataSets database. Specific inclusion and exclusion criteria were adopted for the selection of datasets containing a sufficient number of samples (>10) obtained from both stroke patients and normal controls. Subsequently, the most dysregulated miRNAs associated with the presence of stroke were identified through differential analyses performed in each dataset by using GEO2R, as already done in other similar studies [60,68]. Through these analyses it was possible to identify a list of 21 miRNAs strongly dysregulated in the presence of stroke. All these miRNAs could be potentially involved in the occurrence of stroke, therefore, other studies on the functional role of these miRNAs will be helpful to validate and clarify their effective role in this pathology.

In order to strengthen the data obtained regarding stroke-related miRNAs, a further computational analysis was performed to identify the most dysregulated miRNAs in multiple tumors. Therefore, the miRNA expression data contained in The Cancer Genome Atlas PANCANCER database were downloaded and manually analyzed in order to identify the most dysregulated miRNAs in cancer. The results of these further computational investigations with high stringency ($p<0.01$ Log2FC $\pm$ 1.25) revealed 28 dysregulated miRNAs in all tumors of which 11 were strongly up-regulated and 17 were strongly down-regulated. This further analysis was performed not only to identify miRNAs as potential biomarkers in cancer, but also to filter among the stroke-related miRNAs those also involved in the development and progression of tumors. Indeed, by merging the two lists of dysregulated miRNAs (stroke-associated and cancer-associated miRNAs) it was possible to identify five miRNAs involved in both cancer and stroke that may be used as novel effective biomarkers to predict the risk of stroke in cancer patients. The five miRNAs identified were the miRNAs hsa-miR-

125b-5p, hsa-miR-21-5p, hsa-miR-199a-5p, hsa-miR-503-3p and hsa-miR-140-3p. Of note, all these miRNAs are known to be strongly associated with the development of multiple tumors [60,71,122,123]. Regarding the role of these miRNAs in the development of stroke, multiple studies revealed how hsa-miR-21-5p is up-regulated in ischemic stroke as recently summarized by Bai and Bian [67]. Another miRNA widely studied in stroke patients is hsa-miR-125b-5p as this miRNA can be used as a biomarker to monitor the clinical course of post-ischemic patients [124,125]. Similarly, important functions were also observed for the miRNA hsa-miR-199a-5p in post-ischemic model as this miRNA seems to have a detrimental effect on cognitive functions, therefore, some preliminary studies demonstrated that its inhibition may have protective effects on neurological functions in stroke animal models [126]. More interesting data were obtained for hsa-miR-503-5p which seems to be involved in the pathogenesis of both cancer and cardiovascular diseases. Indeed, this miRNA is able to influence the proliferation of vascular smooth muscle cells and the formation of atherosclerosis thus regulating the key mechanisms responsible for stroke [127–129]. Finally, hsa-miR-140-3p was associated with the regulation of macrophage activities and a protective role in ischemic brain [130,131].

Overall, all these studies confirm the association between the five selected miRNAs and key processes responsible for stroke and stroke-related neurological complications. These literature findings were partially confirmed by other computational investigations performed in this study. Indeed, the five stroke- and cancer-associated miRNAs were strongly involved in the modulation of different key molecular signaling pathways (Hippo, p53, MAPK, etc.) as predicted by the DIANA mirPath analyses performed. In addition, this analysis also revealed that all the five selected miRNAs were involved in the modulation of two key pathways implicated in the pathogenesis of stroke, i.e. the “platelet activation (hsa04611)” and the “complement and coagulation cascades (hsa04610)” pathways [132,133].

The analysis of the five miRNAs in the two latter pathways revealed that these miRNAs regulate the MAPK and PI3K/Akt signal transduction pathways by interacting with multiple genes as well as being able to regulate the CD59 and

SERPINE1 genes within the “Complement and coagulation cascades” pathway. Of note, the regulatory role of miRNAs in the MAPK and PI3K/Akt pathways was already known in stroke patients [119,134]; in addition, CD59 and SERPINE1 are also known to be associated with cardiovascular and cerebrovascular disorders as these two genes are responsible for the inhibition of complement cascade and the inhibition of tissue-type plasminogen activator (PLAT) and urokinase-type plasminogen activator (PLAU), respectively [135,136].

The computational data obtained sustained the effective involvement of the five selected miRNAs hsa-miR-125b-5p, hsa-miR-21-5p, hsa-miR-199a-5p, hsa-miR-503-3p and hsa-miR-140-3p in cancer-related stroke. Therefore, the expression levels of these five miRNAs were investigated in liquid biopsy samples obtained from healthy donors, cancer patients, patients with stroke and patients with concomitant cancer and stroke. To obtain high-sensitive miRNA quantification, the validation analyses on miRNA circulating levels were performed by using the high-sensitive ddPCR technology.

By analyzing the expression levels of the five selected miRNAs by ddPCR, significant variations were observed among groups for the miRNAs hsa-miR125b-5p and hsa-miR199a-5p, while no amplification signals were obtained for the hsa-miR-140-3p due to technical issues. More in detail, a decrement of both hsa-miR-125b-5p and hsa-miR-199a-5p was observed in stroke and cancer patients and patients with only stroke compared to cancer patients. As already mentioned, this data is not surprising as hsa-miR-125b-5p is a well-known tumor suppressor miRNA strongly dysregulated in cancer [71]. However, the data herein obtained demonstrated, for the first time, that its further down-regulation may be associated with cancer-related stroke thus with a worse prognosis in these patients. In this context, it was also demonstrated that hsa-miR-125b-5p actively participates in the pathogenesis of atherosclerosis, vascular inflammation and endothelial damage playing a key role in different cardiovascular diseases [137]. However, in some cases, it is not clear if the down-regulation or up-regulation of this miRNA can be considered a positive or negative prognostic factor in stroke [124].

