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Data-Mining Innovative Approaches in biomedical fields

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Abstract

This dissertation investigates innovative data mining methodologies in the biomedical field, placing special emphasis on the identification and analysis of microRNAs as potential biomarkers for various diseases, devoting specific attention to COVID-19 in the hospitalized elderly population. Using Ingenuity Pathway Analysis, the research examines in detail the complex biological processes and molecular mechanisms influenced by microRNAs, exploring their regulation and potential roles in disease development and response to treatments. The present study not only enriches our understanding of the functions and control of miRNAs in the biomedical context, but also highlights how state-of-the-art bioinformatics tools can facilitate in silico research, opening new horizons for biomarker identification and elucidation of complex biological phenomena. The thesis highlights the importance of combining sophisticated data mining techniques with molecular biology to increase the predictive accuracy of potential biomarkers.

1. Introduction

Biomarker discovery in biomedical research represents a rapidly evolving field that aims to identify molecular signatures for the diagnosis, prognosis, and therapeutic efficacy of diseases [Mayeux, 2004]. Circulating microRNAs (miRNAs), small molecules of noncoding RNA, have emerged as reliable biomarkers for a wide range of clinical conditions, including cardiovascular disease, cancer, dementia, and infectious diseases such as COVID-19 [Mayeux, 2004]. Identification and analysis of miRNA signatures offer crucial insights into disease pathogenesis, particularly in COVID-19, where deregulation of miRNAs is associated with severity and mortality, underscoring their potential as new tools for predicting disease outcomes and tailoring therapeutic strategies [Fulzele et al., 2020].

Population aging is a global phenomenon that reflects the success of public health and socioeconomic development policies in some geographic areas. However, society must adapt to this new scenario to maximize the functional capacity and health of the elderly and promote their social inclusion. In addition, the aging population poses new public health problems, such as a high prevalence of chronic noncommunicable diseases and associated comorbidities [Beard et al., 2016]. The COVID-19 pandemic caused by SARS-CoV-2 virus infection has resulted in high mortality in at-risk populations, such as the elderly, and their social isolation exacerbates the situation. The vulnerability of the elderly population is related to the physiological effects of aging, which affect immune function, promoting morbidity and mortality from infectious diseases [Onder et al., 2020].

Recent advances in next-generation sequencing (NGS) and microarray technologies have greatly enhanced our ability to analyse miRNAs at the genome-wide level. These technologies enable the identification of deregulated miRNAs by comparing samples from diseased and healthy individuals, facilitating biomarker discovery. However, interpreting the large volume of data generated and understanding the functional implications of these miRNAs requires sophisticated analytical approaches [Wang et al., 2009]. The use of databases such as Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) helps determine whether specific miRNAs are significantly enriched in pathways or biological processes associated with observed phenotypes [Kanehisa et al., 2017]. The Ingenuity Pathway Analysis (IPA) tool complements these efforts by providing an integrated platform to explore regulatory networks and pathways involving miRNAs, facilitating a deeper understanding of the roles miRNAs play in biological processes and disease pathways, improving interpretation of complex data, and identifying potential therapeutic targets. This approach exemplifies the significant advances made in biomarker discovery, highlighting the essential role of advanced bioinformatics tools in the modern biomedical research landscape [Krämer et al., 2014].

1.1 Significance of microRNAs as biomarkers

MicroRNAs (miRNAs), about 22 nucleotides long, constitute a highly conserved class of non-coding RNAs through evolutionary history. These miRNAs originate as primary miRNAs (pri-miRNAs), long strands of ribonucleic acid that undergo the process of capping and adenylation by polymerase II within the nucleus. These pri-miRNAs, of about 70 nucleotides, are subsequently processed by Drosha proteins and DiGeorge syndrome critical region 8 (DGCR8). Transferred into the cytoplasm via the 5-RAN exportin complex, these molecules are further finished by endonucleases into mature miRNAs. The function of miRNAs is to bind to the 3' untranslated regions of messenger RNAs (mRNAs), inhibiting their translation or promoting protein catabolism. In addition, miRNAs can be encapsulated in vesicles known as exosomes in the extracellular space or associated with lipoproteins and argonaut proteins for circulation [Backes et al., 2017a].

Long non-coding RNAs (lncRNAs), which regulate the regulatory framework governing gene expression in the transcriptional, post-transcriptional and translational phases, do not code for proteins and exceed 200 nucleotides in length [Huang et al., 2008]. Deviations in lncRNA expression patterns have been linked to a range of human diseases, underscoring the need for a better understanding of disease pathogenesis to advance diagnostic, prognostic, and therapeutic methodologies. lncRNAs are classified into various types, including sense, antisense, bidirectional, intronic, and intergenic, depending on their transcriptional origins with respect to known protein-coding genes. They play a crucial role in the regulation of gene expression and influence fundamental cellular functions and metabolic pathways, particularly through their involvement in chromatin structure modification, epigenetic variation, and subcellular organization [Riffo-Campos et al., 2016].

Circular RNAs (circRNAs), composed of continuous ribonucleotide chains without polyadenylated ends and forming a closed loop structure, are generally considered non-coding maintenance RNAs, although some are capable of encoding specific proteins. Notably, 1976 small circular noncoding RNA fragments, identified as splicing byproducts, have been isolated from plant-infecting viroids. These fragments have been used in the identification of eukaryotic cells, including yeast mitochondrial RNAs and hepatotropic virus δ [Huang et al., 2008].

MicroRNAs (miRNAs) have emerged as key biomolecular entities for their crucial role in the posttranscriptional regulation of gene expression, and their stable presence in various body fluids makes them ideal candidates as non-invasive biomarkers. These small noncoding RNA molecules influence a multitude of cellular processes, from ontogeny to apoptosis, and dysfunction of their expression is often linked to disease pathogenesis [Ambros, 2004; Bartel, 2004]. Characterized by remarkable resistance to RNAase activity and robustness in extracellular

environments, miRNAs are essential for their potential as biomarkers [Mitchell et al., 2008]. Numerous studies have highlighted the utility of circulating miRNAs in blood plasma as reliable diagnostic and prognostic tools for various diseases, including cancer, cardiovascular disease, and neurological disorders [Chen et al., 2008; Creemers et al., 2012; Gupta et al., 2010].

In addition, tissue-specific miRNA expression profiles have provided insights into the tissue origin of diseases, facilitating targeted therapeutic approaches [Lu et al., 2005]. Advances in high-throughput sequencing technologies and miRNA arrays have greatly accelerated the discovery and validation of miRNA signatures as biomarkers for disease detection and monitoring [Mendell and Olson, 2012]. However, challenges such as the standardization of miRNA extraction and quantification methods, as well as the need for large-scale validation studies, remain and must be addressed to fully integrate miRNA-based biomarkers into clinical practice [Moldovan et al., 2014]. Despite these challenges, the potential for miRNAs to revolutionize the field of molecular diagnostics is considerable, underscoring the need for continued research and development in this promising field.

1.2 Regulation of NF-κB:

The transcription factor NF-κB, consisting of the p50/p65 heterodimer, was identified nearly four decades ago as a rapidly induced κ light chain enhancer for activated B lymphocytes, playing a role in the humoral immune response [Sen and Baltimore, 1986; Kaltschmidt et al., 1993; Taganov et al., 2006]. Since then, its recognition sites have been found in numerous gene promoters, and NF-κB has been recognized as an influential promoter of a wide range of gene expression patterns in various cell types, critical in the management of innate and adaptive immune responses. This includes roles in the elimination of waste molecules from the cytoplasm, inflammatory signalling, cell differentiation, growth, oncogenesis, and neuronal degeneration [Taganov et al., 2006; Zhang et al., 2017b; Baltimore, 2019]. Inactive NF-κB exists in a complex with the inhibitory protein IκB in resting cells. However, it can be rapidly activated in response to pro-inflammatory agents such as IL-1β, IL-6, TNFα, and lipopolysaccharide (LPS)-the latter a potent inflammatory stimulant derived from Gram-negative bacteria-along with Aβ peptides, viral components, irradiation, and ROS-inducing substances. These factors lead to degradation of IκB via specific phosphorylation of the IKK complex, ubiquitin proteasome-mediated degradation, and subsequent initiation of NF-κB-driven transcription targeted to pro-inflammatory gene promoters.

It has been noted that a persistent supply of LPS from Gram-negative bacteria into the microbiome could chronically increase NF-κB levels in the central nervous system (CNS), as observed in cultured human neurons and in AD-affected temporal lobe neocortex regions, a primary site of AD pathology [Lukiw and Bazan, 1998;

Zhang et al., 2017a; Zhan et al., 2018; Zhan et al., 2021; Zhao and Lukiw, 2018; Alexandrov et al., 2019; Zhao et al., 2019; Singh, 2022].

Given the central importance of NF-κB pathways in various immunity- and inflammation-related diseases, ranging from cancer to neurodegeneration, significant efforts have been made to design and test both natural and synthetic NF-κB inhibitors. These efforts aim to mitigate NF-κB activation in cells without causing off-target effects. Several therapeutic strategies are under development, such as blocking initial stimulatory signals, targeting phosphorylation pathways that facilitate NF-κB activation, modulating the IκB complex, and preventing DNA binding. These strategies are the subject of intense research and include antioxidants that neutralize ROS, alterations in phosphorylation that affect the transcriptional specificity of NF-κB, genomic editing to limit the abundance of specific NF-κB subunits, and masking of DNA binding sites [Kaur et al., 2015; Barnabei et al., 2021; Jover-Mengual et al., 2021; Pogue and Lukiw, 2021; Christian et al., 2016; Wang et al., 2022; Dai et al., 2020; Katti et al., 2022; Yoon et al., 2022]. In addition, approaches to modulate overregulated miRNAs, targeted delivery systems to minimize off-target effects, and the long-standing use of dietary NF-κB inhibitors to reduce inflammatory signaling have been highlighted [Gilmore and Herscovitch, 2006; Li et al., 2020b; Li et al., 2020c; Olajide and Sarker, 2020; Al-Khayri et al., 2022; Das and Rao, 2022]. About 80 pharmaceutical companies are actively seeking more specific NF-κB inhibitors for clinical use in diseases characterized by excessive NF-κB activity (Future Market Insights, GlobeNewswire, 360 Research Reports)

1.3 Strengthening miRNA Research through Comprehensive Databases and Analytical Tools.

A wide range of resources have enriched our understanding of miRNAs, particularly miRBase [Kozomara et al., 2018], which boasts a large collection of miRNA sequences and functionalities for more than 271 organisms.

miRBase

miRBase is the leading public repository and online resource for microRNA sequencing and annotation (http://mirbase.org/). Established in 2002 (initially named microRNA Registry), miRBase is in charge of microRNA gene nomenclature and has been assigning gene names for new microRNA discoveries ever since. The microRNA gene naming system has been discussed in a number of miRBase publications [Kozomara and Griffiths-Jones, 2014; Kozomara and Griffiths-Jones, 2011; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008; Griffiths-Jones, 2004] and on the miRBase blog (http://mirbase.org/blog/). The miRBase website provides a wide range of information on published microRNAs, including their sequences, biogenesis precursors, genomic coordinates and context, literature references, deep sequencing expression data, and community-driven annotation. miRBase also

serves as a portal for third-party information on microRNA genes and sequences, linking to other resources that include predicted and experimentally validated targets of microRNAs.

The latest version of the miRBase database (v22) contains 38,589 entries representing hairpin-shaped precursor microRNAs from 271 organisms. This represents an increase in sequences by more than one-third from the previous version. These hairpin precursors produce a total of 48,860 different mature microRNA sequences. Vertebrate genomes contain thousands of microRNAs: for example, the human genome contains 1917 annotated hairpin precursors and 2654 mature sequences. The well-annotated genomes of both invertebrates and plants contain hundreds of microRNAs (e.g., Drosophila melanogaster: 258 hairpins, 469 mature sequences; Caenorhabditis elegans: 253 hairpins, 437 mature sequences; Arabidopsis thaliana: 326 hairpins, 428 mature sequences).

This repository, together with miRCarta [Backes et al., 2017a] and mirGeneDB [Fromm et al., 2019], forms the basis of miRNA research, providing insights into both predicted and experimentally validated miRNAs.

miRCarta

miRCarta complements the miRBase database, adding new miRNA candidates, expression data, updated organisms and genomes, serving as an entry point for miRNA researchers. It offers basic functionality such as navigation, which lists all miRNAs and precursors for a selected organism, showing normalized counts of mapped reads from NGS data, with or without mismatches. This allows users to assess whether the expression profile on a precursor (putative or known) seems likely for miRNA expression and to identify true precursors/miRNAs more quickly than false-positive annotations.

The advanced search allows users to narrow query results to specific miRNAs or precursors, validated by certain experiments. Precursor families, supplemented by miRBase content, and precursor genomic clusters are other features offered. Read mapping distribution is visualized for human precursors, showing pileup plots in precursor-specific views and tabular overviews for H. sapiens.

Structural analysis, performed with RNAfold [Lorenz et al., 2011] and visualized with FornaContainer [Kerpedjiev et al., 2015], is available in the precursor-specific views. For annotation, miRCarta provides a combined miRNA and target search using experimentally validated or predicted targets, respectively. If all three databases are selected, the resulting table will contain a column for each database with an input of 0 or 1, which can be used for filtering the results. For potential target pathways, links to the MiRTargetLink

[Hamberg et al., 2016] and miRPathDB [Backes et al., 2017b] tools for miRNAs are included. Links to tissue distribution via the TissueAtlas tool [Ludwig et al., 2016] and disease association via links to miRNAs in miR2Disease [Jiang et al., 2009] and precursors in HMDD [Li et al., 2014] are also provided.

mirGeneDB

MirGeneDB is a manually curated database dedicated to microRNA genes, based on consistent annotation and nomenclature criteria [Fromm et al., 2015]. Originally limited to a few species, MirGeneDB was expanded in its version 2.0 to include 10,899 authentic and consistently named microRNAs, representing 1,275 microRNA families from 45 species, covering every major metazoan group. This expansion benefited from the analysis of more than 400 publicly available small RNA sequencing datasets processed using sRNAbench [Aparicio-Puerta et al., 2019] and miRTrace [Kang et al., 2018], allowing uniform and consistent annotation of miRNAomes for each species using MirMiner [Wheeler et al., 2009].

MirGeneDB 2.0 improved annotations for species such as human, mouse, chicken, and zebrafish, and refined human and zebrafish annotations using available CAGE data [de Rie et al., 2017]. It also made use of data on the absence of Dicer, Drosha, and Exportin 5 to further refine the human annotations. The database places special emphasis on accurate annotation of the 5p and 3p arms of microRNAs, allowing better identification of miRNA isoforms and including features such as Group 2 miRNAs, marked by mono-uridylation at their 3' ends, and processing variants where alternative Drosha/Dicer cuts significantly affect the mature sequence [Manzano et al., 2015].

MirGeneDB stands out for its commitment to annotation quality, aiming to be free of false positivity. Unlike other databases that may allow community annotation, MirGeneDB uses a well-defined set of criteria for microRNA inclusion, ensuring that the nearly complete repertoire of microRNAs from each taxon is added to the database [Fromm et al., 2015; Ambros et al., 2003; Tarver et al., 2013]. This approach allows for consistent and complete annotation, facilitating comparative studies and supporting the utility of microRNAs as reliable phylogenetic markers [Reinhart et al., 2000; Tarver et al., 2018; Kang et al., 2018; Tarver et al., 2013].

In conclusion, MirGeneDB 2.0 offers microRNA researchers a valuable resource for high-quality annotations, a comprehensive dataset, and tools for comparative analyses, supporting evolutionary and functional studies in the field of microRNAs.

Integration of data from diverse sources, such as miRMaster [Fehlmann et al., 2017], TCGA and NCBI SRA, has improved the accuracy of miRNA detection, as illustrated by Alles et al. [Alles et al., 2019], with their validation of 2300 true human mature miRNAs.

miRMaster

MiRMaster is a web-based tool developed to analyze NGS data of small RNAs, including the discovery of novel miRNAs, isomiRs, mutations, and exogenous RNAs. This tool provides a wide range of modules that enable quantification of miRNAs and other non-coding RNAs, identification of new miRNAs, discovery of variants, and analysis of specific motifs. With its ability to process large datasets quickly and accurately, miRMaster proves to be a valuable tool for biomedical research, especially for large-scale studies that aim to better understand miRNA-mediated gene regulation [Fehlmann et al., 2017].

The software is based on the principles of miRDeep2, a renowned prediction tool for miRNAs, but extends its functionality by including a broader feature set to improve prediction accuracy. MiRMaster is designed to facilitate the analysis of high-resolution sequencing data from raw files in FASTQ format, and covers a wide range of analyses, from quantifying miRNA expression to mapping nonhuman RNAs. It also implements application programming interfaces (APIs) for web-based tools, enabling advanced miRNA targetome analyses and miRNA enrichment sets [Fehlmann et al., 2017].

TGCA

The Cancer Genome Atlas (TCGA) project represents a significant initiative in the field of cancer genomics, having molecularly characterized more than 20,000 samples of primary cancer and corresponding normal tissues, covering 33 types of cancer. Jointly launched by the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI), part of the National Institutes of Health (NIH), TCGA aimed to improve understanding of the molecular basis of cancer through advanced genome analysis technologies, including large-scale genome sequencing.

TCGA has greatly advanced the field of cancer genomics, providing a valuable resource for researchers to understand the genetic mechanisms underlying cancer, which can lead to new methods of diagnosis, treatment and prevention. The project used advanced genomic analysis techniques to explore the complex interplay of genetic mutations in cancer, offering insights into tumor classification, molecular aberrations and potential therapeutic targets.

TCGA-generated data have been pivotal in identifying genomic alterations in various cancers, elucidating potential biomarkers for cancer diagnosis and prognosis, and shedding light on potential therapeutic targets. The widespread availability of TCGA data has enabled a wide range of secondary analyses, leading to thousands of studies that extend beyond the original scope of the project, thus amplifying its impact on cancer research [NCI, 2022]

NCBI SRA

The Sequence Read Archive (SRA) is a database maintained by the National Center for Biotechnology Information (NCBI) that archives sequence data obtained from next-generation sequencing technology. Launched in 2009, the SRA includes 9 million records and 12 petabytes of data, representing a vast collection of DNA and RNA sequences that reflect genomic diversity across the tree of life. This database allows researchers to search metadata for these sequences to locate sequence beds for further analysis.

The SRA is part of the International Nucleotide Sequence Database Collaboration (INSDC), which also includes the European Bioinformatics Institute (EBI) and the DNA Database of Japan (DDBJ). Data submitted to any of these organizations are shared among them, making the SRA a publicly available resource of high-throughput sequencing data. It accepts data from all branches of life, as well as metagenomic and environmental surveys, and stores raw sequencing data and alignment information to improve reproducibility and facilitate new discoveries through data analysis.[NIH, 2024]

Specific databases such as HMDD [Huang et al., 2018] and miRCancer [Xie et al., 2013] emphasize the importance of miRNAs in human diseases, emphasizing the importance of accurately identifying authentic miRNA signals among potential false positives within these vast datasets.

HMDD v3.0

HMDD v3.0 is an advanced version of the Human microRNA Disease Database, which has collected 32,281 experimentally supported miRNA-disease association entries, covering 1,102 miRNA genes and 850 diseases from 17,412 articles.

HMDD v3.0 is to offer a comprehensive and accessible web resource where users can search, browse, download, and analyze experimentally supported miRNA-disease associations. Data were collected by analyzing abstracts of microRNA-related publications on PubMed, standardizing miRNA names according to miRBase, and disease names according to Disease Ontology and MeSH terms.

The miRNA-disease associations are grouped into eight types according to the disease hierarchy provided by Disease Ontology. HMDD v3.0 provides detailed evidence codes for each entry, allowing users to assess the level of reliability of miRNA-disease associations. This version of the database is distinguished by accurate standardization of disease names, detailed evidence code classification, and significant accumulation of data, thus greatly enhancing the quality and usefulness of HMDD as a resource for research on miRNA-disease associations [Li et al., 2014; Barupal et al., 2015; Liberzon et al., 2015]

miRCancer

MiRCancer is a specialized database that collects microRNA (miRNA) expression profiles in various forms of human cancer, collected systematically from peer-reviewed publications indexed on PubMed. This resource uses advanced text mining techniques to collect relevant information, ensuring the accuracy of the data through successive manual reviews to ensure reliability. The database is critical for researchers, providing a searchable platform where users can make queries specific to miRNAs or cancer types. It also provides tools for sequence analysis, such as clustering and chi-square analysis, allowing users to perform in-depth analyses on miRNA sequences related to different cancers.