To clarify the conflictual role of hsa-miR-125b-5p in cancer-related stroke a ROC analysis was performed by considering the circulating expression levels of hsa-miR-125b-5p in cancer patients compared to patients with both cancer and stroke. This analysis confirmed the good diagnostic potential of this miRNAs in discriminating patient with cancer and patients with both cancer and stroke, suggesting how the monitoring of the expression levels of this miRNA may be useful to predict an increased risk of cancer-related stroke (AUC=0.8244, 82.3% sensitivity and 75.0% specificity; p=0.0013).

The ROC analysis performed for the circulating expression levels of hsa-miR-199a-5p in cancer patients compared to patients with both cancer and stroke also revealed a good diagnostic value (AUC=0.7887, 83.3% sensitivity 67.9% and specificity; p=0.0042). Of note, hsa-miR-199a-5p is often up-regulated in cancer while it seems to be down-regulated in stroke as revealed by the computational investigations here performed. Therefore, it could be speculated that the fine regulation of the expression levels of this miRNA may influence the prognosis of cancer patients by promoting tumor aggressiveness in certain tumors in the case of miRNA over-expression [70] or vascular and brain disorders in the case of its down-regulation [138].

Overall, the data obtained for these two miRNAs support their involvement in cancer-related stroke. However, due to the low number of patients included in the study, no conclusive results were obtained by analyzing the expression levels of these miRNAs and considering different clinical-pathological features of patients. To partially overcome this limitation of the study, a further computational analysis was performed to investigate the genes targeted by both hsa-miR-125b-5p and hsa-miR-199a-5p and the involvement of these genes in cellular processes related to stroke. For this purpose, a bioinformatics analysis was performed by using miRWalk, a software that allows the identification of genes targeted by specific miRNAs. Through this analysis it was possible to observe how hsa-miR-125b-5p and hsa-miR-199a-5p can concomitantly target a total of 390 genes. Further PPI and GO analyses performed on these genes have finally revealed how the miRNA-targeted genes were involved in a complex PPI network where key hub genes are UBE2D3, STAT6, GPC1 and PIK3C3.

Noteworthy, STAT6, together with inflammatory cytokines, is involved in the resolution and recovery of stroke inflammatory damages [139,140] thus its regulation mediated by miRNAs could influence such resolutive processes. Similarly, GPC1 is actively involved in the protection of endothelial vessel cells from stiffness-mediated dysfunction and disease [141]. The involvement of the members of the PI3K family was already discussed.

By analyzing the pathway interconnected with these miRNA-targeted genes we observed a significant enrichment in pathways associated with neurological disorders and cardiovascular diseases further corroborating the pivotal role of hsa-miR-125b-5p and hsa-miR-199a-5p in stroke.

Finally, Gene Ontology analyses performed through GO Panther confirmed the results described above highlighting how the genes regulated by hsa-miR-125b-5p and hsa-miR-199a-5p were mainly associated with key cellular and molecular pathways known to be involved in the pathogenesis of stroke including Angiogenesis, PDGF signaling pathway, VEGF signaling pathway, Inflammation and chemokine/cytokine signaling pathways, and Endothelin signaling pathway [142,143].

These last data further suggest the involvement of the two selected and validated miRNAs hsa-miR-125b-5p and hsa-miR-199a-5p in the pathogenesis of cancer-related stroke.

Despite the encouraging data here described, the study presents some limitations. First, other computationally selected miRNAs may be involved in the pathogenesis of cancer-related stroke, therefore, further experiments on liquid biopsy samples are needed to test the expression levels of other miRNAs here identified (Table 2). Second, the preliminary findings here observed should be validated in a wider cohort of patients in order to obtain more reliable and statistically significant results. In addition, the patients enrolled in the present study were clinically heterogeneous, therefore for future studies a more homogeneous selection of both cases and controls should be performed.

Finally, additional interesting results could be obtained by also analyzing the mRNA expression or circulating protein levels of some of the factors directly

targeted by hsa-miR-125b-5p and hsa-miR-199a-5p. In this context, RT-qPCR experiments or ELISA assay may be useful to correlate the dysregulation of miRNAs with the alteration of key factors involved in inflammatory and vascular processes.

6 Conclusions

The computational and experimental approaches adopted in this study allowed the identification of a panel of miRNAs potentially associated with both cancer and stroke that can be used as predictive biomarkers in cancer patients at risk for stroke.

The validation of the diagnostic potential of the five selected miRNAs hsa-miR-125b-5p, hsa-miR-21-5p, hsa-miR-199a-5p, hsa-miR-503-5p and hsa-miR-140-3p performed on liquid biopsy samples by using the high-sensitive ddPCR amplification system revealed statistically significant alterations among groups for the miRNAs hsa-miR-125b-5p and hsa-miR-199a-5p. Additional statistical analyses confirmed the diagnostic potential of both hsa-miR-125b-5p and hsa-miR-199a-5p which can be considered good predictive biomarkers of stroke in patients with cancer.

Further computational investigations revealed the pathogenetic role of these two miRNAs in stroke, demonstrating their involvement in the regulation of multiple genes associated with key cellular and molecular pathways responsible for the alteration of the complement and coagulation cascades or the alteration of inflammatory and angiogenetic processes.

Overall, this research project led to the identification of novel candidate biomarkers specific for stroke in cancer patients, potentially filling the gap currently existing in the diagnosis and therapy of this disease. All these results encourage the use of both hsa-miR-125b-5p and hsa-miR-199a-5p for the prediction of cancer-related stroke in cancer patients suggesting how these may represent novel suitable biomarkers for the management of this disease. However, to confirm the real diagnostic potential of hsa-miR-125b-5p and hsa-miR-199a-5p, the data here obtained need to be further validated in a wider cohort of patients collecting clinical-pathological data and evaluating other miRNA-related biomarkers.

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8 APPENDIX A – Supplementary Materials

Supplementary Table 1. Cellular and Molecular Pathways modulated by the five selected miRNAs

No.	KEGG Pathway	p-value	No. Genes	No. miRNAs
1	Fatty acid biosynthesis (hsa00061)	<1e-325	1	1
2	ECM-receptor interaction (hsa04512)	<1e-325	14	2
3	Hippo signaling pathway (hsa04390)	<1e-325	47	4
4	Proteoglycans in cancer (hsa05205)	1.1213E-14	57	4
5	Fatty acid metabolism (hsa01212)	6.9367E-13	10	2
6	Lysine degradation (hsa00310)	1.3286E-10	18	3
7	Pathways in cancer (hsa05200)	4.8001E-09	74	3
8	Pluripotency of stem cells (hsa04550)	5.5853E-08	28	2
9	TGF-beta signaling pathway (hsa04350)	3.7925E-07	22	3
10	Colorectal cancer (hsa05210)	7.3583E-07	18	2
11	Adherens junction (hsa04520)	7.7602E-07	20	3
12	Cell cycle (hsa04110)	3.1153E-06	39	4
13	p53 signaling pathway (hsa04115)	6.9491E-06	27	4
14	Other types of O-glycan biosynthesis (hsa00514)	9.6598E-06	8	2
15	GAG biosynthesis, heparan sulfate/heparin (hsa00534)	2.5456E-05	2	2
16	Oocyte meiosis (hsa04114)	3.3443E-05	16	2
17	Bladder cancer (hsa05219)	4.7637E-05	16	4
18	Bacterial invasion of epithelial cells (hsa05100)	4.8756E-05	22	3
19	Hepatitis B (hsa05161)	5.8915E-05	35	3

20	Fatty acid elongation (hsa00062)	7.8821E-05	6	1
21	Prostate cancer (hsa05215)	0.00012045	16	2
22	Chronic myeloid leukemia (hsa05220)	0.0001323	15	2
23	Focal adhesion (hsa04510)	0.00017014	52	4
24	RNA transport (hsa03013)	0.0002232	31	2
25	Biosynthesis of unsaturated fatty acids (hsa01040)	0.00024713	6	2
26	Endocytosis (hsa04144)	0.00025328	30	3
27	Thyroid hormone signaling pathway (hsa04919)	0.00031957	25	2
28	HTLV-I infection (hsa05166)	0.00079499	27	2
29	Endometrial cancer (hsa05213)	0.00113369	14	2
30	Viral carcinogenesis (hsa05203)	0.00124079	39	3
31	Regulation of actin cytoskeleton (hsa04810)	0.00126278	42	2
32	FoxO signaling pathway (hsa04068)	0.00162262	24	1
33	Ubiquitin mediated proteolysis (hsa04120)	0.00163267	18	2
34	Thyroid cancer (hsa05216)	0.00172257	10	2
35	Glioma (hsa05214)	0.00243063	9	2
36	Calcium reabsorption (hsa04961)	0.00250054	10	3
37	Renal cell carcinoma (hsa05211)	0.00337444	13	2
38	PI3K-Akt signaling pathway (hsa04151)	0.0041269	14	1
39	Melanoma (hsa05218)	0.0042142	11	2
40	ErbB signaling pathway (hsa04012)	0.0045175	10	1
41	Misregulation in cancer (hsa05202)	0.00532961	24	1
42	HIF-1 signaling pathway (hsa04066)	0.00625052	19	2

43	Central carbon metabolism in cancer (hsa05230)	0.00694929	10	2
44	Prolactin signaling pathway (hsa04917)	0.00724537	14	2
45	Small cell lung cancer (hsa05222)	0.00917578	17	3
46	Protein processing in ER (hsa04141)	0.01000062	26	2
47	Rap1 signaling pathway (hsa04015)	0.01067379	11	1
48	Pancreatic cancer (hsa05212)	0.01687203	4	1
49	Amoebiasis (hsa05146)	0.0215843	7	1
50	Choline metabolism in cancer (hsa05231)	0.02613301	11	1
51	Base excision repair (hsa03410)	0.02616709	4	1
52	Non-small cell lung cancer (hsa05223)	0.02643233	9	2
53	MAPK signaling pathway (hsa04010)	0.02695981	49	2
54	Glycerophospholipid metabolism (hsa00564)	0.03411358	14	2
55	Fatty acid degradation (hsa00071)	0.03597035	6	1
56	Pathogenic Escherichia coli infection (hsa05130)	0.04103261	5	1
57	Epstein-Barr virus infection (hsa05169)	0.04748796	39	2

Supplementary Table 2. miRWalk analysis of the genes concomitantly targeted by hsa-miR-125b-5p and hsa-miR-199a-5p with very high interaction levels.