The importance of such a database extends to its role in cancer research, where miRNA expression profiles are crucial to understanding the complex mechanisms of cancer progression and initiation. miRNAs are known to function as oncogenes or tumor suppressors, influencing gene expression involved in cancer pathways. Centralizing miRNA data in miRCancer helps in the identification of potential biomarkers for cancer diagnosis, prognosis, and therapy, enhancing the understanding of miRNA involvement in oncogenic processes [Rajan et al., 2021; Peng and Croce, 2016]

Crucial to the evaluation of gene lists, enrichment analyses often depend on indirect annotations via miRNA target genes to infer involvement in biological pathways. However, databases are emerging that allow direct annotations of miRNAs, facilitating direct association of miRNAs with curated functions and avoiding the limitations of indirect annotations. This approach is demonstrated by Huntley et al. [Huntley et al., 2018; Huntley et al., 2016], who used Gene Ontology for direct annotation of miRNA function.

Complementing these methodologies, Ingenuity Pathway Analysis (IPA) offers a more integrated and sophisticated framework for understanding the involvement of miRNAs in biological processes and disease pathways [Shaath et al., 2021].

Ingenuity Pathway Analysis (IPA)

IPA is an advanced analytical tool that supports researchers in interpreting large datasets from genetic, transcriptional, and proteomic studies. IPA allows users to visualize, integrate and understand biological data within a context of biological pathways and networks. The application uses a large database of molecular interactions, gene functions, and validated biological properties, enabling users to explore the connections between proteins, genes, metabolites, drugs, and diseases [Qiagen, 2024].

Key functionalities of IPA include:

Pathway analysis: IPA can identify the most significant biological pathways associated with experimental data, offering insights into the cellular functions involved and suggesting potentially altered biological mechanisms.

Network analysis: Users can construct interactive networks of protein interactions, showing how genes and proteins interact with each other within cells. This helps identify new therapeutic targets or biomarkers for specific diseases.

Prediction of transcription factor activities: IPA can predict activation or inhibition of transcription factors based on gene expression analysis, offering information on the possible functional consequences of changes in gene expression.

Molecular Connections: IPA can link molecular data to disease processes, pathways and functions, providing a deeper understanding of diseases and potential therapies.

Comparative analysis: Allows comparison of trends and patterns across different datasets, experiments or conditions, facilitating the discovery of common or unique patterns.

Upstream and downstream analysis: Identifies molecules that are potentially responsible for the effects observed in experimental data, allowing users to examine the causes and effects of gene and protein variations.

Visualization tools: Offers a wide range of data visualization options, including graphs, pathway maps and network representations, which help interpret complex biological interactions in an intuitive way.

Extensive and up-to-date database: IPA is supported by one of the largest databases of biological knowledge, which is constantly updated with the latest scientific publications, ensuring that users have access to the latest and most relevant information.

The value of IPA lies in its ability to transform large amounts of biological data into testable understandings and hypotheses, thereby accelerating biomedical and pharmaceutical research. The extensive use of IPA in various fields of biology and medicine is documented in a wide range of scientific publications, attesting to its significant impact in advancing research and therapeutic development.

Using IPA, researchers can explore the complex regulatory networks and pathways associated with miRNAs, improving the interpretation of complex data and identifying potential therapeutic targets. The integration of IPA into miRNA research represents a significant advance, enabling a more in-depth exploration of the roles of miRNAs as biomarkers and their disease-related implications.

1.4 Analysis of NF-kB-responsive microRNAs in inflammatory processes: Implications in age-related diseases

In the context of this thesis, has been applied advanced data mining techniques to investigate the role of the transcription factor NF-kB, which is known for its predominant involvement in inflammatory processes by modulating the expression of pro-inflammatory genes. A particularly interesting aspect is the ability of NF-kB to promote transcriptional activation of post-transcriptional modulators of gene expression, such as noncoding RNAs (miRNAs). Although the role of NF-kB in inflammation-associated gene expression has been extensively studied, the interaction between NF-kB and genes encoding for miRNAs deserves further investigation.

The scientific project covered in this thesis employed a data mining approach to identify miRNAs with potential binding sites for NF-kB at their transcriptional start sites, using in silico analysis using PROmiRNA software. This allowed us to assess the propensity of genomic regions to be cis-regulatory elements of miRNAs, generating a list of 722 human miRNAs, of which 399 were expressed in at least one tissue involved in inflammatory processes. Our selection of "high reliability" hairpins in miRBase identified 68 mature miRNAs, many of them previously identified as inflammamiRs. Identification of target pathways and diseases revealed their involvement in the most common age-related diseases. Taken together, our results strengthen the hypothesis that persistent NF-kB activation may unbalance the transcription of specific inflammamiRNAs, the identification of which could have diagnostic, prognostic, and therapeutic relevance for the most common inflammation- and age-related diseases.

The results of this project have been detailed and discussed in the published scientific article, titled "A Data-Mining Approach to Identify NF-kB-Responsive microRNAs in Tissues Involved in Inflammatory Processes: Potential Relevance in Age-Related Diseases,"[Micolucci et al., 2023] which makes a significant contribution to the

existing literature, opening up new research perspectives in the molecular dynamics that interconnect inflammation, miRNAs and age-related diseases.

2. Aim of the study

The nuclear factor (NF)-kB is a transcription factor (TF) activated by an evolutionarily conserved inflammatory signalling, induced by a wide range of external and internal danger signals [Salminen et al., 2008b; Salminen et al., 2008a; Liu et al., 2022]. The complex modulation of this signalling can be envisaged considering the different activation strategies, well known as “canonical” and “non-canonical” NF-kB activation signalling (reviewed in [Dorrington and Fraser, 2019; Olivieri et al., 2021]). A fine-tuning activation of NF-kB promotes the expression of pro-inflammatory genes and participates in the regulation of survival, activation, and differentiation of innate immune cells and T cells [Liu et al., 2017]. On the contrary, a persistent activation of NF-kB signalling was described in conditions of cellular senescence and organismal aging, as well as in patients affected by the most common age-related degenerative diseases (ARDs) [Adler et al., 2007; Tilstra et al., 2011; Bernal-Lopez et al., 2013]. Many efforts have been made to understand which pathways are regulated by NF-kB and how the NF-kB pathway itself is modulated [Bektas et al., 2014; Rothschild et al., 2018]. It has become clear that not only TFs but also a series of epigenetic factors, including non-coding microRNAs (miRNAs), are involved in the regulation of almost all the human transcriptional programs, both as inhibitors of mRNAs translation and as enhancers of mRNAs transcription [Arora et al., 2013; Iwama, 2013; Elizarova et al., 2021]. Increasing evidence confirmed that these epigenetic factors play key roles in the development and progression of the most common human ARDs [Li et al., 2020d; Peng, 2021].

Regarding the canonical pathway of miRNA processing, that regulates gene expression at the post-transcriptional level, a primary transcript called pri-miRNA is cleaved to a precursor miRNA hairpin structure (pre-miRNA) in the nucleus by the Drosha/Pasha complex and transported into the cytoplasm, where the pre-miRNA is further processed into a miRNA:miRNA* duplex [Akhtar et al., 2015]. After being separated, the mature miRNA is loaded into the Argonaute 2 (Ago 2) containing RNA-induced silencing complexes (RISCs) and drives it to regulate its target mRNAs [Akhtar et al., 2015].

On one hand, a few miRNAs targeting mRNAs belonging to NF-kB pathway have already been identified, highlighting the activation of feedback loops aimed to restrain the inflammatory process triggered by NF-kB. Notably, some miRNAs involved in these feedback circuits were identified as deregulated in ARDs [Olivieri et al., 2021; Lukiw, 2012; Wang et al., 2012; Yang et al., 2015; Markopoulos et al., 2018].

On the other hand, the full elucidation of miRNA biogenesis would be of paramount importance to identify their regulators and the role they might play in complex regulatory networks. Even if computational models were extensively applied to disentangle the complex effects of non-coding RNA in human diseases [Chen et al., 2020], for a long time, the difficulty of experimentally detecting miRNA promoters has limited the ability to identify the NF-kB binding sites in DNA sequences coding for miRNAs. However, the annotation of miRNA promoters, using high-throughput genomic data, allowed us to partially overcome this difficulty [Marsico et al., 2013]. As important transcriptional regulators, miRNAs can upregulate or downregulate many target genes involved in the NF-kB signalling pathway via negative or positive feedback loops, and are involved in several human diseases, too, including the recentCOVID-19pandemic [Wu et al., 2018; Amini-Farsani et al., 2021]. Since

it is conceivable that age-related NF-kB activation could induce an overexpression of NF-kB responsive miRNAs, the identification of such miRNAs, and their targeted mRNAs and pathways, could contribute to clarifying the complex mechanisms that modulate healthy or unhealthy aging trajectories.
In this work, we aimed to: (i) identify all human miRNAs potentially modulated by NF-kB, (ii) select and characterize those NF-kB-responsive miRNAs that are specifically expressed in healthy tissues involved in the modulation of the inflammatory processes (such as cells of the immune system, liver, blood, and bone marrow), (iii) discover their targeted mRNAs and relative pathways, and finally (iv) evaluate the involvement of such pathways in the development of human diseases, including ARDs and severity of COVID-19 pneumonia (case study).

3. Results

3.1. Putative NF-kB Responsive miRNAs

To select NF-kB responsive miRNAs, we analysed the PROmiRNA database [Marsico et al., 2013] , FAN-TOM4 Libraries [Kawaji et al., 2011], “High confidence hairpins” in miRbase [Kozomara and Griffiths-Jones, 2014], and “Human expression dataset” [Betel et al., 2008], following the data-mining process highlighted in the data flow diagram in Figure 1.

We analysed primarily genome-wide PROmiRNA predictions, as well as TF-binding site predictions as reported in [Marsico et al., 2013], to identify miRNAs with potential NF-kB binding sites in their promoter sequences. PROmiRNA is a miRNA promoter recognition method, based on a semi-supervised statistical model trained on multi-tissue deepCAGE FANTOM4 libraries and other sequence features. It is tailored to score the potential of CAGE-enriched genomic regions to be promoters of either intergenic or intragenic miRNAs, thereby modulating miRNA expression in a tissue-specific manner [Marsico et al., 2013]. To identify the TFs that regulate specific miRNAs, for each predicted miRNA transcription start site (TSS), we retrieved the 1 kb cantered on it and used the TRAP approach [Thomas-Chollier et al., 2011] to compute the affinity of TF binding sites for all predicted miRNA promoters using TF models stored in the JASPAR database [Portales-Casamar et al., 2009].

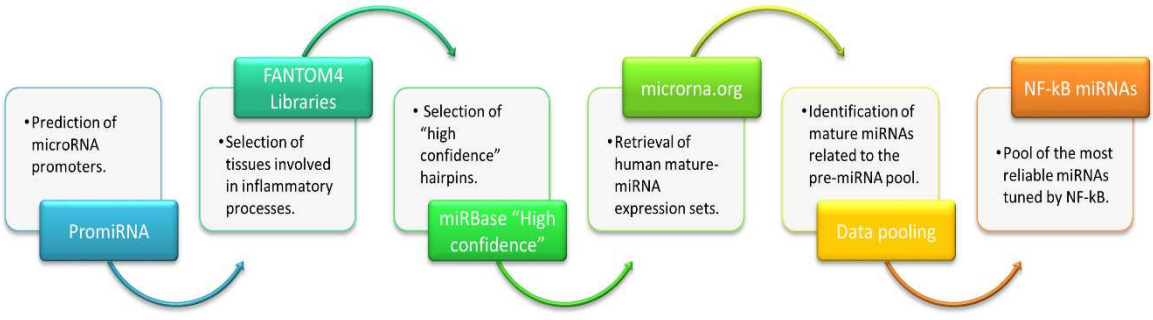

Figure 1. Data flow diagram. Figure depicts the whole data-mining process.

NF-kB appears among the first 10 TFs with the highest affinity for the 1000 bp-long region surrounding the predicted TSSs for 722 miRNA hairpin precursors (Table S1 in Supplementary Materials).

Since tissues show specific miRNA expression patterns, we aimed to highlight the list of putative NF-kB-responsive miRNAs expressed in tissues strictly involved in the modulation of the inflammatory processes, including inflammaging. To achieve this goal, we focused our subsequent research on those miRNAs transcribed in human tissues such as “T cells”, “T cells 2”, “monocytic-cells”, “immune system cells”, “bone marrow”, “blood”, and “liver”. Only the libraries relative to healthy tissues have been taken into consideration. This approach retrieved 399 miRNA hairpin precursors showing “expression at the promoter level” in at least one of these tissues (Table S2 in Supplementary Materials). In general, this is a good indication that the mature forms of these miRNAs are expressed in a specific tissue. However, each step from DNA–RNA transcription to mature miRNA expression can be modulated, thereby modifying or blocking the final expression. Moreover, FANTOM4 libraries are characterized by a certain level of “transcriptional noise”, so we should expect false positives in mature miRNA predictions [Marsico et al., 2013]. Therefore, among these putative NF-kB responsive miRNAs, we selected the “high confidence” hairpins in miRbase [Kozomara and Griffiths-Jones, 2014], retrieving 73 pre-miRNAs (Table S3 in Supplementary Materials). A growing body of evidence suggests that mature sequences derived from both arms of the hairpin might be biologically functional and even that the dominant mature sequence can be processed from opposite arms [Griffiths-Jones et al., 2011]. Following the approach of selecting only the “high confidence” miRNA hairpins and filtering the dataset for “Human Expression dataset” [Betel et al., 2008], 68 “high confidence” expressed miRNAs were identified. This pool of miRNAs, reported in Table 1, constitutes our final set of putative NF-kB responsive miRNAs expressed in healthy tissues linked to inflammatory processes.

3.2. Genomic Features of Putative NF-kB Responsive miRNAs

According to their genomic location, it is possible to distinguish two classes of miRNAs: “intergenic miRNAs” are those located in intergenic regions of the genome, whereas “intra-genic miRNAs” are those embedded in introns or exons of annotated genes [Marsico et al., 2013]. Among the latter, “intronic miRNAs” are those located inside the introns of other genes and can either be co-transcribed with their host gene [Schanen and Li, 2011] or have an independent promoter [Davis and Hata, 2009; Ozsolak et al., 2008; Monteys et al., 2010], whereas intergenic miRNAs can derive from a primary miRNA transcript (pri-miRNAs) located in independent gene units [Marsico et al., 2013; Krol et al., 2010]. Parallelly, it is possible to distinguish different categories of miRNA promoters: “intergenic promoters” are promoters assigned to inter-genic miRNAs; “intragenic promoters” are promoters assigned to intragenic miRNAs and include both “host gene promoters” and “intronic promoters”; finally, “hybrid promoters” are those promoters that fall into intergenic regions upstream of intragenic miRNAs and could not be assigned unambiguously to the miRNA [Marsico et al., 2013].

Table 1. *The sixty-eight putative NF-kB responsive miRNAs expressed in healthy human tissues linked to inflammatory processes. The following attributes are reported: name of the mature miRNAs which derives from the pre-miRNAs previously identified, type of predicted TSS ("intergenic", "host gene", "intronic", or "hybrid"), names of the healthy "Human Expression dataset" libraries in which the miRNAs are expressed (i.e., "liver" and "immune system"), the chromosome where the miRNA precursor is located, the age of the miRNAs corresponding to the predicted TSSs*

Putative NF-kB Responsive miRNAs *	Mature miRNA Expression ** in Tissues Linked to Inflammatory Processes	Prediction of Promoter Location According to PROmiRNA	miRNA Age	Chromosomes
hsa-miR-101	Immune system	intergenic	v	1
hsa-miR-103	Liver and Immune system	host gene-intronic	v	20
hsa-miR-106b	Immune system	host gene	m	7
hsa-miR-10a	Immune system	host gene	v	17
hsa-miR-132	Immune system	intergenic	m	17
hsa-miR-136	Immune system	intergenic	m	14
hsa-miR-140-3p	Liver and Immune system	host gene	v	16
hsa-miR-140-5p	Liver and Immune system	host gene	v	16
hsa-miR-144	Liver and Immune system	intergenic	v	17
hsa-miR-146a	Immune system	intronic	m	5
hsa-miR-148b	Immune system	host gene	m	12
hsa-miR-150	Immune system	intergenic	m	19
hsa-miR-154	Liver	intergenic	m	14
hsa-miR-155	Immune system	intronic	v	21
hsa-miR-15a	Liver and Immune system	host gene-intronic	v	13
hsa-miR-15b	Liver and Immune system	host gene	v	3
hsa-miR-16	Liver and Immune system	host gene-intronic	v	13|3
hsa-miR-17	Liver and Immune system	intronic	v	13
hsa-miR-181b	Immune system	host gene	v	9
hsa-miR-183	Immune system	intergenic	m	7
hsa-miR-186	Liver and Immune system	host gene-intronic	m	1
hsa-miR-188-3p	Immune system	intronic	m	X
hsa-miR-188-5p	Immune system	intronic	m	X
hsa-miR-18a	Immune system	intronic	v	13
hsa-miR-192	Liver and Immune system	intergenic	m	11
hsa-miR-194	Liver and Immune system	host gene-intergenic	m|v	11
hsa-miR-195	Liver	intronic	m	17
hsa-miR-19a	Immune system	intronic	v	13
hsa-miR-19b	Immune system	intronic	m|v	13
hsa-miR-20a	Immune system	intronic	v	13
hsa-miR-221	Immune system	intergenic	v	X
hsa-miR-222	Immune system	intergenic	v	X
hsa-miR-23a	Liver and Immune system	intergenic	m	19

Table 1. *Cont.*

Putative NF-kB Responsive miRNAs *	Mature miRNA Expression ** in Tissues Linked to Inflammatory Processes	Prediction of Promoter Location According to PROmiRNA	miRNA Age	Chromosome
hsa-miR-25	Liver and Immune system	host gene	m	7
hsa-miR-27a	Liver and Immune system	intergenic	m	19
hsa-miR-296-5p	Immune system	intergenic	m	20
hsa-miR-30c	Liver and Immune system	host gene	v	1
hsa-miR-30e	Liver and Immune system	host gene	v	1
hsa-miR-31	Immune system	intronic	v	9
hsa-miR-32	Immune system	intronic	v	9
hsa-miR-339-3p	Immune system	intronic	m	7
hsa-miR-339-5p	Immune system	intronic	m	7
hsa-miR-33a	Immune system	intronic	m	22
hsa-miR-33b	Immune system	host gene-intronic	m	17
hsa-miR-362-5p	Immune system	intronic	m	X
hsa-miR-374a	Immune system	host gene	m	X
hsa-miR-374b	Immune system	intergenic	m	X
hsa-miR-377	Liver	intergenic	m	14
hsa-miR-424	Liver and Immune system	intergenic	m	X
hsa-miR-450a	Immune system	intergenic	m	X
hsa-miR-491-5p	Immune system	intronic	m	9
hsa-miR-500	Liver	intronic	m	X
hsa-miR-503	Immune system	intergenic	m	X
hsa-miR-532-3p	Immune system	intronic	m	X
hsa-miR-542-5p	Immune system	intergenic	m	X
hsa-miR-545	Immune system	host gene	m	X
hsa-miR-548d-5p	Immune system	intronic	p	8
hsa-miR-570	Immune system	intronic	p	3
hsa-miR-616	Immune system	host gene	p	12
hsa-miR-625	Immune system	intronic	p	14
hsa-miR-629	Immune system	intronic	p	15
hsa-miR-708	Immune system	host gene	m	11
hsa-miR-874	Liver and Immune system	intronic	m	5
hsa-miR-885-5p	Liver	intronic	p	3
hsa-miR-92a	Liver and Immune system	intronic	m	13
hsa-miR-93	Immune system	host gene	m	7
hsa-miR-98	Liver and Immune system	intronic	m	X
hsa-miR-99a	Liver and Immune system	intronic	v	21

Note: miRNAs highlighted in bold are those processed starting from two or more pre-miRNA hairpins, each one transcribed starting from two different promoter types. * In PROmiRNA, NF-kB is among the top 10 TFs with the highest affinity for the 1000 bp-long region surrounding the predicted TSSs. ** Mature miRNAs have been selected based on the "Human Expression dataset" (mirorna.org, accessed on 10 January 2023). This selection allows to review mature miRNA expression patterns across the tissues of interest.