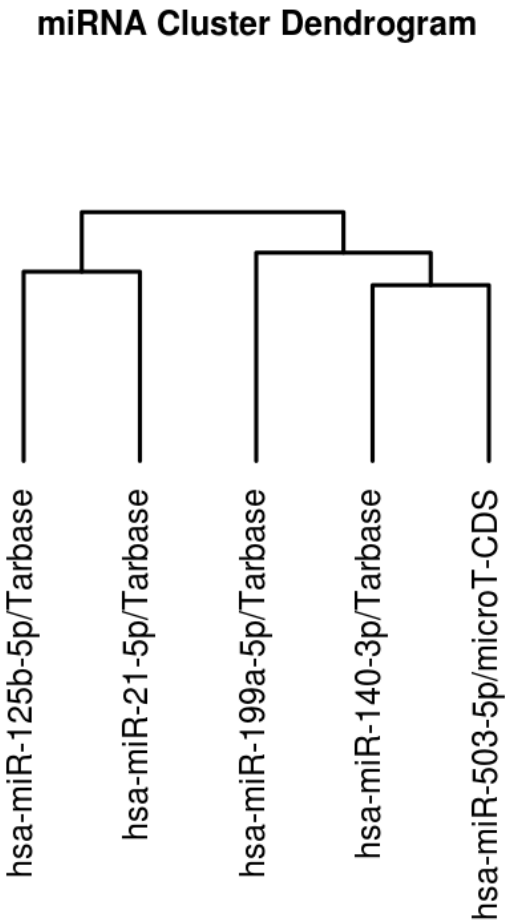
hsa-miR-125b-5p				hsa-miR-199a-5p			
Gene ID	**N. of pairing**	**Gene ID**	**N. of pairing**	**Gene ID**	**N. of pairing**	**Gene ID**	**N. of pairing**
AGO3	17	NFATC3	20	AGO3	20	NFATC3	18
ACTRT3	11	NIPAL4	10	ACTRT3	16	NIPAL4	17
ADAM23	19	NKAIN3	17	ADAM23	16	NKAIN3	17
ADCYAP1R1	18	NME9	15	ADCYAP1R1	21	NME9	21
AFF2	17	NMNAT2	18	AFF2	21	NMNAT2	16
ANAPC7	17	NOBOX	17	ANAPC7	15	NOBOX	20
ANXA13	14	NPTXR	16	ANXA13	17	NPTXR	17
AP1S2	16	NPY4R2	18	AP1S2	16	NPY4R2	19
AP5S1	15	NRG1	17	AP5S1	21	NRG1	16
AREL1	18	NSL1	16	AREL1	19	NSL1	19
ARGFX	17	NTNG2	18	ARGFX	20	NTNG2	17
ARHGAP35	18	NUDT4	16	ARHGAP35	16	NUDT4	17
ARHGAP45	17	NUDT5	11	ARHGAP45	17	NUDT5	19
ARID1B	19	NUMBL	16	ARID1B	18	NUMBL	17
ARPC2	13	OASL	17	ARPC2	18	OASL	14
ARPIN	15	OCRL	18	ARPIN	19	OCRL	18
ARPP19	20	ONECUT2	16	ARPP19	18	ONECUT2	18
ATXN1	16	OPALIN	17	ATXN1	19	OPALIN	16
B3GALNT2	17	OPRM1	16	B3GALNT2	17	OPRM1	16
B4GALT1	18	OR2C3	14	B4GALT1	15	OR2C3	18
B4GAT1	15	OR6A2	14	B4GAT1	18	OR6A2	19
BCL2L1	18	ORMDL3	15	BCL2L1	19	ORMDL3	19
BEND6	10	OXNAD1	17	BEND6	12	OXNAD1	14
BTBD7	16	P2RY2	18	BTBD7	12	P2RY2	19
BTN2A1	18	P3R3URF	19	BTN2A1	16	P3R3URF	13
C12orf43	17	PAQR6	15	C12orf43	14	PAQR6	19
C1orf50	18	PAQR7	14	C1orf50	18	PAQR7	21
C21orf62	12	PAQR9	15	C21orf62	20	PAQR9	14
C2orf72	15	PCDH9	19	C2orf72	19	PCDH9	15
CACNA1E	18	PDE10A	11	CACNA1E	16	PDE10A	21
CALHM5	16	PDGFRA	12	CALHM5	18	PDGFRA	20
CALN1	16	PDK3	14	CALN1	20	PDK3	20
CAPN13	16	PEX5L	16	CAPN13	14	PEX5L	20
CARD8	16	PGPEP1	17	CARD8	19	PGPEP1	17
CAV2	13	PHLDA1	18	CAV2	14	PHLDA1	17
CD2BP2	11	PIGL	15	CD2BP2	19	PIGL	17