As shown in Table1, among the promoter locations of the 68 putative NF-kB responsive miRNAs, 19 are "intergenic", 15 are "host gene", and 28 "intronic". Interestingly, miR-15a, miR-16, miR-103, miR-186, and miR-33b can be modulated by both "host gene" and "intronic" promoters, whereas miR-194 is regulated by both

“host gene” and “intergenic promoters”. Growing evidence indicates that alternative promoters are a mechanism for creating diversity in miRNA transcriptional regulation, as ascertained for protein-coding genes [Carninci et al., 2006].
Regarding the phylogenesis of the 68 putative NF-kB responsive miRNAs, we showed that 22 miRNAs are conserved up to the vertebrate lineage (v), 38 miRNAs are conserved up to the mammal lineage (m), miR-194 and miR-19b up to the mammal and vertebrate lineage, and, finally, only 6 miRNAs are conserved in the primate lineage (p).

3.3. Characterization of the Interplay Linking NF-kB, miRNAs, and Their Host Genes

To better characterize miRNAs that share the promoters of the host gene and to determine whether those host genes are also known to be regulated by NF-kB, multiple assessments were conducted. Firstly, we retrieved available information regarding the host genes and their intragenic miRNAs, as reported in Table 2, whereas expression correlation plots between miRNAs and their host gene are shown in Figure S1 (Supplementary Material).

No experimental evidence was found regarding the host gene of hsa-mir-374a, hsa-mir-545, and hsa-mir-15a. All the others are intronic miRNAs of genes involved in various biological processes ranging from DNA replication to differentiation:

- NFYC (Nuclear transcription factor Y subunit gamma) is a component of the sequence-specific heterotrimeric transcription factor (NF-Y) which specifically recognizes a 5’- CCAAT-3’ box motif found in the promoters of its target genes. NF- Y can function as both an activator and a repressor, depending on its interacting cofactors [Nakshatri et al., 1996];
- ZRANB2 (Zinc finger Ran-binding domain-containing protein 2) is a splicing factor required for alternative splicing of TRA2B/SFRS10 transcripts. May interfere with constitutive 5’-splice site selection [Adams et al., 2001];
- IARS2 (Isoleucine--tRNA ligase, mitochondrial) is a nuclear gene encoding mitochondrial isoleucyl–tRNA synthetase on which depends the translation of mitochondrial-encoded proteins [Schwartzentruber et al., 2014];
- SMC4 (Structural maintenance of chromosomes protein 4) is the central component of the condensing complex, a complex required for the conversion of interphase chromatin into mitotic-like condense chromosomes [Kimura et al., 2001];
- MCM7 (DNA replication licensing factor MCM7) acts as a component of the MCM2-7 complex (MCM complex) which is the replicative helicase essential for “once per cell cycle” DNA replication initiation and elongation in eukaryotic cells. It is the core component of CDC45-MCM-GINS (CMG) helicase, the molecular machine that unwinds template DNA during replication, and around which the replisome is built [Rzechorzek et al., 2020; Jones et al., 2021; Jenkyn-Bedford et al., 2021; Baris et al., 2022; Ishimi and Irie, 2015; Ishimi, 1997].
- NR6A1 (Nuclear receptor subfamily 6 group A member 1) is an orphan nuclear receptor that binds to a response element containing the sequence 5’-TCAAGGTCA-3’. By similarity, it may be involved in the regulation of gene expression in germ cell development during gametogenesis. It is involved in

regulating embryonic stem cell differentiation, reproduction, and neuronal differentiation [Wang et al., 2015];

- TENM4 (Teneurin-4) is involved in neural development, regulating the establishment of proper connectivity within the nervous system. It plays a role in the establishment of the anterior-posterior axis during gastrulation. Also, it regulates the differentiation and cellular process formation of oligodendrocytes and myelination of small-diameter axons in the central nervous system (CNS) [Hor et al., 2015];
- COPZ1 (Coatomer subunit zeta-1) is a cytosolic protein complex involved in intracellular trafficking, endosome maturation, lipid homeostasis, and autophagy [Beck et al., 2009; Razi et al., 2009]. It is associated with iron metabolism through the regulation of transferrin [Ward et al., 1991; Zhang et al., 2021];
- DDIT3 (DNA damage-inducible transcript 3 protein) is a multifunctional transcription factor in endoplasmic reticulum stress response. It plays an essential role in the response to a wide variety of cell stresses and induces cell cycle arrest and apoptosis [Yamaguchi and Wang, 2004; Ohoka et al., 2005; Oliveira et al., 2009];
- WWP2 (NEDD4-like E3 ubiquitin-protein ligase WWP2) plays an important role in protein ubiquitination and inhibits activation-induced T-cell death by catalyzing EGR2 ubiquitination [Chen et al., 2009]. In human embryonic stem cells, WWP2 promotes the degradation of transcription factor OCT4 which not only plays an essential role in maintaining the pluripotent and self-renewing state of embryonic stem cells but also acts as a cell fate determinant through a gene dosage effect [Yamaguchi and Wang, 2004];
- HOXB3 (Homeobox protein Hox-B3) is a sequence-specific transcription factor that is part of a developmental regulatory system that provides cells with specific positional identities on the anterior-posterior axis. Therefore, it may regulate gene expression, morphogenesis, and differentiation [Verma et al., 2022];
- SREBF1(Sterol regulatory element-binding protein 1) is a precursor of the transcription factor form (Processed sterol regulatory element-binding protein 1), which is embedded in the endoplasmic reticulum membrane [Xu et al., 2020]. Its processed form is a key transcription factor that regulates the expression of genes involved in cholesterol biosynthesis and lipid homeostasis [Xu et al., 2020; Yokoyama et al., 1993; Amemiya-Kudo et al., 2002];
- PANK2 (Pantothenate kinase 2) is the mitochondrial isoform that catalyses the phosphorylation of pantothenate to generate 4'-phosphopantothenate in the first and rate-determining step of coenzyme A (CoA) synthesis [Kotzbauer et al., 2005; Leonardi et al., 2007b; Leonardi et al., 2007a; Zhang et al., 2006]. It is required for angiogenic activity of the umbilical vein of endothelial cells (HUVEC) [Pagani et al., 2018].

Notably, five genes out of thirteen are engaged in transcription regulation (NR6A1, DDIT3, HOXB3, SREBF1, and NFYC), and only three are considered housekeeping genes (NFYC, ZRANB2, and COPZ1).
Experimentally validated interactions shared among the three groups of molecules, namely i) the 21 NF-kB responsive miRNAs sharing the host gene promoter, ii) their

host genes, and iii) the three TF members (NFKB1, REL, and RELA) are depicted in Figure 2.

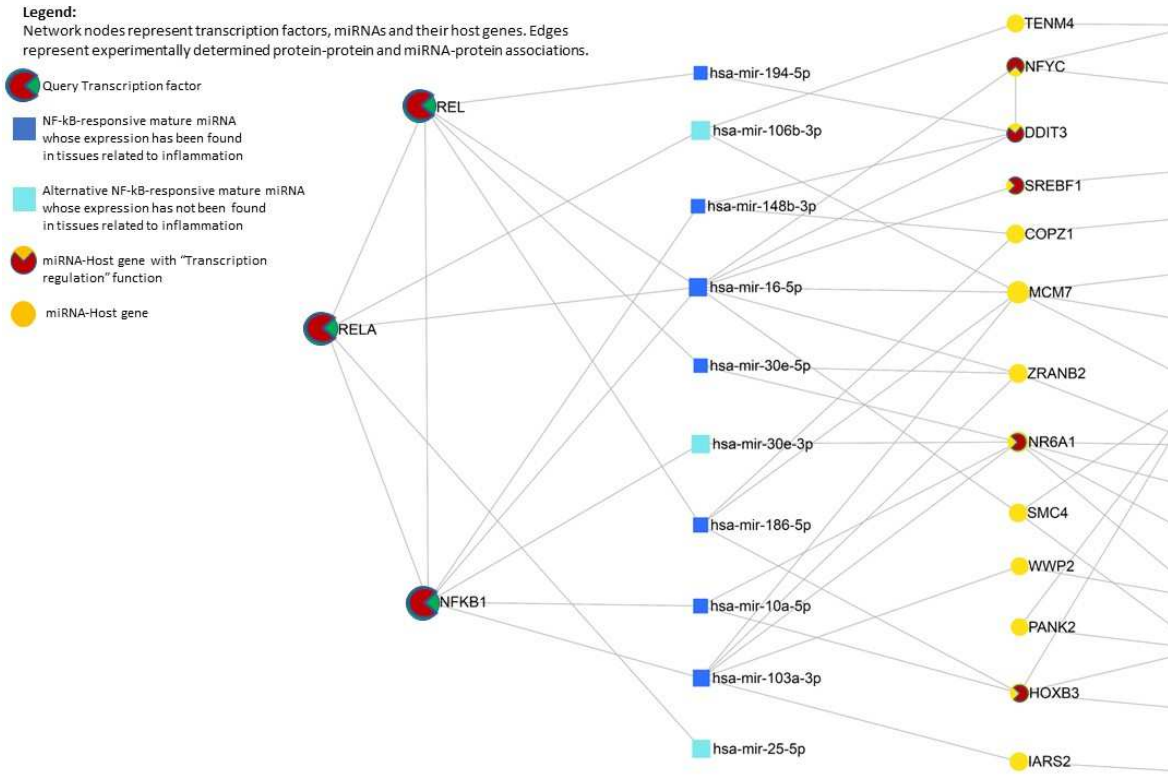

Figure 2. *TF–miRNA co-regulatory network from experimentally validated data. In this visualization, a tripartite layout has been chosen. This provides an easy abstraction of relations between different types of molecular entities in complex networks composed of several types of nodes, such as miRNAs, genes, and TFs [Chang et al., 2020; Pavlopoulos et al., 2018]*

Important nodes can be identified on the basis of their node centrality measures, such as degree and betweenness. The degree of a node is the total number of connections to other nodes. High-degree nodes are considered important “hubs” in a network [Fan and Xia, 2018; Zhu et al., 2007]. The betweenness measures the number of shortest paths going through a node, taking into consideration the global network structure. Nodes with higher betweenness are important “bottlenecks” in a network [Fan and Xia, 2018; Zhu et al., 2007]. Nodes identified by NFKB1, REL, miR-16-5p, miR-103a-3p, and NR6A1 have high degree centrality values; whereas RELA, miR-10a-5p, and miR-30e-5p represent nodes that occur between two dense clusters and have a high betweenness centrality even if their degree centrality values are not high.

Therefore, we performed an explorative evaluation of known and potential Pro-tein-Protein Interactions among REL, RELA, NFKB1, and miRNA-host genes (Figure 3) by querying STRING Database [Szklarczyk et al., 2021; Szklarczyk et al., 2018; Szklarczyk et al., 2016].

The STRING network shows that almost all host gene proteins have some degree of interaction. Experimental and biochemical data confirm the functional association of NFKB1, REL, and RELA. On the other hand, the higher confidence interaction values suggest a functional link between DDIT3, NFYC, MCM7, and SREBF1, as well as be-tween IARS2, SMC4, and WWP2. Of note, experimental evidence in Figure 2 indicated that NFBK1, REL, RELA, DDIT3, NFYC, MCM7, SREBF1, and SMC4 are all targets of miR-16-5p, but miR-103a-3p, in turn, regulates IARS2, MCM7, and WWP2.

Table 2. Host genes and intragenic miRNAs information.

miRNA Hairpin	Host Gene Information				Intragenic miRNA Information			
	Ch	Host Gene	Entrez Gene ID	Host Gene Biological Process	Intron n°	Distance from Upstream Exon	Direction	Mature miRNA ID in miRBase 22.1 Release (In Parentheses Previous IDs) §
hsa-mir-30c-1 [H]	1	NFYC	4802	Transcription regulation ‡	4	4038	sense	miR-30c-5p (**miR-30c**); miR-30c-1-3p (miR-30c-1*)
hsa-mir-30e [H]	1	NFYC	4802	Transcription regulation ‡	4	1109	sense	miR-30e-5p (**miR-30e**); miR-30e-3p (miR-30e*)
hsa-mir-186 [H, I]	1	ZRANB2	9406	mRNA processing ‡	8	1560	sense	miR-186-5p (**miR-186**); miR-186-3p (miR-186*)
hsa-mir-194-1 [H, Ig]	1, 11	IARS2	55699	Protein biosynthesis	12	6996	antisense	miR-194-5p (**miR-194**); miR-194-3p (miR-194*)
hsa-mir-15b [H]	3	SMC4	10051	DNA condensation	3	84	sense	miR-15b-5p (**miR-15b**); miR-15b-3p (miR-15b*)
hsa-mir-16-2 [H, I]	3, 13	SMC4	10051	DNA condensation	3	241	sense	miR-16-5p (**miR-16**); miR-16-2-3p (miR-16-2*)
hsa-mir-106b [H]	7	MCM7	4176	DNA replication	13	99	sense	miR-106b-5p (**miR-106b**); miR-106b-3p (miR-106b*)
hsa-mir-25 [H]	7	MCM7	4176	DNA replication	13	530	sense	miR-25-5p (miR-25*); miR-25-3p (**miR-25**)
hsa-mir-93 [H]	7	MCM7	4176	DNA replication	13	326	sense	miR-93-5p (**miR-93**); miR-93-3p (miR-93*)
hsa-mir-181b-2 [H]	9	NR6A1	2649	Transcription regulation	2	139,140	antisense	miR-181b-5p (**miR-181b**); miR-181b-2-3p
hsa-mir-708 [H]	11	TENM4	26011	Differentiation	1	38,400	sense	miR-708-5p (**miR-708**); miR-708-3p (miR-708*)
hsa-mir-148b [H]	12	COPZ1	22818	Protein transport ‡	1	12,035	sense	miR-148b-5p (miR-148b*); miR-148b-3p (**miR-148b**)
hsa-mir-616 [H]	12	DDIT3	1649	Transcription regulation	1	1159	sense	miR-616-5p (miR-616, miR-616*); miR-616-3p (**miR-616**)
hsa-mir-140 [H]	16	WWP2	11060	Ubl conjugation pathway	6	1191	sense	**miR-140-5p** (miR-140); **miR-140-3p**

Table 2. *Cont.*

miRNA Hairpin	Host Gene Information					Intragenic miRNA Information		
	Ch	Host Gene	Entrez Gene ID	Host Gene Biological Process	Intron n°	Distance from Upstream Exon	Direction	Mature miRNA ID in miRBase 22.1 Release (In Parentheses Previous IDs) §
hsa-mir-10a [H]	17	HOXB3	3213	Transcription regulation	1	2202	sense	miR-10a-5p (**miR-10a**); miR-10a-3p (miR-10a*)
hsa-mir-33b [H, I]	17	SREBF1	6720	Transcription regulation	12	314	sense	miR-33b-5p (**miR-33b**); miR-33b-3p (miR-33b*)
hsa-mir-103a-2 [H, I]	20	PANK2	80025	Coenzyme A biosynthesis	5	444	sense	miR-103a-2-5p (miR-103-2*; miR-103a-2*); miR-103a-3p (**miR-103**, miR-103a)

Note: Promoter location according to PROmiRNA: [H] host gene; [I] intronic; [Ig] intergenic. **Ch**: chromosome number; in bold, the locus of interest if more than one is indicated. ‡ Genes classified as housekeeping gene in Housekeeping and Reference Transcript Atlas. § In the last column, mature miRNAs nomenclature has been harmonized throughout miRBase database; miRNA nomenclature used in Table 1 has been highlighted in bold.

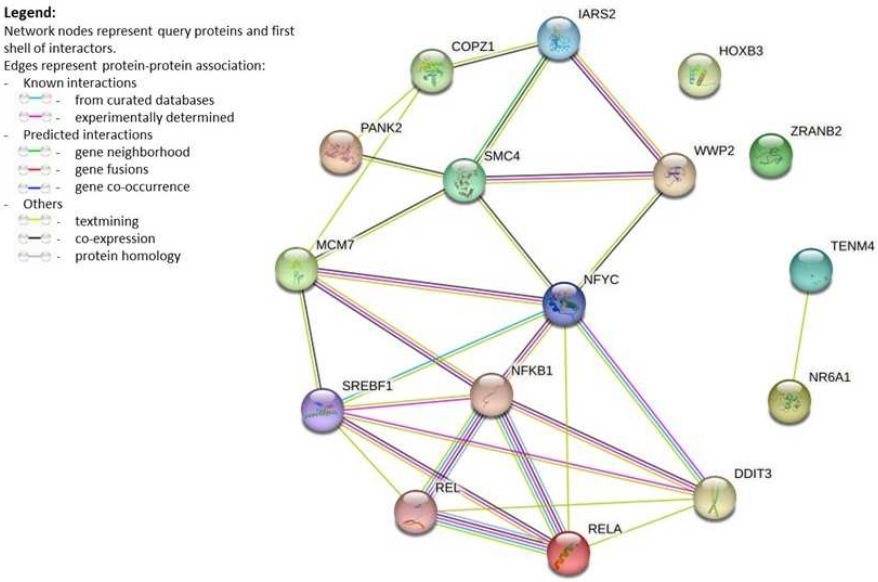

Figure 3. *Protein-Protein Interaction Network. Network nodes represent proteins: splice isoforms or post-translational modifications are collapsed, i.e., each node represents all the proteins produced by a single, protein-coding gene locus. Edges represent protein-protein associations and are meant to be specific and meaningful, i.e., proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding to each other [Szklarczyk et al., 2021; Szklarczyk et al., 2018; Szklarczyk et al., 2016]. The greater the number of edges shared between two nodes, the greater the confidence of the interaction score. The line colour indicates the type of interaction evidence*.

Finally, the significantly differentially expressed host genes in age-related conditions have been identified (Table 3). Worth a mention is the downregulation of DDIT3, SMC4, and TENM4 in replicative senescence of human fibroblasts; the upregulation of SMC4 and MCM7 after Vitamin C treatment; the upregulation of HOXB3 and TENM4 in Alzheimer's disease; and the deregulation of DDIT3 and SMC4 in Covid-19 disease.

Table 3. Differentially expressed miRNA-host genes in age-related diseases.

Differentially Expressed Gene	Cell/Tissue	Treatment/Condition	log2 Fold Change	*p*-Value	*q*-Value	DOI
DDIT3	Human diploid fibroblasts IMR90	Replicative senescence	−1.09474	1.55×10^{-23}	2.63×10^{-22}	10.1093/nar/gkz555
DDIT3	Lung	COVID-19 vs. Control	3.04617	1.11×10^{-17}	2.55×10^{-16}	10.1038/s41556-021-00796-6
HOXB3	Human induced pluripotent stem (iPS) cell-derived neural progenitor cells	Alzheimer's disease	1.30000	5.10×10^{-4}	3.40×10^{-2}	10.1016/j.celrep.2019.01.023
MCM7	Human arterial endothelial cell	Ionizing radiation	−1.07367	2.66×10^{-33}	1.94×10^{-31}	10.1093/nar/gkz555
MCM7	Human diploid fibroblasts WI38	Ionizing radiation	−1.60665	1.53×10^{-10}	5.99×10^{-9}	10.1093/nar/gkz555
MCM7	WRN-/- mesenchymal stem cell	Vitamin C	1.90980	5.08×10^{-51}		10.1007/s13238-016-0278-1
SMC4	Human arterial endothelial cell	Ionizing radiation	−1.14304	5.14×10^{-38}	4.56×10^{-36}	10.1093/nar/gkz555
SMC4	Human diploid fibroblasts WI38	Ionizing radiation	−2.31474	1.24×10^{-14}	8.41×10^{-13}	10.1093/nar/gkz555
SMC4	Lung	COVID19 vs. Control	−2.85324	4.44×10^{-10}	3.79×10^{-9}	10.1038/s41556-021-00796-6
SMC4	Human diploid fibroblasts WI38	Replicative senescence	−1.44078	8.14×10^{-7}	1.75×10^{-5}	10.1093/nar/gkz555
SMC4	WRN-/- mesenchymal stem cell	Vitamin C	2.18610	4.24×10^{-55}		10.1007/s13238-016-0278-1
TENM4	Human diploid fibroblasts WI38	Replicative senescence	−4.84098		1.97×10^{-52}	10.1093/nar/gkz555
TENM4	Human diploid fibroblasts IMR90	Ionizing radiation	−1.63819	3.26×10^{-23}	5.65×10^{-22}	10.1093/nar/gkz555
TENM4	Human diploid fibroblasts WI38	Ionizing radiation	1.82630	6.17×10^{-12}	3.09×10^{-10}	10.1093/nar/gkz555
TENM4	Human diploid fibroblasts IMR90	Replicative senescence	−1.03845	1.66×10^{-10}	1.24×10^{-9}	10.1093/nar/gkz555
TENM4	Human induced pluripotent stem (iPS) cell-derived neurons	Alzheimer's disease	1.70000	5.50×10^{-4}	1.70×10^{-2}	10.1016/j.celrep.2019.01.023

3.4. Pathways targeted by the 68 putative NF-kB responsive miRNAs

By performing an Ingenuity Pathway Analysis (IPA) Target Filter Analysis, we identified mRNAs targeted by the putative NF-kB responsive miRNAs. 18095 mRNAs were retrieved, of which 9613 were experimentally observed or highly predicted. The significance was reported as p-value in Table S4. The let7a-5p was the miRNAs with associated the highest number of mRNA targets (2014 targets).
Then, we performed a network analysis focusing on putative NF-kB responsive miRNAs targeting mRNAs coding for molecules belonging to the NF-kB pathways (Figure 4).

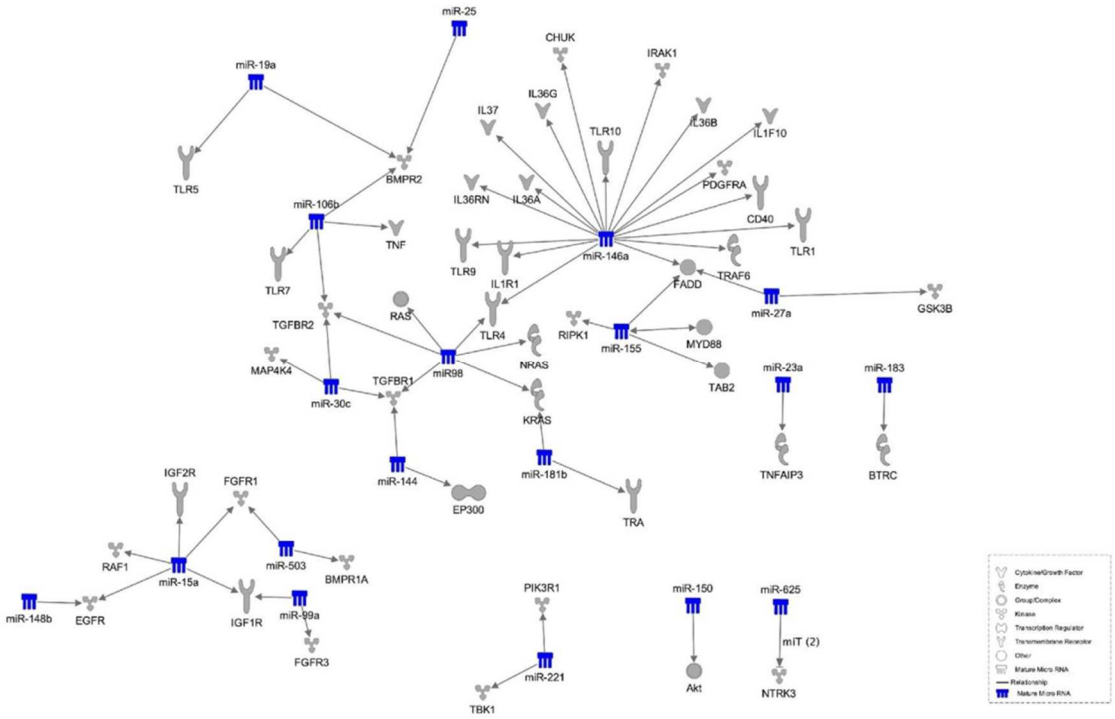

Figure 4. IPA Path Designer NF-kB Target network. Molecules belonging to NF-kB pathway targeted by NF-kB responsive miRNAs.

Interestingly, the NF-kB responsive miRNAs do not directly target genes coding for the NF-kB different subunits, but most of them are able to target genes coding for molecules belonging to NF-kB activation pathways, such as TLR and MYD88. This re-sult is very interesting, considering that the modulation of NF-kB biological activity is related to its activation, rather than to the modulation of NF-kB subunits expression.
Further, to discover the main diseases and functions associated with the selected miRNAs dataset we performed an IPA Core Analysis (Figure 5). The Diseases and Functions are shown by bar chart, sorted by their -log p-value (p-value from Fisher's Exact test).

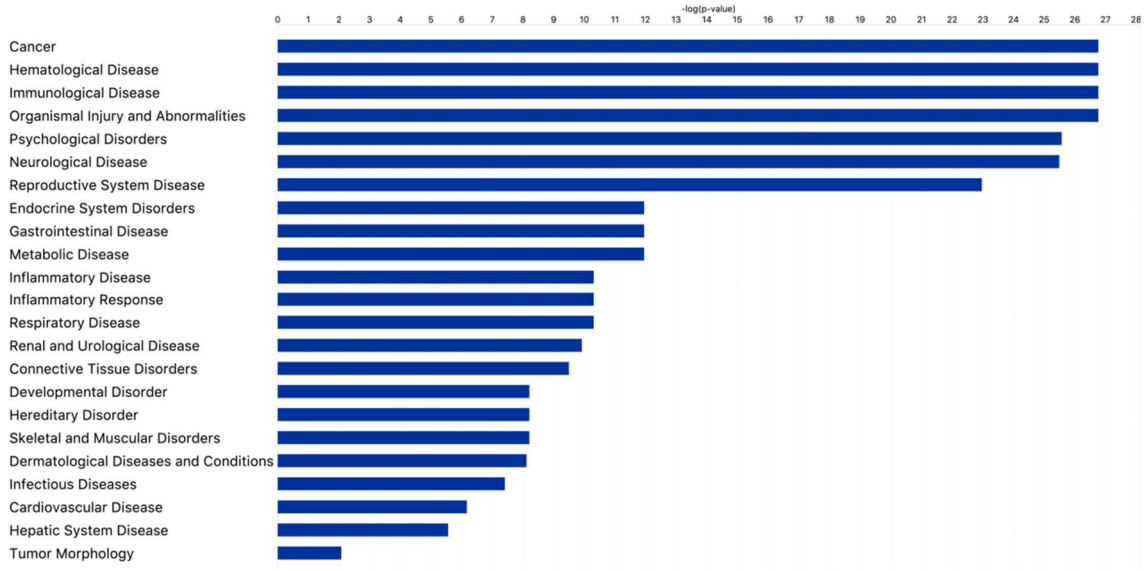

Figure 5. *Most relevant human diseases associated with molecular pathways targeted by putative NF-kB responsive miR-NAs. The Diseases and Functions associated with molecular pathways targeted by putative NF-kB responsive miRNAs are shown by the bar chart, sorted by their -log p-value (Fisher's Exact test p-value). 23 relevant human diseases are listed*.

Cancers, immunological diseases, neurological diseases, and metabolic diseases, all well-recognized as inflammatory-based diseases, are among the diseases associated with the highest probability with NF-kB responsive miRNAs. Focusing on metabolic diseases, the most affected diseases are the non-insulin dependent diabetes mellitus (-log p-value 11.955), Alzheimer disease (-log p-value 9.532), and diabetes mellitus (-log p-value 7.680).

To better explain the association of identified NF-kB putative responsive miRNAs with these human diseases, we depicted miRNAs-diseases relationship in Figure 6. Figure 6 panel A depicts NF-KB putative responsive miRNAs associated with metabolic diseases, whereas Figure 6, panels B, C and D show the association between identified NF-kB responsive miRNAs and cardiovascular diseases, neurological diseases, and cancer, respectively.

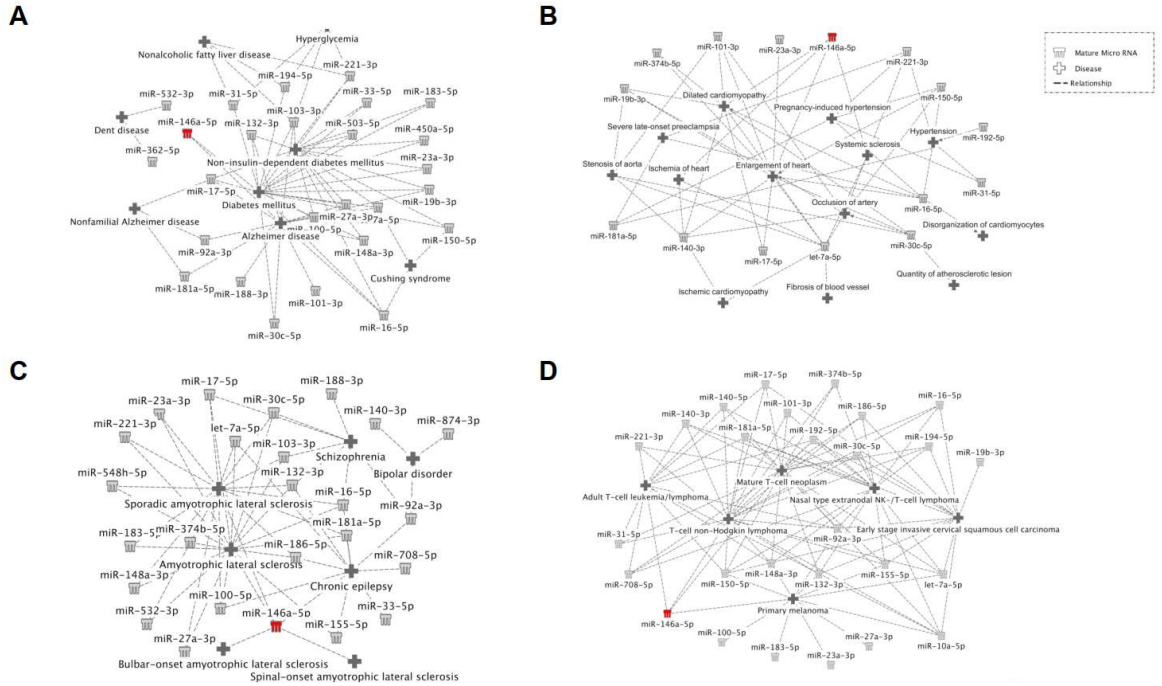

Figure 6. *IPA Diseases Networks. Diseases Networks targeted by NF-kB responsive miRNAs. Metabolic disease (panel A), cardiovascular diseases (panel B) neurological diseases (panel C) and cancer (panel D).*

3.5. The 68 putative NF-kB responsive miRNAs and previously identified inflammamiRs

To test whether the 68 putative NF-kB responsive miRNAs could have a biological value in the context of the previous evidence, we compared our results with those already present in the literature. Among these 68 miRNAs, 21 have been experimentally validated to be transcribed by NF-kB1: miR-16-2 [75], miR-10a [76], miR-140-3p, miR-140-5p [77], miR-148b [78], miR-15b [79], miR-186 [80], miR-146a, miR-155, miR-19b, miR-20a, miR-19a, miR-17, miR-221, miR-222, miR-18a, miR-92a, miR-101, miR-23a, miR-27a, and miR-30c [Markopoulos et al., 2018].
In addition, we have chosen as a reference all available data on the miRNAs relevant to aging, inflammation, and immunity, that can be referred as inflammamiRs [Prattichizzo et al., 2017]. A detailed comparison table has been provided in Table S5.
Figure 7A shows the “word cloud” with the 68 “high confidence” expressed miRNAs. The more features a specific miRNA holds (such as: the number of promoter types, the number of miRNA precursors, if it is expressed in more than one tissue, and finally if it is known to target NF-kB), the bigger and bolder it appears in the figure. Figure 7B depicts a Venn diagram modified from [Prattichizzo et al., 2017], displaying the miRNAs related to inflammation, immunity, and aging based on their circulating shuttles. In the inner circles are grouped exosome-associated miRNAs, while in the outer circles the circulating miRNAs associated with Ago-2, HDL, or other microparticles. In this version, it is important to note that bold characters indicate miRNAs overlapping among the two groups. Most of the 68 high-confidence NF-kB responsive miRNAs (reported in panel A) were previously identified as circulating miRNAs associated with aging, immunological functions, and inflammation, i.e. inflammaging [Prattichizzo et al., 2017]. Only 3 miRNAs, such as miR-154, -377, and -885-5p, were not retrieved in previous analysis [Prattichizzo et al., 2017]. However,

based on recent literature, all of them are related to NF-kB/inflammation pathways [Kim et al., 2021; Liu et al., 2018; Solé et al., 2023]. All the 68 NF-kB responsive miRNAs are therefore included in the Venn diagram reported in panel B, highlighting that these miRNAs identified as tissues expressed miRNAs are also detectable in blood and most of them were identified inside extracellular vesicles, i.e., exosomes (miRNAs depicted in inner circles Fig.7 panel B).

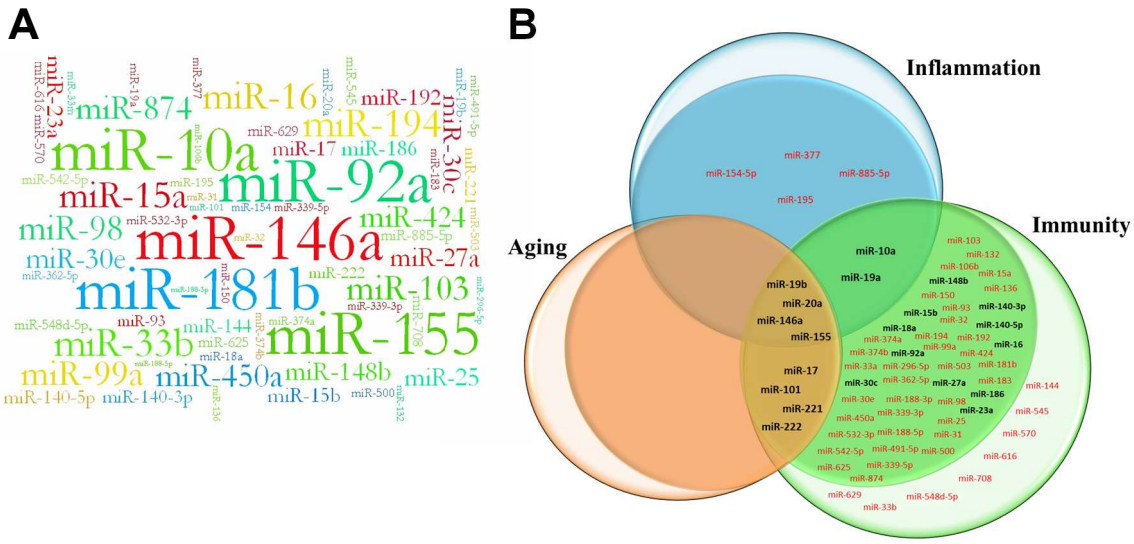

Figure 7. *(A) Word Cloud of the 68 putative NF-kB responsive miRNAs. The "word cloud" has been used to highlight the values of a miRNA based on its characteristics (such as: the number of promoter types, the number of miRNA precursors, if it is ex-pressed in more than one tissue, and finally if it is known to target NF-kB). The more features a specific miRNA holds, the bigger and bolder it appears in the "word cloud". This Word cloud has been drawn using Wordaizer version 6.0 APP Helmond (www.apphelmond.com). (B) Venn diagram showing the NF-kB and inflammamiRs research in context. Modified version of the Venn diagram from [Prattichizzo et al., 2017]. The Venn diagram displays the 68 NF-kB responsive miRNAs related to inflammation, immunity, and aging based on their circulating shuttles. In bold, the 21 experimentally validated miRNAs; in red, the 47 not yet experimentally validated miRNAs. In the inner circles are grouped exosome-associated miRNAs, while in the outer circles the circulating miRNAs associated with Ago-2, HDL, or other microparticles.*

3.6. mRNAs targeted by the 68 putative NF-kB responsive miRNAs belonging to pathways involved in aging process and/or age-related diseases.

By further analysing the IPA Target Filter Analysis results, we finally identified the mRNAs, either experimentally validated or highly predicted, to be targeted by the 68 putative NF-kB responsive miRNAs, belonging to pathways related to aging or to the most common age-related conditions. Among the 9613 mRNAs predicted to be target-ed by such NF-kB responsive miRNAs, 189 mRNAs targeted by 46 out of 68 miRNAs, were associated to "cellular senescence pathway" (Table S6

Supplementary Materials). In addition, out of the 9613, quite all, 8599 mRNAs were related to diseases reported in Figure 6, such as metabolic diseases, cardiovascular diseases, neurological diseases, and cancer. All these conditions share an inflammatory etiopathogenesis and are prototypical ARD.

3.6.1. Association between clinical and blood-based features with lung involvement and survival

Seventy-three patients with COVID-19 pneumonia were evaluated by chest CT and blood sampling at admission. The median age was 85 years, with an interquartile range (IQR) of 82-90. The female:male ratio was 1.35.

As baseline clinical characteristics, the distribution of myocardial infarction, dementia, chronic kidney disease (CKD), hypertension, stroke, COPD, atrial fibrillation, cancer, congestive heart failure (CHF), diabetes, Charlson index (0 to 2), and median values with IQR of oxygen saturation, days from infection to admission, and days from symptoms to admission were assessed. Mean values with IQR of white blood cells (WBC), neutrophils, lymphocytes, monocytes, and platelets (PLT) were included as baseline blood characteristics, neutrophil-lymphocyte ratio (NLR), neutrophil-lymphocyte derived ratio (dNLR), platelet-lymphocyte ratio (PLR), lymphocyte-monocyte ratio (LMR), miR-483-5p, miR-320b, cfDNA [pg/µl], CD163, elastase.

Exploring the association between these features and survival status, and CT score, percentage of healthy lung (HL%), percentage of ground-glass opacity (GGO%), percentage of lung consolidation (LC%) (Table 4), a significant association was found between radiological parameters and some clinical features, but not with blood-based ones. For example, patients with low CT scores were more often female. A low HL% was associated with a higher frequency of CKD, stroke and moderate-severe severity on CT. Regarding survival, deceased patients were older and had more frequent stroke, COPD and shorter time from infection to admission, but were less frequently diabetic.

Table 4. Patient's characteristics according to survival status.