CD40	12	PIK3AP1	18	CD40	18	PIK3AP1	21
CDC6	14	PIK3C3	15	CDC6	17	PIK3C3	19
CDH13	15	PIK3R1	14	CDH13	13	PIK3R1	19
CDH4	14	PIK3R3	19	CDH4	19	PIK3R3	13
CDHR1	16	PKHD1L1	18	CDHR1	18	PKHD1L1	15
CEP350	10	PLCXD1	15	CEP350	16	PLCXD1	17
CEP57L1	16	PLEKHG3	20	CEP57L1	18	PLEKHG3	17
CERT1	14	PMAIP1	15	CERT1	18	PMAIP1	18
CFLAR	14	POFUT2	12	CFLAR	14	POFUT2	17
CHCHD7	16	POU2F1	17	CHCHD7	17	POU2F1	19
CHRNG	19	POU5F1	16	CHRNG	16	POU5F1	21
CHST6	12	PPT1	18	CHST6	18	PPT1	14
CIAO2A	17	PRDM11	18	CIAO2A	19	PRDM11	18
CIITA	16	PRDM2	18	CIITA	17	PRDM2	18
CLMN	14	PRDM6	19	CLMN	19	PRDM6	13
CLSTN2	21	PRLR	11	CLSTN2	17	PRLR	20
CNBD2	16	PRRG3	11	CNBD2	14	PRRG3	17
CNR1	19	PSEN1	17	CNR1	15	PSEN1	17
CNTLN	17	PSMC1	19	CNTLN	20	PSMC1	18
COA7	10	PSMF1	17	COA7	20	PSMF1	21
COL20A1	17	PUS7L	13	COL20A1	15	PUS7L	15
COPS8	9	PXN	20	COPS8	19	PXN	19
CORO6	10	RAET1E	20	CORO6	21	RAET1E	17
COX18	18	RASL10B	12	COX18	21	RASL10B	18
CPLX2	17	RBFOX1	14	CPLX2	16	RBFOX1	15
CREG2	12	RBM14	14	CREG2	19	RBM14	16
CTNND1	16	RBM23	16	CTNND1	18	RBM23	19
CYP2U1	19	RCSD1	16	CYP2U1	15	RCSD1	20
CYREN	19	RECQL5	16	CYREN	18	RECQL5	17
DAG1	16	RFFL	15	DAG1	12	RFFL	16
DCTD	15	RHEB	12	DCTD	19	RHEB	14
DCTN5	16	RNF115	19	DCTN5	18	RNF115	18
DENND2C	17	RNF145	13	DENND2C	19	RNF145	20
DERL2	13	RNF152	16	DERL2	20	RNF152	15
DGKG	16	RPGRIP1L	15	DGKG	20	RPGRIP1L	20
DISC1	15	RPS27L	18	DISC1	18	RPS27L	18
DNM2	13	RTN4	17	DNM2	16	RTN4	12
DPF3	14	RUBCNL	16	DPF3	23	RUBCNL	20
DPYSL3	16	SAMD4A	16	DPYSL3	19	SAMD4A	20
DSC2	18	SCAI	16	DSC2	22	SCAI	16