	Total N=76	**Survived N=54**	**Deceased N=22**	**p**
Female Gender, n(%)	44(57.9%)	31(57.4%)	13(59.1%)	0.893
Infarction, n(%)	7(9.2%)	4(7.4%)	3(13.6%)	0.394
Dementia, n(%)	23(30.3%)	17(31.5%)	6(27.3%)	0.717
CKD, n(%)	14(18.4%)	8(14.8%)	6(27.3%)	0.204
Hypertension, n(%)	50(65.8%)	36(66.7%)	14(63.6%)	0.801
Stroke, n(%)	10(13.2%)	3(5.6%)	7(31.8%)	**0.002**
COPD, n(%)	10(13.2%)	3(5.6%)	7(31.8%)	**0.002**
Atrial Fibrillation, n(%)	20(26.3%)	13(24.1%)	7(31.8%)	0.487
Cancer, n(%)	16(21.1%)	13(24.1%)	3(13.6%)	0.311
CHF, n(%)	22(28.9%)	14(25.9%)	8(36.4%)	0.363
Diabetes, n(%)	19(25%)	18(33.3%)	1(4.5%)	**0.009**
Charlson index, n(%)				0.856
0	38(50.0%)	26(48.2%)	12(54.6%)	
1	24(31.6%)	18(33.3%)	6(27.3%)	
2	14(18.4%)	10(18.5%)	4(18.2%)	
CT severity, n(%)				0.167
Mild (<8)	38(50%)	29(53.7%)	9(40.9%)	
Moderate (9-15)	31(40.8%)	22(40.7%)	9(40.9%)	
Severe (>15)	6(7.9%)	2(3.7%)	4(18.2%)	
NA	1(1.3%)	1(1.9%)	0(0%)	
Age, median(IQR)	86(82-90)	85(82-90)	90(87-93)	**0.003**
Saturation, median(IQR)	97(95-98)	97(95-98)	97(95-98)	0.826
Days from Infection to Admission, median(IQR)	2(0-3)	2(0-3)	1(0-1)	**0.025**
Days from Contagion to Admission, median(IQR)	10(8-14)	11(8-14)	8(8-17)	0.574
Days from Symptoms to Admission, median(IQR)	5(1-8)	5.5(1-8)	4(1-8)	0.815
WBC, median(IQR)	8.5(5.9-11.8)	7.9(4.9-10.1)	13.7(7.1-16.9)	**0.006**
Neutrophils ($\times 10^3$/ µL), median (IQR)	44.5(20.1-61.8)	51.7(28.2-61.8)	21.1(15.1-59.5)	0.103
Lymphocytes ($\times 10^3$/ µL), median (IQR)	1.2(0.8-1.8)	1.2(0.9-1.7)	0.9(0.5-1.8)	0.087
Monocytes ($\times 10^3$/ mm^3), median(IQR)	0.5(0.4-0.7)	0.6(0.4-0.7)	0.4(0.3-0.5)	**0.033**
PLT, median(IQR)	233(177-297)	246.5(180-301)	218(177-257)	0.208
NLR, median(IQR)	34.4(17.5-53.9)	35.0(21.0-51.0)	27.5(15.3-57.3)	0.686
dNLR, median(IQR)	-1.2(-1.2--1.1)	-1.2(-1.2--1.1)	-1.1(-1.2-6.1)	0.073
PLR, median(IQR)	194.7(129.1-328.8)	188.4(131.2-283.0)	242.5(127.4-384.4)	0.210
LMR, median(IQR)	2.2(1.6-3.4)	2.3(1.7-3.4)	2.0(1.2-3.3)	0.353
miR-483-5p, median(IQR)	0.1(0.0-0.2)	0.1(0.0-0.2)	0.1(0.0-0.2)	0.330
CT Total Score, median(IQR)	8(5.5-12)	8(5-11)	10.5(6-15)	0.143
HL (l) , median(IQR)	1.5(1.0-2.3)	1.8(1.1-2.5)	1.1(0.7-2.0)	**0.046**
GGO (l) , median(IQR)	0.9(0.6-1.2)	0.9(0.6-1.2)	0.8(0.5-1.2)	0.388
Consolidation (l) , median(IQR)	0.2(0.1-0.3)	0.2(0.1-0.3)	0.3(0.1-0.4)	0.238
Lung volume (l) , median(IQR)	2.8(2.2-3.5)	2.9(2.4-3.8)	2.5(1.7-2.9)	**0.018**
HL (%), median(IQR)	59.9(38.5-72.8)	61.0(42.8-72.8)	45.0(35.8-70.5)	0.254
GGO (%), median(IQR)	32.5(21.1-46.4)	30.7(22.7-45.3)	38.5(19.6-48.7)	0.590
Consolidation (%), median(IQR)	7.5(4.2-13.6)	6.7(3.5-11.7)	10.0(4.9-16.8)	**0.050**

3.6.2. Correlation between radiological parameters and microRNAs

The correlation between the 4 radiological parameters and two biological biomarkers, miR-483-5p and cfDNA, which showed a p-value <0.1 in univariate analysis, potentially associated with hospital mortality for COVID-19 [Giuliani et al., 2022; Cardelli et al., 2022], was examined. A slight increase in miR-483-5p levels was observed with higher GGO% (correlation 0.28; p = 0.018) and a slight decrease with higher HL% (correlation -0.27; p = 0.023). No significant associations were found between miR-483-5p and the other radiological parameters, nor correlations between cfDNA and radiological parameters (Table 5). Scatter plots were provided to illustrate statistically significant correlations (Figure 8). Significant enrichment of lung disease-related biological processes associated with miR-483-5p was observed in the PAH analysis (Figure 9).

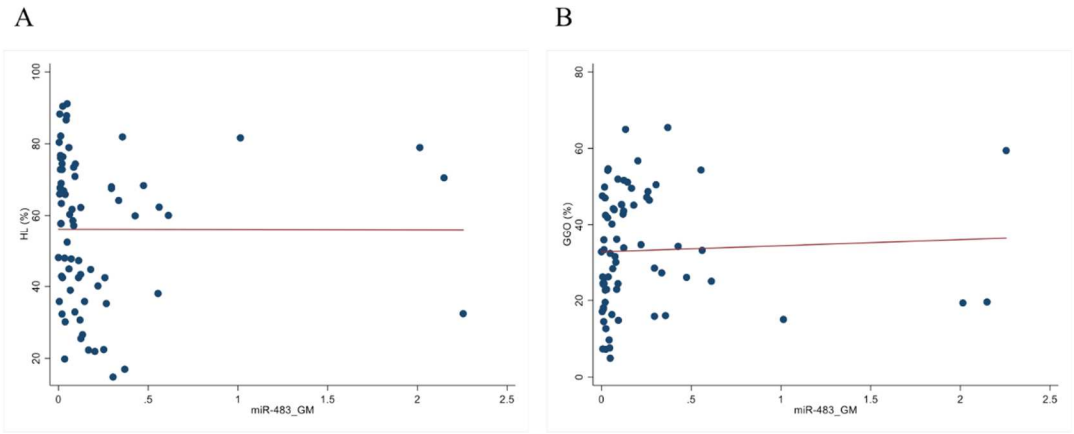

Figure 8. *Scatter plots with regression line of the correlations between miR-483-5p and HL% (A) and GGO% (B).*

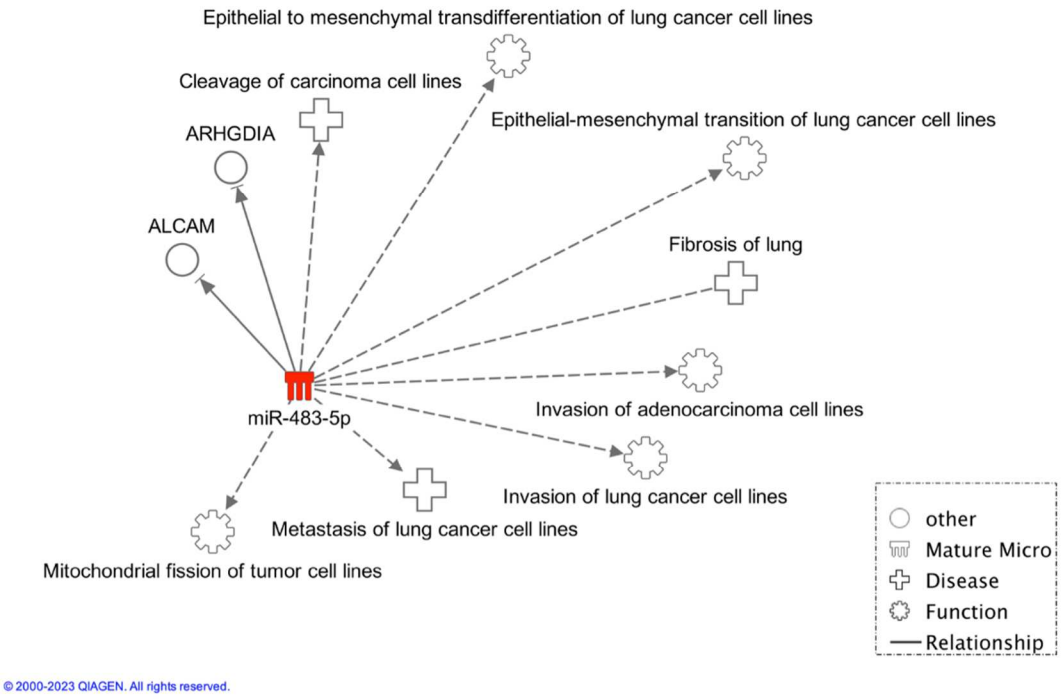

Figure 9. *Relationship generated by the Ingenuity Pathway Analysis programme (IPA). The shown associations among the miR-483-5p and the Diseases and Functions have been selected as related to lung diseases and functions***.**

Table 5. Coefficients of the correlation between radiological parameters and microRNAs.

	miR-483-5p		miR-320b	
	Coefficient	p	Coefficient	p
CT Total Score	0.02	0.888	-0.03	0.782
HL (%)	**-0.28**	**0.017**	-0.07	0.582
GGO (%)	**0.29**	**0.013**	0.05	0.675
Consolidation (%)	0.06	0.631	-0.04	0.736

4. Discussion

NF-kB is an ubiquitously and evolutive conserved TF activated by a plethora of external and internal proinflammatory stimuli [Fukata et al., 2009; Salminen et al., 2012; Hayden and Ghosh, 2012]. The crucial role as a mediator of the inflammatory responses, together with the finding that the activation or inhibition of NF-kB can induce or reverse respectively the main features of aged organisms, has brought NF-kB under consideration as a key TF that drives the biological aging process [García-García et al., 2021]. In this framework, the identification of genes modulated by NF-kB can be considered a cutting-edge issue [Bass et al., 2021; Kizilirmak et al., 2022; Liu et al., 2020].
NF-kB-responsive genes were extensively investigated, whereas NF-kB-responsive genes for non-coding RNAs were only recently highlighted.
Here we demonstrated that applying a data-mining approach, it has been possible to select the most reliable NF-kB responsive miRNAs. Most notably, the availability of data on TFs binding sites on human miRNAs sequences constituted a starting point and the foundation for studying all human miRNAs with potential NF-kB binding sites in their promoter regions.
Some years ago, it has been advanced a general hypothesis that the aging process and the development of the most common ARDs could be fostered by a low-grade, chronic, systemic inflammatory process named “inflammaging” [Franceschi et al., 2000]. Inflammaging, which is principally sustained by the activation of the innate immune cells, is paralleled by the increased burden of senescent cells acquiring a senescence-associated secretory phenotype (SASP), that turns senescent cells into proinflammatory cells [Salminen et al., 2012; Franceschi et al., 2000; Laberge et al., 2012; Freund et al., 2010; Fulop et al., 2018].
In immune cells and tissues obtained from patients affected by the most common ARDs, NF-kB is commonly constitutively activated [Songkiatisak et al., 2022]. Of note, NF-kB activation should be an inducible, but transient event in physiological conditions. However, despite the presence of multiple checks and balances that control NF-kB activation, in cellular and organismal aging, as well as in many ARDs, NF-kB activation becomes persistent [Josephson et al., 2021; Haga and Okada, 2022]. In this study, using PROmiRNA software and a data mining approach, we provide a list of 73 putative “high confidence” pre-miRNAs sequences corresponding to 68 NF-kB responsive mature miRNAs sequences.
Likewise, we highlighted the presence of distinct types of promoters that can regulate NF-kB responsive miRNAs.
Thirty-three miRNAs of the 68 high confidence expressed miRNAs identified have an “intronic” promoter and 5 of these have both an “intronic” and “host gene” promoter, whereas only one microRNA (miR-194) shares both “intergenic” and “host-gene” promoter. Alternative promoters are a common mechanism to create diversity in the transcriptional regulation of microRNA [Carninci et al., 2006].
It has been demonstrated that “intronic” promoters convey an additional degree of freedom over intragenic microRNA transcriptional regulation by virtue of some peculiar characteristics, thus allowing the modulation of miRNA expression levels in a tissue- and condition-specific manner [Marsico et al., 2013]. Besides the other features, in this context, it is important to stress that: “intronic” promoters can explain cases of poor correlation between host gene and microRNA expression, functioning as a real alternative promoter [Marsico et al., 2013]. As shown in Figure S1, the expression levels of NF-kB-miRNAs modulated by both “host gene” and “intronic” promoters (i.e., miR-16, miR-103, miR-186, and miR-33b) or by both “host gene” and “intergenic promoters” (i.e., miR-194) are not correlated with the expression levels of their host gene. Whereas mostly of the miRNAs that share the host gene promoters are characterized by directly (e.g., miR-15b) or inversely (e.g., miR-30c, miR-616, and miR-93) correlated transcription levels.

“Intronic” promoters are expressed in a tissue-specific manner, but “host gene” promoters are considered primarily for housekeeping gene regulation [Marsico et al., 2013]. Housekeeping genes are required for the maintenance of essential functions of any cell type, so they are expected to be constitutively expressed in all cells and at any development stage [Hounkpe et al., 2020]. Among the NF-kB-miRNA host genes, COPZ1, NFYC, and ZRANB2 have been catalogued as housekeeping genes (Table 2).
“Intronic” promoters are mainly triggered by tissue-specific master regulator TFs, instead TFs of “host gene” promoters broadly overlap with those of protein coding genes and can be considered mainly for housekeeping. (“Intergenic” promoters are regulated by a combination of intronic-specific and host-gene specific TFs). This suggests a different evolutionary mechanism [Marsico et al., 2013]. In this study, the expression levels of the three housekeeping host gene (COPZ1, ZRANB2, and NFYC) and their miRNAs (respectively miR-148b-3p, miR-186-5p, and lastly miR-30c-5p and miR-30e-5p) are mainly inversely correlated or not showing clear correlation trends (Figure S1).
“Intronic” microRNA promoters are less evolutionarily conserved than either “intergenic” and “host gene” promoters [Marsico et al., 2013].
Conversely, evolutionarily conserved miRNAs are more likely to be regulated by an “intronic” promoter [Marsico et al., 2013].
Moreover, those intragenic miRNAs that share the promoters of the host gene, interact with their own host genes (miR-16-2::MSC4; miR-106b::MCM7, miR-181b-2::NR6A1, miR-708::TENM4, miR-148b::COPZ1, and miR-10a::HOXB3), but also with the other functionally related host genes creating a complex regulatory mechanism (Figure 2). NFKB1, REL, miR-16-5p, miR-103a-3p, and NR6A1 are the most important hub nodes in the network, whereas miR-10a-5p connects the hub nodes identified by NFKB1, NR6A1, and HOXB3 as well as miR-30e-5p connects REL, NR6A1, and ZRAMB2 hubs. Interestingly, in the network it is possible to identify a clear TF-miRNA feed-forward loop involving DDIT3, miR-16-5p, and NFYC. In a TF-miRNA feed-forward loop, TF and miRNA co-regulate the target genes: in a “coherent” feed-forward loop the TF and miRNA have the same effects on their common targets, whereas in an “incoherent” feed-forward loop, the TF and miRNA carry out opposing effects, which precisely fine-tune gene expressions to minimize noise an maintain stability [Chang et al., 2020; Bracken et al., 2016]. TF-miRNA feed-forward loops have a specific function in noise buffering effect, which can minimize the response of stochastic signalling noise to maintain steady-state target level [Inui et al., 2010; Xie et al., 2019]. Disruption of feed-forward loops could lead to serious dysregulations at the origin of diseases and cancers, e.g., the interference in NF-kB/miR-19/CYLD loop can induce T-cell leukemogenesis [Xie et al., 2019; Ye et al., 2012]. Therefore, investigating the regulatory motifs among DDIT3, 16-5p, and NFYC could provide valuable insights to dissect the molecular mechanisms underlying biological processes and diseases triggered by NF-kB constitutive activation.
Protein–protein interaction analysis of protein-coding host genes, revealed that most of them could be functionally related (Figure 3). Beyond the well-known functional association of NFKB1, REL (cREL), and RELA, several data highlighted the association with the endoplasmic reticulum stress providing opportunities to fine-tune cellular stress responses [Schmitz et al., 2018]. In the framework of atherosclerosis, multiple links between NF-kB and ER stress were suggested. A disturbed flow can cause endoplasmic reticulum stress leading to SREBF1 activation with nuclear localization, and to DDIT3 expression triggered by endoplasmic reticulum stress response elements [Le et al., 2017]. NFYC is a subunit of a trimeric complex (NFY) known to interact with several TFs to enable the synergistic activation of specific classes of promoters. NFY directly controls the expression of TF genes such as P53 (DNA-damage), XBP1, CHOP/DDIT3 (endoplasmic reticulum stress), and HSF1 (Heat shock) [Dolfini et al., 2012; Vaiman et al., 2013]. Of note, experimental data

have shown the upregulation of both SMC4 and MCM7 in mesenchymal stem cells after Vitamin C treatment, the downregulation of DDIT3, SMC4, and TENM4 in replicative senescence of human fibroblasts; the upregulation of HOXB3 and TENM4 in Alzheimer's disease; and finally, the deregulation of DDIT3 and SMC4 in Covid-19 disease (Table 3).
In this scenario, targeting NF-kB signalling is becoming a promising strategy for drugs development and ARDs treatment [Liu et al., 2020; Mato-Basalo et al., 2021].
Almost all the 68 miRNAs that we identified in our current analysis were previously associated with inflammaging process and with the most common ARDs, such as metabolic diseases, cardiovascular diseases, neurodegenerative diseases, and cancers [Slota and Booth, 2019; Grants et al., 2020].
Out of the 9613 mRNAs targeted by the 68 NF-kB responsive miRNAs, quite all, 8599 mRNAs were related to such diseases. Of note, 189 mRNAs were associated with "cellular senescence pathway", which is recognized as the main culprit of the aging process.
Most of the NF-kB responsive miRNAs are involved in a negative feedback loop to restrain exacerbated inflammation [Olivieri et al., 2021; Ward et al., 1991; Leonardi et al., 2007a; Zhang et al., 2006; Pagani et al., 2018; Chang et al., 2020; Pavlopoulos et al., 2018; Fan and Xia, 2018].
Notably, the identified NF-kB responsive miRNAs are not able to directly modulate gene expression of NF-kB subunits but are able to target molecules belonging to NF-kB activation pathways (canonical and non-canonical pathway). Interestingly, among the NF-kB-responsive miRNAs genes identified with our approach, the most relevant examples of mRNAs that can target molecules belonging to the NF-kB canonical and-non-canonical pathways or related molecules are miR-146a and miR-155. In fact, MiR-146a and miR-155, control NF-kB activity during inflammation by a combinatory action without targeting directly NF-KB subunits [Mann et al., 2018]. MiR-155 is rapidly upregulated by NF-kB during the early phase inflammatory response through a positive feedback loop necessary for signal amplification. MiR-146a is rather gradually upregulated by NF-kB and forms a negative feedback loop attenuating NF-kB activity in the late phase of inflammation. The combined action of these two positive (NF-kB::miR-155) and negative (NF-kB::miR-146a) NF-kB-miRNA regulatory loops provides optimal NF-kB activity during inflammatory stimuli, and eventually lead to the resolution of the inflammatory response in physiological condition.
Another example is miR-16 that targets the IKKα/β complex of the NF-kB canonical pathway polarizing macrophages toward an M2 phenotype [Khalife et al., 2019].These results are in line with the known modulation of NF-kB biological activity, based on based on the activation and not on the expression of its subunits [Olivieri et al., 2021].
Interestingly, all the 68 NF-kB responsive miRNAs are detectable in blood and most of them were identified inside extracellular vesicles, i.e., exosomes. Exosomes are currently considered as a crucial intercellular cross-talk mechanism, acting both at paracrine and systemic levels [Mensà et al., 2020].This result highlights the complexity of the feed-back loops between NF-kB activation in specific tissues, the expression of NF-kB responsive miRNAs and their release in the bloodstream as a systemic intercellular communication mechanism. A further level of complexity can be envisaged considering that NF-kB is known to indirectly regulate miRNA expression through the modulation of other transcription factors. NF-kB can modulate AP-1 transcription factor [Fujioka et al., 2004], which in turn is able to modulate different miRNAs genes, i.e. miR-21[del Mar Díaz-González et al., 2019].
Of note, our analysis confirmed previous evidence on the potential role of some miRNAs in physiological and pathological aging. Of note, among the 68 miRNAs, 21 were already experimentally identified as NF-kB responsive, reinforcing the reliability of ours results. Our data also highlight the potential value of the 47 NF-kB putative responsive miRNAs (listed in figure 7, panel B) that are yet to be experimentally validated.

Our results are of interest in the framework of the research on the biomarkers/drugs of aging and inflammation related diseases. If NF-kB responsive miRNAs are hyper-transcribed in tissues involved in the modulation of inflammatory responses, the hypothesis that circulating miRNAs could be useful tools to track the trajectories of healthy or un-healthy aging is reinforced [Olivieri et al., 2017; Zhavoronkov et al., 2019; Hamdan et al., 2021; Kinser and Pincus, 2020; Rovčanin Dragović et al., 2022] and possible therapeutic strategies based on the inhibition of those miRNAs could be further tested.

The case study compared chest CT parameters in COVID-19 pneumonia and novel circulating biomarkers of immune cell activation/inflammation, such as circulating MicroRNAs previously identified as biomarkers of mortality risk in elderly patients with COVID-19. Two microRNAs, miR-320b and miR-483-5p, already analysed in a cohort of 116 COVID-19 patients, were selected for the study [Giuliani et al., 2022]. In addition, lung injury was associated with several deregulated microRNAs; in particular, up-regulation of miR-150 was correlated with lung improvement at hospital discharge [Bueno et al., 2022]. The results confirm the prognostic value of CT score and lung involvement, which has already been described in the literature [Jayachandran et al., 2022; Li et al., 2020a; Zakariaee et al., 2022; Colombi et al., 2020]. A significant association between LC% and risk of death was reported by Li et al . In the current study, LC% emerged as frequent in cases with the worst prognosis, confirming its negative prognostic role associated with death [Cardelli et al., 2022]. The negative correlation between HL% and mortality reflects the risk of death related to the reduction of healthy lung parenchyma. Recent results provide support for the involvement of miR-483-5p in infectious diseases. It was observed that elevated levels of miR-483-5p showed more than 90% sensitivity and specificity in discriminating between patients with COVID-19 and healthy subjects. In another study on pulmonary tuberculosis (TB), six serum miRNAs, including miR-483-5p, were identified as specific for pulmonary TB patients compared with non-TB patients. In addition, miR-483-5p was found to be increased in pediatric pneumonia and severe pneumonia. [Giannella et al., 2022; Zhang et al., 2013; Feng et al., 2021; Huang et al., 2017]. The positive correlation between GGO% and miR-483-5p in early stages could indicate intense activation of immunity associated with GGO%. However, no association was found between miR-483-5p and lung consolidation, suggesting that miR-483-5p might mainly reflect activation of GGO-associated immune responses. It can be reasonably assumed that the intensity of the early immune response, together with increased GGO%, may contribute to a percentage increase in lung consolidation (LC%) at a later stage, which has been correlated with poorer prognosis in both early and late stages, as documented in the existing literature. [Jayachandran et al., 2022; Francone et al., 2020; Li et al., 2020a; Zakariaee et al., 2022; Li et al., 2020e; Colombi et al., 2020]. The main limitation of this case study is the small sample size, which might limit the generalization of the results. Further studies with larger samples and a longitudinal approach are needed to explore the role of miR-483-5p and other microRNAs in lung involvement and disease progression in COVID-19.

5. Conclusions

The transcription factor NF-κB is universally recognized as a crucial regulator in inflammatory responses, as it influences the expression of inflammation-associated genes. In addition to this, it has a complex role in transcriptional activation of gene expression genesis through post-transcriptional modulators, such as noncoding RNAs, including microRNAs (miRNAs). Although the involvement of NF-κB in the regulation of inflammation-related genes is well documented, the dynamics between NF-κB and miRNA-producing genes requires further investigation. In our study, has been employed PROmiRNA software to conduct an in silico analysis to predict miRNA promoters, allowing us to assess the likelihood that specific genomic regions are cis-regulatory elements for miRNAs. From this analysis, has been compiled a list of 722 human miRNAs, of which 399 were active in at least one inflammatory tissue. Using miRBase, have been then identified 68 mature miRNAs of "high reliability," many of which have already been categorized as inflamma-miRs. Our research on the targeted pathways and diseases associated with these miRNAs reveals their significant role in prevalent age-related diseases. Our findings support the hypothesis that sustained NF-κB activity may impair the transcriptional balance of specific inflammatory miRNAs, suggesting that identification of these miRNAs could be crucial for the diagnosis, prognosis, and treatment of common inflammatory and age-related conditions. In the case study, has been evaluated the potential of microRNAs as biomarkers for COVID-19 disease. Has been used IPA analysis to confirm the role of miR-483-5p and selected lung-related diseases and functions. In this dissertation, has been demonstrated overall that established data mining methodologies can reveal the most reliable microRNAs (miRNAs) that play crucial roles in regulating specific biological pathways of interest. Unraveling the interactions between miRNAs and NF-κB represents a critical avenue of research to understand the complex disruptions of various metabolic pathways associated with both normal and pathological aging. Further research is imperative to confirm the importance of identifying these miRNAs, which could have diagnostic, prognostic, and therapeutic value for common inflammatory and age-related conditions. Through our case studies, has been highlighted the importance of using state-of-the-art data mining techniques and incorporating new bioinformatics tools. These methodologies improve our in silico understanding and facilitate the integration of literature and clinical data, simplifying the understanding of complex biological processes. Our focused approach aims to refine biomarker discovery, thereby contributing to the advancement of precision medicine.

Furthermore, the assessment of miR-483-5p-expressed inflammation and the degree of lung involvement seem to be promising tools for prognostic evaluation of COVID-19 pneumonia in elderly patients. Their integration could improve the understanding of the disease and contribute to better patient outcomes.

6. Methodology

6.1. Data-mining process

In the field of Knowledge Discovery in Databases (KDD), a data-mining approach is used to extract meaningful information and to develop significant relationships among variables stored in large data sets [Sahu et al., 2011]. In this study, we have mined and integrated data from multiple databases to select NF-kB responsive miRNAs, and the process has been tailored based on the research question. Four main steps can be distinguished:

6.1.1. Database selection

The following data sources have been investigated to retrieve the data and develop the study: PROmiRNA [Marsico et al., 2013], FANTOM4 libraries [Kawaji et al., 2011; Betel et al., 2008], "High confidence human hair-pins" in miRBase [Kozomara and Griffiths-Jones, 2014], and "Human Expression" dataset (microrna.org) [Betel et al., 2008].

- **PROmiRNA** provides an interesting approach for miRNA promoter annotation based on a semi-supervised statistical model trained on deepCAGE data and sequence features [Marsico et al., 2013]. It has been used to identify all human miRNAs potentially modulated by NF-kB, i.e.: "NF-kappaB", "NFKB1", "REL", and "RELA".

- **FANTOM4** libraries, generated by the FANTOM4 project [Kawaji et al., 2011], collect a wide range of genome-scale data from several tissues. The analysis of FANTOM4 libraries retrieved those miRNAs showing "expression at the promoter level" in different human tissues. The following libraries from healthy tissues have been selected: "blood", "bone marrow", "immune system cells", "liver", "monocytic-cells", "T cells", and "T cells 2".

- **miRBase** database is the public repository for all published miRNA sequences and associated annotations [Kozomara and Griffiths-Jones, 2014; Kozomara et al., 2018; Kozomara and Griffiths-Jones, 2011; Griffiths-Jones et al., 2008; Griffiths-Jones et al., 2006; Griffiths-Jones, 2004]. "High confidence human hairpins" dataset [Kozomara and Griffiths-Jones, 2014] has been downloaded to identify all human miRBase entries with high confidence levels assessed using the deep sequencing data sets collated in miRbase (Datasets have been downloaded from this link: *https://www.mirbase.org/blog/2014/07/high-confidence-mirna-set-available-for-mirbase-21/).*
- Finally, **microRNA.org** [Betel et al., 2008], a comprehensive resource of microRNA target pre-dictions and expression profiles, has been searched to extract the "Human Ex-pression dataset", meaning the mature microRNA expression profiles in various tissues as presented by Landgraf et al. [Landgraf et al., 2007]. Expressed miRNAs from the following healthy tissues have been selected (library names for each sample type are indicated in brackets): liver (hsa_Liver), pluripotent hematopoietic stem cell (hsa_HSC-CD34), B cells from peripheral blood (hsa_B-cell-CD19, hsa_B-cell-CD19-2, hsa_B-cell-CD19-pool), T-lymphocytes (hsa_T-cell-CD4, hsa_T-cell-CD4-2, hsa_T-cell-

CD4-effector, hsa_T-cell-CD4-memory, hsa_T-cell-CD4-naïve, hsa_T-cell-CD8, hsa_T-cell-CD8-2 hsa_T-cell-CD8-naïve), NK cells (hsa_NK-CD56), monocytes (hsa_Monocytes-CD14), granulocytes (hsa_Granulocytes-CD15), and Dendritic cells (hsa_DC-unstim). Libraries from cell lines, tumor samples, genetic disorders, and so on, have been discharged. (Datasets have been downloaded from this link: http://www.microrna.org/microrna/getDownloads.do).

6.1.2. Data extraction and integration

This phase includes downloading, extracting, filtering, and combining the data from the databases previously identified. The integration of multiple datasets has been possible through the following steps.

6.1.3. Data cleaning and transformation

Because the data originates from multiple sources, the integration often involves converting data format, cleaning, removal of incorrect data, generating new variables, resolving redundancy, and checking against miRNA nomenclature consistency both between miRNAs name originating in different miRBase versions, and between the names of pri-miRNAs and the mature forms. This issue has been manually curated by comparing miRNA names in miRBase database version 21.

6.1.4. Assessment of the results

This is the final stage of a KDD process involving the translation of aggregated data into comprehensible knowledge. The validity and reliability of the data have been tested by comparing the results obtained in the data-mining process with those already published in the literature.
The whole data-mining process is illustrated in the Data Flow Diagram in Figure 1. Data obtained at each intermediate step are provided in Supplementary Tables (S1, S2, S3 Supplementary materials). The final miRNA-pool is reported in Table 1.

6.2. Bioinformatic evaluations

6.2.1. Evaluation of miRNA-Host Gene-Transcription Factor interactions

Host gene and intragenic miRNAs information (Table 2) as well as expression correlation data between miRNAs and their host gene (Figure S1) have been retrieved from MiRIAD, a database integrating microRNA inter- and intragenic data (*https://www.miriad-database.org/*)[Hinske et al., 2014]. In Table 2, host gene biological process have been obtained from UniProt database (Release 2022_05) (https://www.uniprot.org/) [Consortium, 2022]; the Housekeeping and Reference Transcript Atlas (HRT Atlas v1.0) (*https://housekeeping.unicamp.br/*) [Hounkpe et al., 2020] has been investigated to discover those host gene cataloged as housekeeping genes.
Experimentally validated interactions shared among NFKB1, REL, RELA, the NF-kB-responsive miRNAs sharing the host gene promoter, and their host genes, have been identified (Figure 2) by querying: i) DIANA-TarBase v8

(*http://www.microrna.gr/tarbase*) retrieving experimentally supported miRNA-gene interactions [Karagkouni et al., 2017]; ii) TRRUST v2 (*www.grnpedia.org/trrust*) a manually curated data-base of transcriptional regulatory interactions [Han et al., 2018]; iii) and STRING v10 to highlight the protein-protein interactions, with the constrain to retrieve only experimental evidences [Szklarczyk et al., 2015]. The whole process, including the final network creation and visualization, has been handled using miRNet (version 2.0), a miRNA-centric network visual analytics platform (*https://www.mirnet.ca/*) [Chang et al., 2020; Fan and Xia, 2018; Fan et al., 2016].
STRING database Version 11.5 (*https://string-db.org/*) has been used to discover known and potential interactions among REL, RELA, NFKB1, and miRNA-host gene proteins (Figure 3). STRING is a database of predicted and known protein-protein interactions. The interactions include direct (physical) and indirect (functional) associations; these stem from knowledge transfer between organisms, from interactions aggregated from other (primary) databases, and from computational prediction [Szklarczyk et al., 2021; Szklarczyk et al., 2016]. The network has been created by setting a minimum required interaction score of 0.15.
The RNA-seq datasets in Aging Atlas (*https://ngdc.cncb.ac.cn/aging/index*) have been examined to explore age-related changes in host gene expression [Consortium, 2021]. Table 3 shows differentially expressed host genes in strictly age-related conditions, only those genes showing |log2FC| > 1 and q-value < 0.005 (or p-value < 0.005 if q-value was not provided) have been reported. Data relative to particular experimental conditions (e.g., gene knockdown) have not been reported.

6.2.2. Ingenuity Pathway Analysis

Bioinformatic evaluations (networks and diseases analysis) were performed by the Ingenuity Pathway Analysis software (Qiagen, Hilden, Germany). The putative NF-kB responsive miRNAs identified through the data-mining process were analyzed to ex-plore the Experimentally Observed or High Predicted mRNA targets via the microRNA Target Filter Analysis.
Furthermore, an IPA Core Analysis was performed to define the associated Dis-eases and Functions. Direct and Indirect Relationships from the Ingenuity knowledge Base (gene only) datasets were considered. We filtered only molecules and/or relationships experimentally observed in any tissue from human, rat, or mouse. Across the observations, 51 miRNAs were ready to be analyzed (Table S4) [Krämer et al., 2014]. All the networks and diseases and biological functions were assessed using IPA software (Qiagen, Hilden, Germany).

6.3. Case study. Analysis of COVID-19-relevant microRNAs in the hospitalized elderly population: the use of IPA to discover novel miRNA markers.

Population aging is a global phenomenon that reflects the success of public health and socioeconomic development policies in various geographic regions. However, societal adaptation to this reality is essential to maximize the health and functionality of the elderly and promote their social inclusion. Aging presents challenges, such as the high prevalence of chronic diseases and comorbidities. The COVID-19 pandemic

has caused high mortality in the elderly, compounded by their social isolation. The vulnerability of the elderly is related to the physiological effects of aging, with a higher incidence of infectious diseases. Frailty is more evident in the hospitalized elderly, with more severe symptoms of COVID-19. Daily activities of the elderly are further compromised by pneumonia, especially in elderly patients with COVID-19 and history of chronic diseases [Piccininni et al., 2020; Faraji and Metz, 2021; Knopp et al., 2020; Trotter et al., 2008; Miyashita et al., 2022]

6.4. Imaging parameters for the severity of COVID-19 pneumonia.

Several chest CT abnormalities in COVID-19 pneumonia have been reported in various studies; conclusions may vary depending on the stage and severity of the disease. Chest CT can accurately assess lung injury, and the use of the severity score (CTSS) has been shown to be useful in the objective assessment of lung involvement [Jayachandran et al., 2022]. This score could be useful in predicting short-term outcome [Francone et al., 2020]. Common abnormalities include ground-glass opacities (GGO), consolidations, and "crazy pavement" patterns [Li et al., 2020a]. Lesions are mainly described as bilateral, multifocal with a peripheral or multilobular distribution, mainly involving the lower lobes [Churruca et al., 2021]. Other abnormalities, such as lymph node enlargement and effusions, may indicate severe inflammation [Li et al., 2020a].

6.5. MicroRNAs in the diagnosis and prognosis of COVID-19

MicroRNAs (miRNAs) are small noncoding RNA molecules that play key roles in various biological processes, targeting complementary mRNA and leading to suppression of protein synthesis [Visacri et al., 2021]. Circulating miRNAs have been proposed as biomarkers for diagnosis and/or prognosis as well as for understanding the pathophysiology of clinical conditions such as cardiovascular disease, cancer, and dementia, including COVID-19 [Viereck and Thum, 2017; Wang et al., 2018; Blount et al., 2022; Condrat et al., 2020]. The identification of miRNA signatures is crucial in providing clues to the pathogenesis of COVID-19, highlighting correlations between severe disease and impaired pathways related to inflammation, interferon responses, organ damage, and cardiovascular failure [Giannella et al., 2022; De Gonzalo-Calvo et al., 2021]. The association between miRNA levels and COVID-19 outcome could develop models for early prediction of severity and personalized therapeutic strategies [De Gonzalo-Calvo et al., 2021]. Recently, miR-483-5p and miR-320b were identified as biomarkers associated with increased risk of mortality in elderly patients hospitalized for COVID-19 [Giuliani et al., 2022]. However, the association between miRNAs and lung conditions in the elderly has not been investigated [Giuliani et al., 2022].

6.6. Circulating cfDNA and other biomarkers in the diagnosis and prognosis of COVID-19

Circulating cell-free DNA (cfDNA) originates from apoptotic or necrotic cells or from neutrophil extracellular trap (NET) formation [Barnes et al., 2020; Thierry and Roch, 2020; Zuo et al., 2020]. Although its plasma concentration is generally low in healthy subjects, it increases significantly in pathological conditions, including tumors and inflammatory diseases [Kustanovich et al., 2019; Han and Lo, 2021]. In the context of COVID-19, plasma cfDNA has been associated with systemic inflammation and disease progression [Cheng et al., 2021; Storci et al., 2021]. In a recent study, has been found that elevated cfDNA levels in the plasma of elderly patients hospitalized for COVID-19 are associated with an increased risk of in-hospital mortality, while reduced cfDNA integrity correlates with a worse prognosis [Cardelli et al., 2022]. In addition, increased levels of circulating proteins associated with neutrophil (neutrophil elastase, LL-37) and macrophage (sCD163) activation have been linked to an increased risk of in-hospital mortality in patients [Cardelli et al., 2022].

6.7. Study design and patient recruitment for the case study on COVID-19.

This is a single-centre retrospective study of a cohort of patients with COVID-19 pneumonia undergoing computed tomography (CT) scan of the chest on admission, with collection of clinical data and serum samples.
The patients were recruited as part of the Report-Age COVID-19 project, an observational study conducted at the Italian National Center for Aging (IRCCS INRCA), Ancona, Italy. Approval was obtained from the IRCCS INRCA Ethics Committee (Reference ID: CE-INRCA-20008), and the study protocol was registered in the ClinicalTrials.gov database (Reference ID: NCT04348396). The study protocol was performed in accordance with local and international guidelines as well as the Declaration of Helsinki. Seventy-three patients were selected from the database based on the availability of plasma and chest CT samples at hospital admission.

6.8. Blood sample collection

Peripheral venous blood was processed within 2 h after collection. Blood cell count samples were centrifuged at 2'500 x g at 4°C for 15 min. Thereafter, plasma and serum were immediately processed for routine analysis or divided into 500-µl aliquots and stored at -80°C for further analysis.

6.9. Biological Parameters.

Biological parameters included total white blood cell, monocyte and platelet counts, measured by standard automated procedures. Serum concentrations of CRP, D-dimer, sodium, potassium and procalcitonin were assessed by standard procedures. Glomerular filtration rate (GFR) was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation[Levey et al., 2009]. Circulating levels of microRNAs were analyzed as previously described [Giuliani et al., 2022], including total RNA extraction from plasma, miRNA retrotranscription, and cDNA amplification by qRT-PCR. Starbase website was used to identify miR-483-5p target genes, with a threshold of at least 3 prediction programs used to filter miRNA targets. IPA was employed to explore mRNAs targets of miR-483-5p and associated

biological functions and diseases. Circulating nucleic acids were extracted from plasma using a QIAamp Viral RNA Mini Kit, stored at -80°C and analyzed by Cell-free DNA Screen Tape Assay to estimate the concentration of double-stranded DNA fragments. NE and CD163 levels were measured by ELISA kits, following a previously published procedure [Cardelli et al., 2022].

6.10. Statistical Analyses

Regarding descriptive statistics, normality in the distribution of continuous variables was assessed by the Shapiro-Wilk test; mean and standard deviation or median and interquartile range were reported, depending on the distribution. Comparison of variables between groups was done by unpaired Student's t test or Mann-Whitney's U test, as appropriate. Categorical variables were expressed as absolute numbers and percentages, and statistical significance was assessed by Chi-square test. Image-derived parameters (e.g., CT Score, HL%, GGO%, and % consolidation) were dichotomized into values below or above (or equal to) the median of the same variable. The association of image-derived parameters with mortality during hospitalization was explored by Kaplan-Meyer curves and evaluated by log-rank test for equality. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) were estimated by four proportional Cox regression models. To test the possible association between biological and image-derived parameters, Spearman correlation coefficients were calculated for all pairs of variables with $p < 0.01$, reporting correlation coefficients (rho) and significance levels (p). For statistically significant correlations, two-dimensional scatter plots with regression line were drawn. A two-tailed p value <0.05 was considered significant. Data were analysed using the statistical software package STATA version 15.1 for Windows (StataCorp, College Station, TX, USA).

7. Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24065123/s1. Reference [Russo et al., 2017] is cited in the Supplementary Materials.