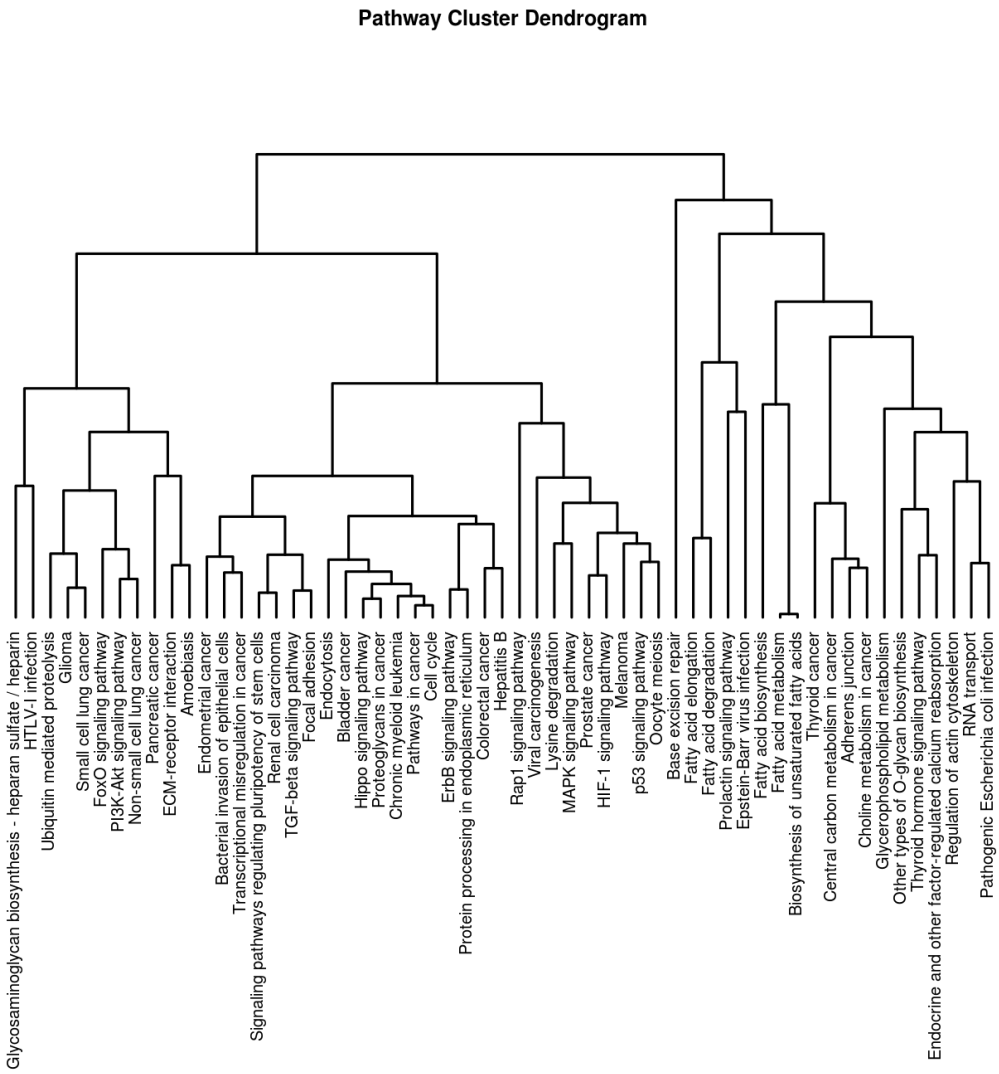
DSE	17	SCD5	16	DSE	14	SCD5	18
DYRK1A	19	SDHC	10	DYRK1A	14	SDHC	20
EBF3	12	SEC14L2	13	EBF3	18	SEC14L2	17
EIF2AK1	16	SERINC3	19	EIF2AK1	17	SERINC3	20
EIF5	17	SERPINB12	14	EIF5	18	SERPINB12	18
ENSA	14	SESN1	13	ENSA	18	SESN1	15
EOLA2	11	SHB	16	EOLA2	15	SHB	19
EPHA10	21	SHISA6	19	EPHA10	18	SHISA6	15
EPN2	17	SHOX	10	EPN2	21	SHOX	21
ETS1	17	SIAH3	15	ETS1	16	SIAH3	18
F11R	15	SINHCAF	14	F11R	20	SINHCAF	15
FABP2	12	SLC24A2	19	FABP2	19	SLC24A2	21
FAM126A	16	SLC24A4	17	FAM126A	19	SLC24A4	21
FAM241B	10	SLC30A4	16	FAM241B	20	SLC30A4	20
FAM53C	18	SLC30A6	17	FAM53C	20	SLC30A6	15
FAM98B	17	SLC35C2	16	FAM98B	18	SLC35C2	19
FARSB	17	SLC41A1	17	FARSB	21	SLC41A1	19
FBN3	18	SLC5A3	12	FBN3	18	SLC5A3	19
FGF1	12	SLC7A2	18	FGF1	14	SLC7A2	19
FHIP1A	16	SLC7A6OS	15	FHIP1A	18	SLC7A6OS	16
FLRT2	19	SLCO5A1	17	FLRT2	17	SLCO5A1	17
FLT1	15	SMAD2	11	FLT1	19	SMAD2	16
FOXP1	13	SNX22	16	FOXP1	20	SNX22	16
FRMD5	19	SOGA1	19	FRMD5	21	SOGA1	23
FSTL1	14	SOX5	15	FSTL1	16	SOX5	18
FURIN	14	SP4	16	FURIN	18	SP4	14
FUT7	16	SPATS2	14	FUT7	15	SPATS2	15
FXYD3	15	SPECC1	11	FXYD3	19	SPECC1	21
GABRA2	11	SPN	11	GABRA2	17	SPN	18
GABRQ	18	SPRY3	17	GABRQ	18	SPRY3	15
GALNT6	16	SPRYD4	18	GALNT6	19	SPRYD4	20
GATAD2B	17	SRGAP1	17	GATAD2B	16	SRGAP1	17
GATD1	18	SRSF10	16	GATD1	19	SRSF10	20
GGCX	17	ST6GAL2	12	GGCX	18	ST6GAL2	18
GIPR	18	ST8SIA1	12	GIPR	20	ST8SIA1	20
GJB1	17	ST8SIA5	17	GJB1	17	ST8SIA5	21
GLRA2	16	STAT2	11	GLRA2	19	STAT2	18
GMEB1	18	STAT6	20	GMEB1	20	STAT6	19
GP2	13	STIM1	11	GP2	17	STIM1	17
GPC1	16	STIMATE	20	GPC1	15	STIMATE	17

GRIA4	18	STK32B	16	GRIA4	19	STK32B	16
GRIK3	17	STK35	10	GRIK3	21	STK35	13
GTDC1	15	STMN4	13	GTDC1	20	STMN4	19
GTF2A1	16	STOX2	14	GTF2A1	19	STOX2	18
GUCY1A1	16	STRN	13	GUCY1A1	17	STRN	15
H6PD	12	STX3	17	H6PD	18	STX3	14
HAPLN4	20	SUFU	20	HAPLN4	20	SUFU	17
HCN4	15	SYN3	20	HCN4	17	SYN3	20
HDAC9	11	SYNM	20	HDAC9	14	SYNM	17
HDHD2	16	SYT12	18	HDHD2	15	SYT12	19
HDLBP	17	SYT2	19	HDLBP	18	SYT2	19
HEBP2	18	TAF5L	17	HEBP2	18	TAF5L	18
HLCS	16	TANC2	15	HLCS	18	TANC2	18
HMGCR	11	TAOK1	16	HMGCR	17	TAOK1	17
HMGXB4	12	TBC1D16	19	HMGXB4	16	TBC1D16	20
IGF2BP1	16	TET2	19	IGF2BP1	16	TET2	19
IL13	18	TIGD3	18	IL13	20	TIGD3	18
INPP4A	19	TIGIT	15	INPP4A	17	TIGIT	18
IP6K1	22	TLR10	17	IP6K1	18	TLR10	19
IST1	12	TLR4	14	IST1	16	TLR4	16
ITGA10	13	TMEM135	15	ITGA10	17	TMEM135	20
ITGA2	17	TMEM161A	11	ITGA2	18	TMEM161A	18
ITM2B	18	TMEM164	20	ITM2B	19	TMEM164	19
JAKMIP2	18	TMEM168	19	JAKMIP2	17	TMEM168	17
JPH2	20	TMEM199	14	JPH2	18	TMEM199	16
KCNA6	13	TMEM30B	12	KCNA6	18	TMEM30B	16
KCNG4	17	TMEM59	18	KCNG4	20	TMEM59	19
KCNK10	18	TMEM8B	15	KCNK10	17	TMEM8B	20
KCNK6	16	TOR1AIP2	18	KCNK6	19	TOR1AIP2	17
KCNQ1	16	TPPP2	11	KCNQ1	18	TPPP2	20
KCNQ2	19	TPST2	13	KCNQ2	19	TPST2	18
KCTD11	21	TRAPPC5	18	KCTD11	17	TRAPPC5	20
KCTD21	11	TREM1	16	KCTD21	20	TREM1	19
KIAA0895L	13	TRIM36	13	KIAA0895L	21	TRIM36	16
KIAA1549	18	TRPV3	18	KIAA1549	14	TRPV3	23
KIF2C	14	TSKU	15	KIF2C	18	TSKU	18
KLF4	14	TSPAN18	16	KLF4	18	TSPAN18	18
LAMTOR3	17	UBE2D3	14	LAMTOR3	14	UBE2D3	12
LARP4	12	UBE2S	13	LARP4	17	UBE2S	15
LCLAT1	10	UBIAD1	17	LCLAT1	19	UBIAD1	15