- **Figure S1.** Expression correlation plots between NF-kB-miRNAs and their host gene. (Database URL: http://www.miriad-database.org).;

- **Table S1.** Seven-hundred-twenty-two pre-miRNAs transcribed in different human tissues and predicted to have miR-NA-promoters containing putative NF-kB binding sequences. The following attributes are reported: name of the pre-miRNA corresponding to the predicted TSS, type of predicted TSS ("intergenic", "host gene", "intronic" or "hybrid"), the FANTOM4 libraries, the chromosome where the miRNA precursor is located, the age of the miRNAs corresponding to the predicted TSSs. Note: each pair of pre-mRNAs highlighted in bold are the hairpin precursors of the same mature miRNA but transcribed starting from two different promoter types. * In PROmiRNA, NF-kB is among the top 10 transcription factors with highest affinity for the 1000 bp-long region surrounding the predicted TSSs of the reported miRNAs. § All human tissues retrieved from FANTOM4 Li-braries, where the pre-miRNAs have an "expression at the promoter level". "v" indicates that the miRNA is conserved up to the vertebrate lineage; "m" indicates that the miRNA is conserved up to the mammal lineage and "p" indicates that the miRNA is conserved only in the primate lineage.;

- **Table S2.** Three-hundred-ninety-nine pre-miRNAs transcribed in healthy human tissues linked to inflammatory processes and predicted to have miRNA-promoters containing putative NF-kB binding sequences. The following attributes are reported: name of the pre-miRNA corresponding to the predicted TSS, type of predicted TSS ("intergenic", "host gene", "intronic" or "hybrid"), the healthy FANTOM4 libraries selected for analysis (i.e., liver, blood, bone marrow, immune system cells, monocytic-cells, T cells, and T cells 2), the chromosome where the miRNA precursor is located, the age of the miRNAs corresponding to the predicted TSSs. Note: each pair of pre-mRNAs highlighted in bold are the hairpin precursors of the same mature miRNA but transcribed starting from two different promoter types. * In PROmiRNA, NF-kB is among the top 10 transcription factors with highest affinity for the 1000 bp-long region surrounding the predicted TSSs of the reported miRNAs. § Healthy human tissues linked to inflammatory processes and retrieved from FANTOM4 Libraries, where the pre-miRNAs have an "expression at the promoter level". "v" indicates that the miRNA is conserved up to the vertebrate lineage; "m" indicates that the miRNA is conserved up to the mammal lineage and "p" indicates that the miRNA is conserved only in the primate lineage.;

- **Table S3.** Seventy-three "high confidence" pre-miRNAs transcribed in healthy human tissues linked to inflammatory processes and predicted to have miRNA-promoters containing putative NF-kB binding sequences. The following attributes are reported: name of the pre-miRNA corresponding to the

predicted TSS, type of predicted TSS (“intergenic”, “host gene”, “intronic” or “hybrid”), the healthy FANTOM4 libraries selected for analysis i.e., “liver” and “immune system” (the latter includes blood, bone marrow, immune system cells, monocytic-cells, T cells, and T cells 2 libraries), the chromosome where the miRNA precursor is located, the age of the miRNAs corresponding to the predicted TSSs. Note: each pair of pre-mRNAs highlighted in bold are the hairpin precursors of the same mature miRNA but transcribed starting from two different promoter types. * In PROmiRNA, NF-kB is among the top 10 transcription factors with highest affinity for the 1000 bp-long region surrounding the predicted TSSs of the reported miRNAs. ** Pre-miRNAs have been selected based on the “High Confidence dataset” (miRBase.org). The main intention of this selection is to provide a subset of miRNA precursors that you can be positive are real. § Healthy human tissues linked to inflammatory processes and retrieved from FANTOM4 Libraries, where the pre-miRNAs have an “expression at the promoter level”. “v” indicates that the miRNA is conserved up to the vertebrate lineage; “m” indicates that the miRNA is conserved up to the mammal lineage and “p” indicates that the miRNA is conserved only in the primate lineage.;

- **Table S4**. IPA Core Analysis. Diseases and Functions associated to the putative NF-kB responsive miRNAs.;

- **Table S5.** Comparison between NF-kB responsive miRNAs and miRNAs relevant to aging, inflammation, and immunity. The following attributes are reported: miRNA names and their group type as retrieved from [81], NF-kB-responsive miRNAs, names of the healthy “Human Expression dataset” libraries in which the miRNAs are expressed (i.e., “liver” and “immune system”), the miRandola extracellular RNA (exRNA) form retrieved from http://mirandola.iit.cnr.it/ [Russo et al., 2017].

- **Table S6**. List of mRNAs resulting from Target Filter Analysis belonging to the IPA canonical pathway 'cellular senescence.

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A Data-Mining Approach to Identify NF-kB-Responsive microRNAs in Tissues Involved in Inflammatory Processes: Potential Relevance in Age-Related Diseases

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Abstract: The nuclear factor NF-kB is the master transcription factor in the inflammatory process by modulating the expression of pro-inflammatory genes. However, an additional level of complexity is the ability to promote the transcriptional activation of post-transcriptional modulators of gene expression as non-coding RNA (i.e., miRNAs). While NF-kB's role in inflammation-associated gene expression has been extensively investigated, the interplay between NF-kB and genes coding for miRNAs still deserves investigation. To identify miRNAs with potential NF-kB binding sites in their transcription start site, we predicted miRNA promoters by an in silico analysis using the PROmiRNA software, which allowed us to score the genomic region's propensity to be miRNA cis-regulatory elements. A list of 722 human miRNAs was generated, of which 399 were expressed in at least one tissue involved in the inflammatory processes. The selection of "high-confidence" hairpins in miRbase identified 68 mature miRNAs, most of them previously identified as inflammamiRs. The identification of targeted pathways/diseases highlighted their involvement in the most common age-related diseases. Overall, our results reinforce the hypothesis that persistent activation of NF-kB could unbalance the transcription of specific inflammamiRNAs. The identification of such miRNAs could be of diagnostic/prognostic/therapeutic relevance for the most common inflammatory-related and age-related diseases.

Keywords: NF-kB; microRNAs; inflammation; data-mining

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1. Introduction

The nuclear factor (NF)-kB is a transcription factor (TF) activated by an evolutionarily conserved inflammatory signaling, induced by a wide range of external and internal danger signals [1–3]. The complex modulation of this signaling can be envisaged considering the different activation strategies, well known as "canonical" and "non-canonical" NF-kB activation signaling (reviewed in [4,5]). A fine-tuning activation of NF-kB promotes the expression of pro-inflammatory genes and participates in the regulation of survival, activation, and differentiation of innate immune cells and T cells [6]. On the contrary, a persistent activation of NF-kB signaling was described in conditions of cellular senescence

and organismal aging, as well as in patients affected by the most common age-related degenerative diseases (ARDs) [7–9]. Many efforts have been made to understand which pathways are regulated by NF-kB and how the NF-kB pathway itself is modulated [10,11]. It has become clear that not only TFs but also a series of epigenetic factors, including non-coding microRNAs (miRNAs), are involved in the regulation of almost all the human transcriptional programs, both as inhibitors of mRNAs translation and as enhancers of mRNAs transcription [12–14]. Increasing evidence confirmed that these epigenetic factors play key roles in the development and progression of the most common human ARDs [15,16].

Regarding the canonical pathway of miRNA processing, that regulates gene expression at the post-transcriptional level, a primary transcript called pri-miRNA is cleaved to a precursor miRNA hairpin structure (pre-miRNA) in the nucleus by the Drosha/Pasha complex and transported into the cytoplasm, where the pre-miRNA is further processed into a miRNA:miRNA* duplex [17]. After being separated, the mature miRNA is loaded into the Argonaute 2 (Ago 2) containing RNA-induced silencing complexes (RISCs) and drives it to regulate its target mRNAs [17].

On one hand, a few miRNAs targeting mRNAs belonging to NF-kB pathway have already been identified, highlighting the activation of feedback loops aimed to restrain the inflammatory process triggered by NF-kB. Notably, some miRNAs involved in these feedback circuits were identified as deregulated in ARDs [5,18–21].

On the other hand, the full elucidation of miRNA biogenesis would be of paramount importance to identify their regulators and the role they might play in complex regulatory networks. Even if computational models were extensively applied to disentangle the complex effects of non-coding RNA in human diseases [22], for a long time, the difficulty of experimentally detecting miRNA promoters has limited the ability to identify the NF-kB binding sites in DNA sequences coding for miRNAs. However, the annotation of miRNA promoters, using high-throughput genomic data, allowed us to partially overcome this difficulty [23]. As important transcriptional regulators, miRNAs can upregulate or downregulate many target genes involved in the NF-kB signaling pathway via negative or positive feedback loops, and are involved in several human diseases, too, including the recent COVID-19 pandemic [24,25]. Since it is conceivable that age-related NF-kB activation could induce an overexpression of NF-kB responsive miRNAs, the identification of such miRNAs, and their targeted mRNAs and pathways, could contribute to clarifying the complex mechanisms that modulate healthy or unhealthy aging trajectories.

In this work, we aimed to: (i) identify all human miRNAs potentially modulated by NF-kB, (ii) select and characterize those NF-kB-responsive miRNAs that are specifically expressed in healthy tissues involved in the modulation of the inflammatory processes (such as cells of the immune system, liver, blood, and bone marrow), (iii) discover their targeted mRNAs and relative pathways, and finally (iv) evaluate the involvement of such pathways in the development of human diseases, including ARDs.

2. Results

2.1. Putative NF-kB Responsive miRNAs

To select NF-kB responsive miRNAs, we analyzed the PROmiRNA database [23], FANTOM4 Libraries [26], "High confidence hairpins" in miRbase [27], and "Human expression dataset" [28], following the data-mining process highlighted in the data flow diagram in Figure 1.

We analyzed primarily genome-wide PROmiRNA predictions, as well as TF-binding site predictions as reported in [23], to identify miRNAs with potential NF-kB binding sites in their promoter sequences. PROmiRNA is a miRNA promoter recognition method, based on a semi-supervised statistical model trained on multi-tissue deepCAGE FANTOM4 libraries and other sequence features. It is tailored to score the potential of CAGE-enriched genomic regions to be promoters of either intergenic or intragenic miRNAs, thereby modulating miRNA expression in a tissue-specific manner [23]. To identify the TFs that regulate specific miRNAs, for each predicted miRNA transcription start site (TSS), we retrieved the 1 kb

centered on it and used the TRAP approach [29] to compute the affinity of TF binding sites for all predicted miRNA promoters using TF models stored in the JASPAR database [30].

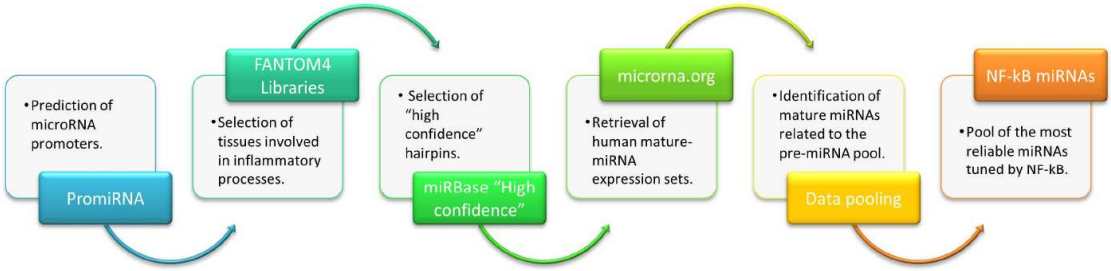

Figure 1. Data flow diagram. Figure depicts the whole data-mining process.

NF-kB appears among the first 10 TFs with the highest affinity for the 1000 bp-long region surrounding the predicted TSSs for 722 miRNA hairpin precursors (Table S1).

Since tissues show specific miRNA expression patterns, we aimed to highlight the list of putative NF-kB-responsive miRNAs expressed in tissues strictly involved in the modulation of the inflammatory processes, including inflammaging. To achieve this goal, we focused our subsequent research on those miRNAs transcribed in human tissues such as "T cells", "T cells 2", "monocytic-cells", "immune system cells", "bone marrow", "blood", and "liver". Only the libraries relative to healthy tissues have been taken into consideration. This approach retrieved 399 miRNA hairpin precursors showing "expression at the promoter level" in at least one of these tissues (Table S2). In general, this is a good indication that the mature forms of these miRNAs are expressed in a specific tissue. However, each step from DNA–RNA transcription to mature miRNA expression can be modulated, thereby modifying or blocking the final expression. Moreover, FANTOM4 libraries are characterized by a certain level of "transcriptional noise", so we should expect false positives in mature miRNA predictions [23]. Therefore, among these putative NF-kB responsive miRNAs, we selected the "high confidence" hairpins in miRbase [27], retrieving 73 pre-miRNAs (Table S3). A growing body of evidence suggests that mature sequences derived from both arms of the hairpin might be biologically functional and even that the dominant mature sequence can be processed from opposite arms [31,32]. Following the approach of selecting only the "high confidence" miRNA hairpins and filtering the dataset for "Human Expression dataset" [28], 68 "high confidence" expressed miRNAs were identified. This pool of miRNAs, reported in Table 1, constitutes our final set of putative NF-kB responsive miRNAs expressed in healthy tissues linked to inflammatory processes.

2.2. Genomic Features of Putative NF-kB Responsive miRNAs

According to their genomic location, it is possible to distinguish two classes of miRNAs: "intergenic miRNAs" are those located in intergenic regions of the genome, whereas "intragenic miRNAs" are those embedded in introns or exons of annotated genes [23]. Among the latter, "intronic miRNAs" are those located inside the introns of other genes and can either be co-transcribed with their host gene [33] or have an independent promoter [34–36], whereas intergenic miRNAs can derive from a primary miRNA transcript (pri-miRNAs) located in independent gene units [23,37]. Parallelly, it is possible to distinguish different categories of miRNA promoters: "intergenic promoters" are promoters assigned to intergenic miRNAs; "intragenic promoters" are promoters assigned to intragenic miRNAs and include both "host gene promoters" and "intronic promoters"; finally, "hybrid promoters" are those promoters that fall into intergenic regions upstream of intragenic miRNAs and could not be assigned unambiguously to the miRNA [23].

Table 1. The sixty-eight putative NF-kB responsive miRNAs expressed in healthy human tissues linked to inflammatory processes. The following attributes are reported: name of the mature miRNAs which derives from the pre-miRNAs previously identified, type of predicted TSS ("intergenic", "host gene", "intronic", or "hybrid"), names of the healthy "Human Expression dataset" libraries in which the miRNAs are expressed (i.e., "liver" and "immune system"), the chromosome where the miRNA precursor is located, the age of the miRNAs corresponding to the predicted TSSs.

Putative NF-kB Responsive miRNAs *	Mature miRNA Expression ** in Tissues Linked to Inflammatory Processes	Prediction of Promoter Location According to PROmiRNA	miRNA Age	Chromosomes
hsa-miR-101	Immune system	intergenic	v	1
hsa-miR-103	Liver and Immune system	host gene-intronic	v	20
hsa-miR-106b	Immune system	host gene	m	7
hsa-miR-10a	Immune system	host gene	v	17
hsa-miR-132	Immune system	intergenic	m	17
hsa-miR-136	Immune system	intergenic	m	14
hsa-miR-140-3p	Liver and Immune system	host gene	v	16
hsa-miR-140-5p	Liver and Immune system	host gene	v	16
hsa-miR-144	Liver and Immune system	intergenic	v	17
hsa-miR-146a	Immune system	intronic	m	5
hsa-miR-148b	Immune system	host gene	m	12
hsa-miR-150	Immune system	intergenic	m	19
hsa-miR-154	Liver	intergenic	m	14
hsa-miR-155	Immune system	intronic	v	21
hsa-miR-15a	Liver and Immune system	host gene-intronic	v	13
hsa-miR-15b	Liver and Immune system	host gene	v	3
hsa-miR-16	Liver and Immune system	host gene-intronic	v	13 | 3
hsa-miR-17	Liver and Immune system	intronic	v	13
hsa-miR-181b	Immune system	host gene	v	9
hsa-miR-183	Immune system	intergenic	m	7
hsa-miR-186	Liver and Immune system	host gene-intronic	m	1
hsa-miR-188-3p	Immune system	intronic	m	X
hsa-miR-188-5p	Immune system	intronic	m	X
hsa-miR-18a	Immune system	intronic	v	13
hsa-miR-192	Liver and Immune system	intergenic	m	11
hsa-miR-194	Liver and Immune system	host gene-intergenic	m | v	11
hsa-miR-195	Liver	intronic	m	17
hsa-miR-19a	Immune system	intronic	v	13
hsa-miR-19b	Immune system	intronic	m | v	13
hsa-miR-20a	Immune system	intronic	v	13
hsa-miR-221	Immune system	intergenic	v	X
hsa-miR-222	Immune system	intergenic	v	X
hsa-miR-23a	Liver and Immune system	intergenic	m	19

Table 1. *Cont.*

Putative NF-kB Responsive miRNAs *	Mature miRNA Expression ** in Tissues Linked to Inflammatory Processes	Prediction of Promoter Location According to PROmiRNA	miRNA Age	Chromosomes
hsa-miR-25	Liver and Immune system	host gene	m	7
hsa-miR-27a	Liver and Immune system	intergenic	m	19
hsa-miR-296-5p	Immune system	intergenic	m	20
hsa-miR-30c	Liver and Immune system	host gene	v	1
hsa-miR-30e	Liver and Immune system	host gene	v	1
hsa-miR-31	Immune system	intronic	v	9
hsa-miR-32	Immune system	intronic	v	9
hsa-miR-339-3p	Immune system	intronic	m	7
hsa-miR-339-5p	Immune system	intronic	m	7
hsa-miR-33a	Immune system	intronic	m	22
hsa-miR-33b	Immune system	host gene-intronic	m	17
hsa-miR-362-5p	Immune system	intronic	m	X
hsa-miR-374a	Immune system	host gene	m	X
hsa-miR-374b	Immune system	intergenic	m	X
hsa-miR-377	Liver	intergenic	m	14
hsa-miR-424	Liver and Immune system	intergenic	m	X
hsa-miR-450a	Immune system	intergenic	m	X
hsa-miR-491-5p	Immune system	intronic	m	9
hsa-miR-500	Liver	intronic	m	X
hsa-miR-503	Immune system	intergenic	m	X
hsa-miR-532-3p	Immune system	intronic	m	X
hsa-miR-542-5p	Immune system	intergenic	m	X
hsa-miR-545	Immune system	host gene	m	X
hsa-miR-548d-5p	Immune system	intronic	p	8
hsa-miR-570	Immune system	intronic	p	3
hsa-miR-616	Immune system	host gene	p	12
hsa-miR-625	Immune system	intronic	p	14
hsa-miR-629	Immune system	intronic	p	15
hsa-miR-708	Immune system	host gene	m	11
hsa-miR-874	Liver and Immune system	intronic	m	5
hsa-miR-885-5p	Liver	intronic	p	3
hsa-miR-92a	Liver and Immune system	intronic	m	13
hsa-miR-93	Immune system	host gene	m	7
hsa-miR-98	Liver and Immune system	intronic	m	X
hsa-miR-99a	Liver and Immune system	intronic	v	21

Note: miRNAs highlighted in bold are those processed starting from two or more pre-miRNA hairpins, each one transcribed starting from two different promoter types. * In PROmiRNA, NF-kB is among the top 10 TFs with the highest affinity for the 1000 bp-long region surrounding the predicted TSSs. ** Mature miRNAs have been selected based on the "Human Expression dataset" (mirorna.org, accessed on 10 January 2023). This selection allows to review mature miRNA expression patterns across the tissues of interest.

As shown in Table 1, among the promoter locations of the 68 putative NF-kB responsive miRNAs, 19 are "intergenic", 15 are "host gene", and 28 "intronic". Interestingly, miR-15a, miR-16, miR-103, miR-186, and miR-33b can be modulated by both "host gene" and "intronic" promoters, whereas miR-194 is regulated by both "host gene" and "intergenic promoters". Growing evidence indicates that alternative promoters are a mechanism for creating diversity in miRNA transcriptional regulation, as ascertained for protein-coding genes [38].

Regarding the phylogenesis of the 68 putative NF-kB responsive miRNAs, we showed that 22 miRNAs are conserved up to the vertebrate lineage (v), 38 miRNAs are conserved up to the mammal lineage (m), miR-194 and miR-19b up to the mammal and vertebrate lineage, and, finally, only 6 miRNAs are conserved in the primate lineage (p).

2.3. Characterization of the Interplay Linking NF-kB, miRNAs, and Their Host Genes

To better characterize miRNAs that share the promoters of the host gene and to determine whether those host genes are also known to be regulated by NF-kB, multiple assessments were conducted. Firstly, we retrieved available information regarding the host genes and their intragenic miRNAs, as reported in Table 2, whereas expression correlation plots between miRNAs and their host gene are shown in Figure S1.