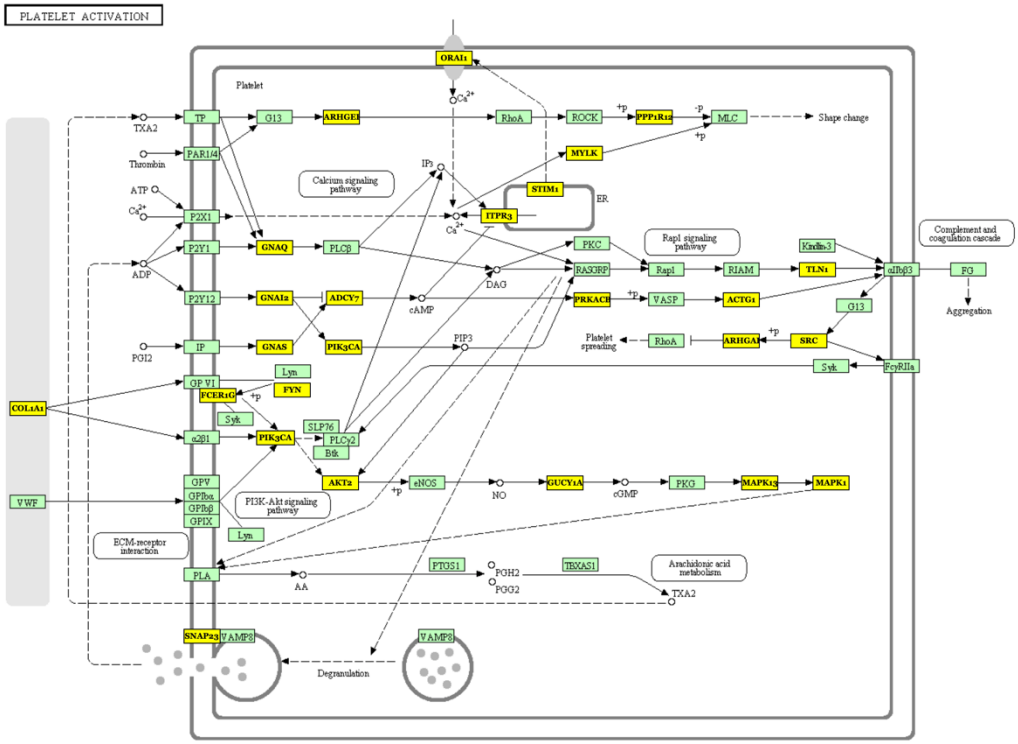
LCOR	19	UBXN7	12	LCOR	17	UBXN7	18
LDB3	12	UCN2	17	LDB3	16	UCN2	16
LDLRAD2	17	UNC5CL	14	LDLRAD2	20	UNC5CL	19
LIFR	17	USP36	19	LIFR	17	USP36	17
LMO2	17	VASH1	16	LMO2	16	VASH1	19
LNPK	18	VCL	14	LNPK	19	VCL	18
LRP10	17	VPS53	19	LRP10	20	VPS53	19
LRP11	18	WASHC2A	14	LRP11	20	WASHC2A	19
LRRC10B	12	WASL	18	LRRC10B	15	WASL	17
LRRC2	16	WDFY2	18	LRRC2	20	WDFY2	17
MAFG	16	WIPF2	16	MAFG	19	WIPF2	21
MAP3K20	15	WNT6	11	MAP3K20	21	WNT6	13
MAP3K9	17	XKR7	16	MAP3K9	17	XKR7	21
MARCHF8	10	YAF2	13	MARCHF8	19	YAF2	21
MARVELD3	12	ZBTB24	16	MARVELD3	17	ZBTB24	20
MAVS	15	ZBTB8B	16	MAVS	16	ZBTB8B	16
MEAF6	14	ZC2HC1C	15	MEAF6	19	ZC2HC1C	20
MED28	18	ZC3H12B	17	MED28	17	ZC3H12B	15
METTL21A	12	ZFAND2A	12	METTL21A	20	ZFAND2A	20
METTL8	14	ZFP37	22	METTL8	15	ZFP37	18
MLH3	16	ZFYVE26	15	MLH3	17	ZFYVE26	17
MOCS3	16	ZFYVE27	13	MOCS3	20	ZFYVE27	20
MPZL1	17	ZHX3	18	MPZL1	19	ZHX3	17
MRPL43	17	ZIC4	17	MRPL43	13	ZIC4	18
MXRA7	16	ZNF142	15	MXRA7	17	ZNF142	21
MYO6	18	ZNF207	10	MYO6	15	ZNF207	15
MYORG	13	ZNF268	11	MYORG	14	ZNF268	17
MYRF	12	ZNF285	12	MYRF	19	ZNF285	18
N4BP2L1	10	ZNF397	16	N4BP2L1	18	ZNF397	18
NBPF10	15	ZNF436	16	NBPF10	20	ZNF436	17
NBPF6	14	ZNF442	15	NBPF6	20	ZNF442	19
NCBP2	17	ZNF502	14	NCBP2	20	ZNF502	15
NCKAP1	17	ZNF512	13	NCKAP1	19	ZNF512	18
NCKAP1L	11	ZNF544	15	NCKAP1L	15	ZNF544	21
NCS1	18	ZNF576	16	NCS1	18	ZNF576	18
NDST1	13	ZNF71	15	NDST1	19	ZNF71	18
NDUFV3	13	ZNF736	20	NDUFV3	15	ZNF736	22
NEBL	14	ZNF827	18	NEBL	15	ZNF827	18
NFASC	15	ZSCAN20	15	NFASC	17	ZSCAN20	18



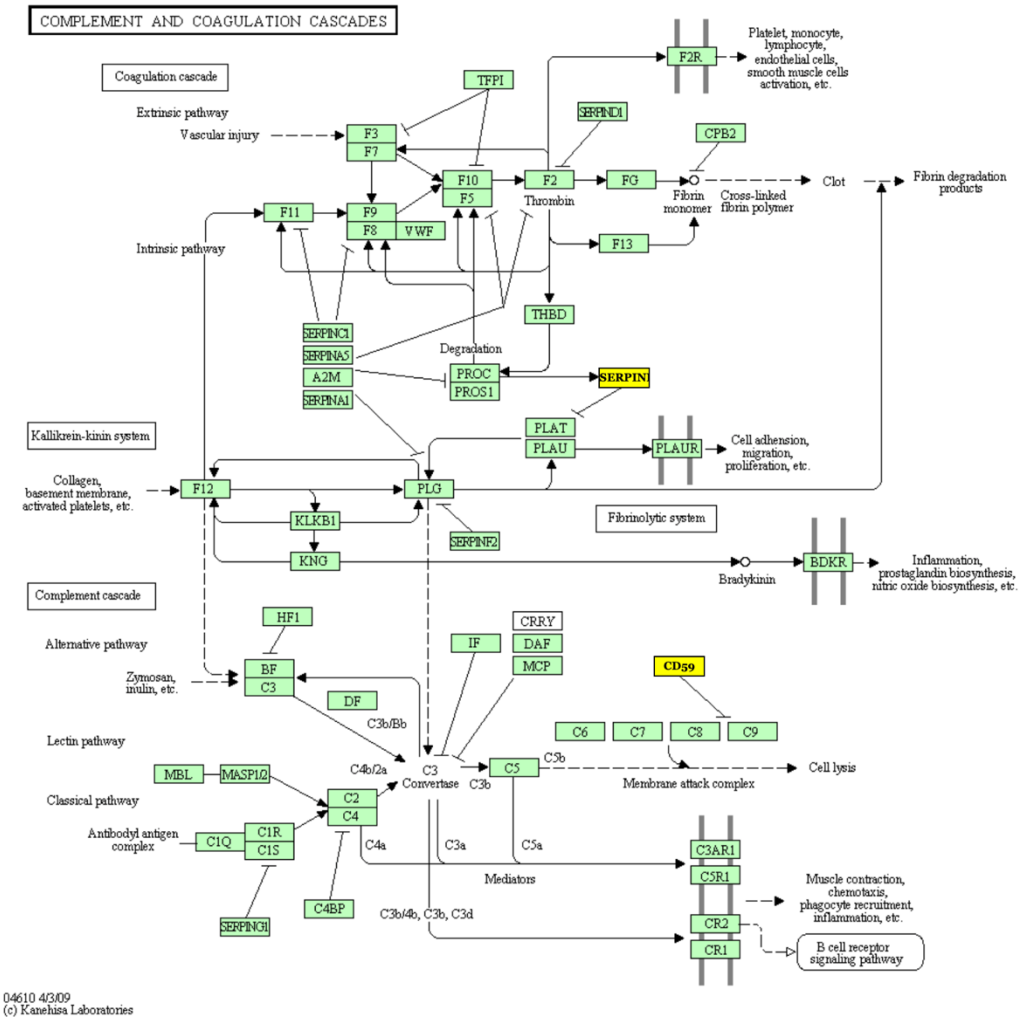
Supplementary Figure 1. Dendrogram of miRNA cluster.



Supplementary Figure 2. Dendrogram of miRNA-modulated pathway cluster.



Supplementary Figure 3. Genes targeted by miRNAs within the Platelet activation pathway (hsa04611).



Supplementary Figure 4. Genes targeted by miRNAs within the Complement and coagulation cascades pathway (hsa04610).