No experimental evidence was found regarding the host gene of hsa-mir-374a, hsa-mir-545, or hsa-mir-15a. All the others are intronic miRNAs of genes involved in various biological processes ranging from DNA replication to differentiation:

- NFYC (Nuclear transcription factor Y subunit gamma) is a component of the sequence-specific heterotrimeric TF (NF-Y) which specifically recognizes a 5′- CCAAT-3′ box motif found in the promoters of its target genes. NF-Y can function as both an activator and a repressor, depending on its interacting cofactors [39];
- ZRANB2 (Zinc finger Ran-binding domain-containing protein 2) is a splicing factor required for alternative splicing of TRA2B/SFRS10 transcripts. May interfere with constitutive 5′-splice site selection [40];
- IARS2 (Isoleucine-tRNA ligase, mitochondrial) is a nuclear gene encoding mitochondrial isoleucyl-tRNA synthetase on which depends the translation of mitochondrial-encoded proteins [41];
- SMC4 (Structural maintenance of chromosomes protein 4) is the central component of the condensin complex, a complex required for the conversion of interphase chromatin into mitotic-like condense chromosomes [42];
- MCM7 (DNA replication licensing factor MCM7) acts as a component of the MCM2-7 complex (MCM complex) which is the replicative helicase essential for "once per cell cycle" DNA replication initiation and elongation in eukaryotic cells. It is the core component of CDC45-MCM-GINS (CMG) helicase, the molecular machine that unwinds template DNA during replication, and around which the replisome is built [43–48];
- NR6A1 (Nuclear receptor subfamily 6 group A member 1) is an orphan nuclear receptor that binds to a response element containing the sequence 5′-TCAAGGTCA-3′. By similarity, it may be involved in the regulation of gene expression in germ cell development during gametogenesis. It is involved in regulating embryonic stem cell differentiation, reproduction, and neuronal differentiation [49];
- TENM4 (Teneurin-4) is involved in neural development, regulating the establishment of proper connectivity within the nervous system. It plays a role in the establishment of the anterior–posterior axis during gastrulation. Moreover, it regulates the differentiation and cellular process formation of oligodendrocytes and myelination of small-diameter axons in the central nervous system (CNS) [50];
- COPZ1 (Coatomer subunit zeta-1) is a cytosolic protein complex involved in intracellular trafficking, endosome maturation, lipid homeostasis, and autophagy [51,52]. It is associated with iron metabolism through the regulation of transferrin [53,54];

- DDIT3 (DNA damage-inducible transcript 3 protein) is a multifunctional TF in endoplasmic reticulum stress response. It plays an essential role in the response to a wide variety of cell stresses and induces cell cycle arrest and apoptosis [55–57];
- WWP2 (NEDD4-like E3 ubiquitin-protein ligase WWP2) plays an important role in protein ubiquitination and inhibits activation-induced T cell death by catalyzing EGR2 ubiquitination [58]. In human embryonic stem cells, WWP2 promotes the degradation of TF OCT4, which not only plays an essential role in maintaining the pluripotent and self-renewing state of embryonic stem cells but also acts as a cell fate determinant through a gene dosage effect [55];
- HOXB3 (Homeobox protein Hox-B3) is a sequence-specific TF that is part of a developmental regulatory system that provides cells with specific positional identities on the anterior–posterior axis. Therefore, it may regulate gene expression, morphogenesis, and differentiation [59];
- SREBF1 (Sterol regulatory element-binding protein 1) is a precursor of the TF form (Processed sterol regulatory element-binding protein 1), which is embedded in the endoplasmic reticulum membrane [60]. Its processed form is a key TF that regulates the expression of genes involved in cholesterol biosynthesis and lipid homeostasis [60–62];
- PANK2 (Pantothenate kinase 2) is the mitochondrial isoform that catalyzes the phosphorylation of pantothenate to generate 4′-phosphopantothenate in the first and rate-determining step of coenzyme A (CoA) synthesis [63–66]. It is required for angiogenic activity of the umbilical vein of endothelial cells (HUVEC) [67].

Notably, five genes out of thirteen are engaged in transcription regulation (NR6A1, DDIT3, HOXB3, SREBF1, and NFYC), and only three are considered housekeeping genes (NFYC, ZRANB2, and COPZ1).

Experimentally validated interactions shared among the three groups of molecules, namely (i) the 21 NF-kB responsive miRNAs sharing the host gene promoter, (ii) their host genes, and (iii) the three TF members (NFKB1, REL, and RELA) are depicted in Figure 2.

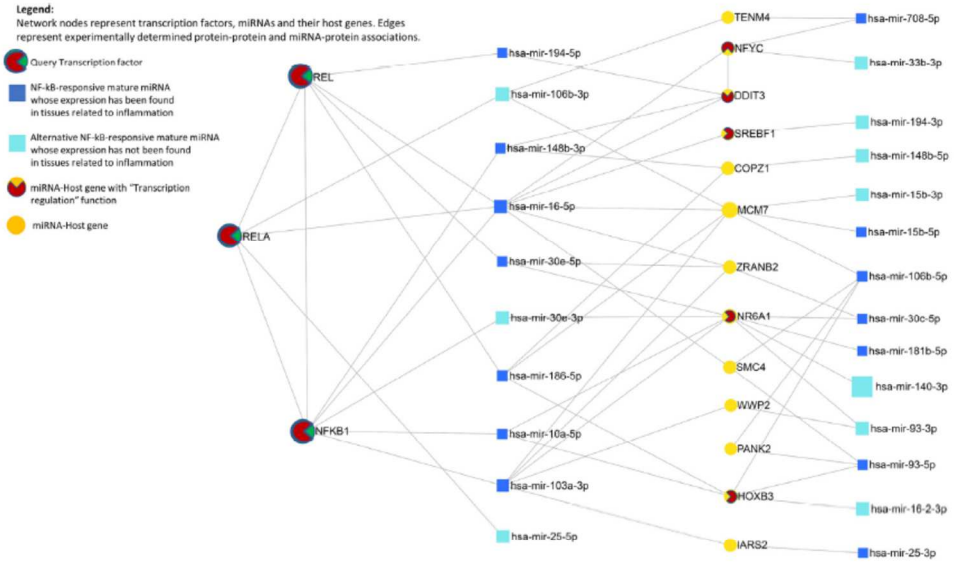

Figure 2. TF–miRNA co-regulatory network from experimentally validated data. In this visualization, a tripartite layout has been chosen. This provides an easy abstraction of relations between different types of molecular entities in complex networks composed of several types of nodes, such as miRNAs, genes, and TFs [68,69].

Table 2. Host genes and intragenic miRNAs information.

miRNA Hairpin	Host Gene Information					Intragenic miRNA Information		
	Ch	Host Gene	Entrez Gene ID	Host Gene Biological Process	Intron n°	Distance from Upstream Exon	Direction	Mature miRNA ID in miRBase 22.1 Release (In Parentheses Previous IDs) §
hsa-mir-30c-1 [H]	1	NFYC	4802	Transcription regulation ‡	4	4038	sense	miR-30c-5p (**miR-30c**); miR-30c-1-3p (miR-30c-1*)
hsa-mir-30e [H]	1	NFYC	4802	Transcription regulation ‡	4	1109	sense	miR-30e-5p (**miR-30e**); miR-30e-3p (miR-30e*)
hsa-mir-186 [H, I]	1	ZRANB2	9406	mRNA processing ‡	8	1560	sense	miR-186-5p (**miR-186**); miR-186-3p (miR-186*)
hsa-mir-194-1 [H, Ig]	1, 11	IARS2	55699	Protein biosynthesis	12	6996	antisense	miR-194-5p (**miR-194**); miR-194-3p (miR-194*)
hsa-mir-15b [H]	3	SMC4	10051	DNA condensation	3	84	sense	miR-15b-5p (**miR-15b**); miR-15b-3p (miR-15b*)
hsa-mir-16-2 [H, I]	3, 13	SMC4	10051	DNA condensation	3	241	sense	miR-16-5p (**miR-16**); miR-16-2-3p (miR-16-2*)
hsa-mir-106b [H]	7	MCM7	4176	DNA replication	13	99	sense	miR-106b-5p (**miR-106b**); miR-106b-3p (miR-106b*)
hsa-mir-25 [H]	7	MCM7	4176	DNA replication	13	530	sense	miR-25-5p (miR-25*); miR-25-3p (**miR-25**)
hsa-mir-93 [H]	7	MCM7	4176	DNA replication	13	326	sense	miR-93-5p (**miR-93**); miR-93-3p (miR-93*)
hsa-mir-181b-2 [H]	9	NR6A1	2649	Transcription regulation	2	139,140	antisense	miR-181b-5p (**miR-181b**); miR-181b-2-3p
hsa-mir-708 [H]	11	TENM4	26011	Differentiation	1	38,400	sense	miR-708-5p (**miR-708**); miR-708-3p (miR-708*)
hsa-mir-148b [H]	12	COPZ1	22818	Protein transport ‡	1	12,035	sense	miR-148b-5p (miR-148b*); miR-148b-3p (**miR-148b**)
hsa-mir-616 [H]	12	DDIT3	1649	Transcription regulation	1	1159	sense	miR-616-5p (miR-616, miR-616*); miR-616-3p (**miR-616**)
hsa-mir-140 [H]	16	WWP2	11060	Ubl conjugation pathway	6	1191	sense	**miR-140-5p** (miR-140); **miR-140-3p**

Table 2. *Cont.*

miRNA Hairpin	Host Gene Information				Intragenic miRNA Information			
	Ch	Host Gene	Entrez Gene ID	Host Gene Biological Process	Intron n°	Distance from Upstream Exon	Direction	Mature miRNA ID in miRBase 22.1 Release (In Parentheses Previous IDs) §
hsa-mir-10a [H]	17	HOXB3	3213	Transcription regulation	1	2202	sense	miR-10a-5p (**miR-10a**); miR-10a-3p (miR-10a*)
hsa-mir-33b [H, I]	17	SREBF1	6720	Transcription regulation	12	314	sense	miR-33b-5p (**miR-33b**); miR-33b-3p (miR-33b*)
hsa-mir-103a-2 [H, I]	20	PANK2	80025	Coenzyme A biosynthesis	5	444	sense	miR-103a-2-5p (miR-103-2*; miR-103a-2*); miR-103a-3p (**miR-103**, miR-103a)

Note: Promoter location according to PROmiRNA: [H] host gene; [I] intronic; [Ig] intergenic. Ch: chromosome number; in bold, the locus of interest if more than one is indicated. ‡ Genes classified as housekeeping gene in Housekeeping and Reference Transcript Atlas. § In the last column, mature miRNAs nomenclature has been harmonized throughout miRBase database; miRNA nomenclature used in Table 1 has been highlighted in bold.

Important nodes can be identified on the basis of their node centrality measures, such as degree and betweenness. The degree of a node is the total number of connections to other nodes. High-degree nodes are considered important "hubs" in a network [70,71]. The betweenness measures the number of shortest paths going through a node, taking into consideration the global network structure. Nodes with higher betweenness are important "bottlenecks" in a network [70,71]. Nodes identified by NFKB1, REL, miR-16-5p, miR-103a-3p, and NR6A1 have high degree centrality values, whereas RELA, miR-10a-5p, and miR-30e-5p represent nodes that occur between two dense clusters and have a high betweenness centrality even if their degree centrality values are not high.

Therefore, we performed an explorative evaluation of known and potential protein–protein interactions among REL, RELA, NFKB1, and miRNA-host genes (Figure 3) by querying the STRING Database [72–74].

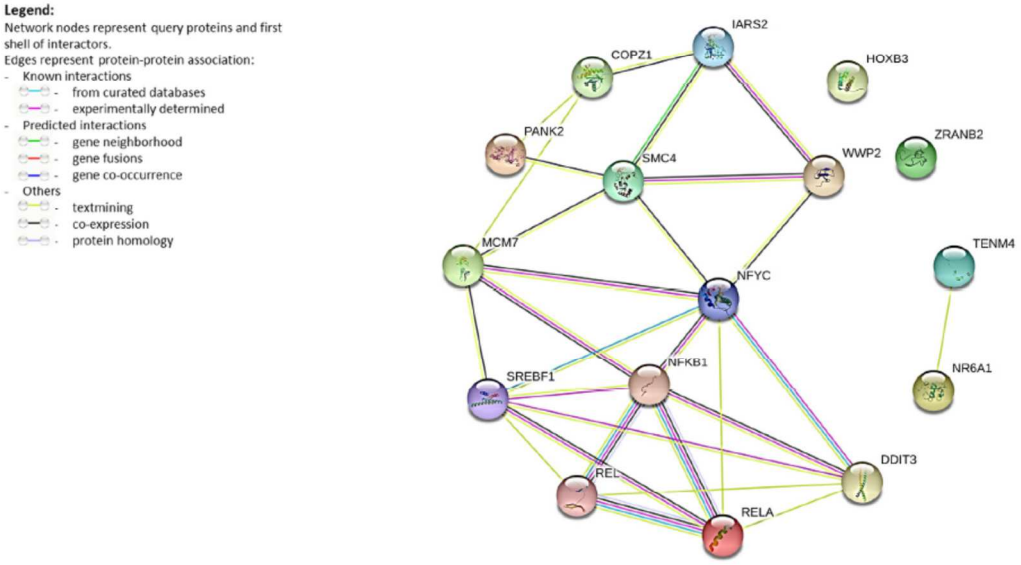

Figure 3. Protein–protein interaction network. Network nodes represent proteins: splice isoforms or post-translational modifications are collapsed, i.e., each node represents all the proteins produced by a single, protein-coding gene locus. Edges represent protein–protein associations and are meant to be specific and meaningful, i.e., proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding to each other [72–74]. The greater the number of edges shared between two nodes, the greater the confidence of the interaction score. The line color indicates the type of interaction evidence.

The STRING network shows that almost all host gene proteins have some degree of interaction. Experimental and biochemical data confirm the functional association of NFKB1, REL, and RELA. On the other hand, the higher confidence interaction values suggest a functional link between DDIT3, NFYC, MCM7, and SREBF1, as well as between IARS2, SMC4, and WWP2. Of note, experimental evidence in Figure 2 indicated that NFBK1, REL, RELA, DDIT3, NFYC, MCM7, SREBF1, and SMC4 are all targets of miR-16-5p, but miR-103a-3p, in turn, regulates IARS2, MCM7, and WWP2.

Finally, the significantly differentially expressed host genes in ARDs have been identified (Table 3). Worth a mention is the downregulation of DDIT3, SMC4, and TENM4 in replicative senescence of human fibroblasts; the upregulation of SMC4 and MCM7 after vitamin C treatment; the upregulation of HOXB3 and TENM4 in Alzheimer's disease; and the deregulation of DDIT3 and SMC4 in COVID-19 disease.

Table 3. Differentially expressed miRNA-host genes in age-related diseases.

Differentially Expressed Gene	Cell/Tissue	Treatment/Condition	log2 Fold Change	*p*-Value	*q*-Value	DOI
DDIT3	Human diploid fibroblasts IMR90	Replicative senescence	−1.09474	1.55×10^{-23}	2.63×10^{-22}	10.1093/nar/gkz555
DDIT3	Lung	COVID-19 vs. Control	3.04617	1.11×10^{-17}	2.55×10^{-16}	10.1038/s41556-021-00796-6
HOXB3	Human induced pluripotent stem (iPS) cell-derived neural progenitor cells	Alzheimer's disease	1.30000	5.10×10^{-4}	3.40×10^{-2}	10.1016/j.celrep.2019.01.023
MCM7	Human arterial endothelial cell	Ionizing radiation	−1.07367	2.66×10^{-33}	1.94×10^{-31}	10.1093/nar/gkz555
MCM7	Human diploid fibroblasts WI38	Ionizing radiation	−1.60665	1.53×10^{-10}	5.99×10^{-9}	10.1093/nar/gkz555
MCM7	WRN-/- mesenchymal stem cell	Vitamin C	1.90980	5.08×10^{-51}		10.1007/s13238-016-0278-1
SMC4	Human arterial endothelial cell	Ionizing radiation	−1.14304	5.14×10^{-38}	4.56×10^{-36}	10.1093/nar/gkz555
SMC4	Human diploid fibroblasts WI38	Ionizing radiation	−2.31474	1.24×10^{-14}	8.41×10^{-13}	10.1093/nar/gkz555
SMC4	Lung	COVID19 vs. Control	−2.85324	4.44×10^{-10}	3.79×10^{-9}	10.1038/s41556-021-00796-6
SMC4	Human diploid fibroblasts WI38	Replicative senescence	−1.44078	8.14×10^{-7}	1.75×10^{-5}	10.1093/nar/gkz555
SMC4	WRN-/- mesenchymal stem cell	Vitamin C	2.18610	4.24×10^{-55}		10.1007/s13238-016-0278-1
TENM4	Human diploid fibroblasts WI38	Replicative senescence	−4.84098		1.97×10^{-52}	10.1093/nar/gkz555
TENM4	Human diploid fibroblasts IMR90	Ionizing radiation	−1.63819	3.26×10^{-23}	5.65×10^{-22}	10.1093/nar/gkz555
TENM4	Human diploid fibroblasts WI38	Ionizing radiation	1.82630	6.17×10^{-12}	3.09×10^{-10}	10.1093/nar/gkz555
TENM4	Human diploid fibroblasts IMR90	Replicative senescence	−1.03845	1.66×10^{-10}	1.24×10^{-9}	10.1093/nar/gkz555
TENM4	Human induced pluripotent stem (iPS) cell-derived neurons	Alzheimer's disease	1.70000	5.50×10^{-4}	1.70×10^{-2}	10.1016/j.celrep.2019.01.023

2.4. Pathways Targeted by the 68 Putative NF-kB Responsive miRNAs

By performing an Ingenuity Pathway Analysis (IPA) Target Filter Analysis, we identified mRNAs targeted by the putative NF-kB responsive miRNAs. A total of 18,095 mRNAs were retrieved, of which 9613 were experimentally observed or highly predicted. The significance was reported as *p*-value in Table S4. The let-7a-5p was the miRNAs with the highest associated number of mRNA targets (2014 targets).

Then we performed a network analysis focusing on putative NF-kB responsive miRNAs targeting mRNAs coding for molecules belonging to the NF-kB pathways (Figure 4).

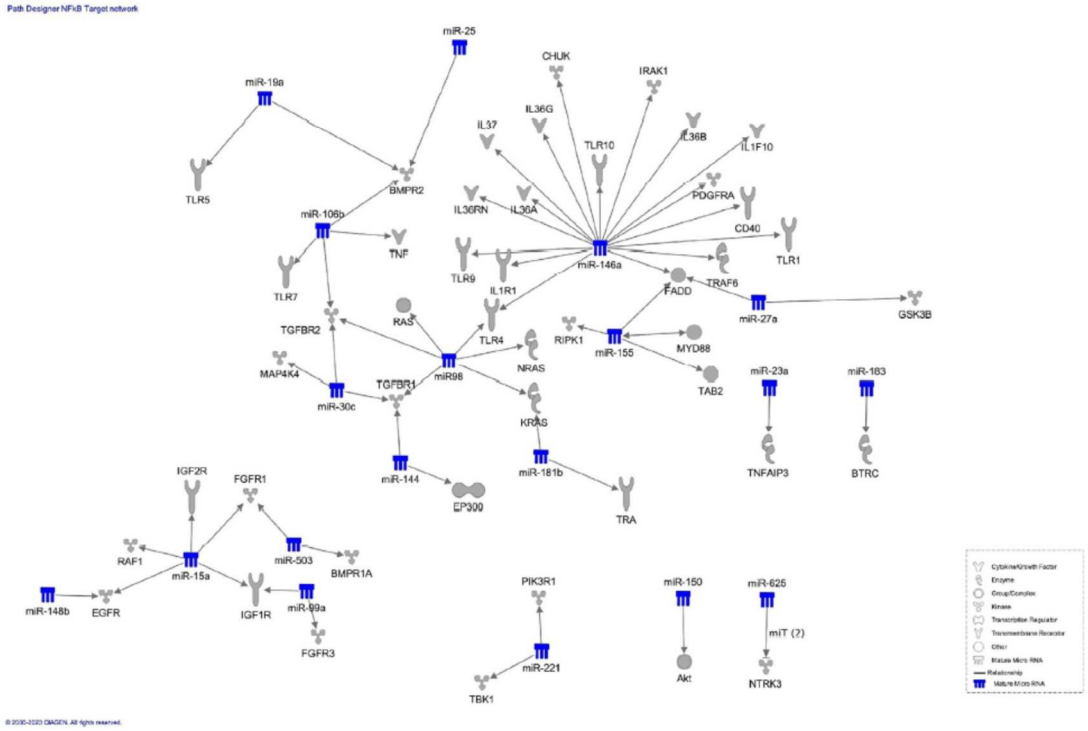

Figure 4. IPA Path Designer NF-kB target network. Molecules belonging to NF-kB pathway targeted by NF-kB responsive miRNAs. © 2000–2023 QIAGEN.

Interestingly, the NF-kB responsive miRNAs do not directly target genes coding for the NF-kB different subunits, but most of them are able to target genes coding for molecules belonging to NF-kB activation pathways, such as TLR and MYD88. This result is very interesting, considering that the modulation of NF-kB biological activity is related to its activation, rather than to the modulation of NF-kB subunits expression.

Further, to discover the main diseases and functions associated with the selected miRNAs dataset, we performed an IPA Core Analysis (Figure 5). The diseases and functions are shown by bar chart, sorted by their −log *p*-value (*p*-value from Fisher's Exact test).

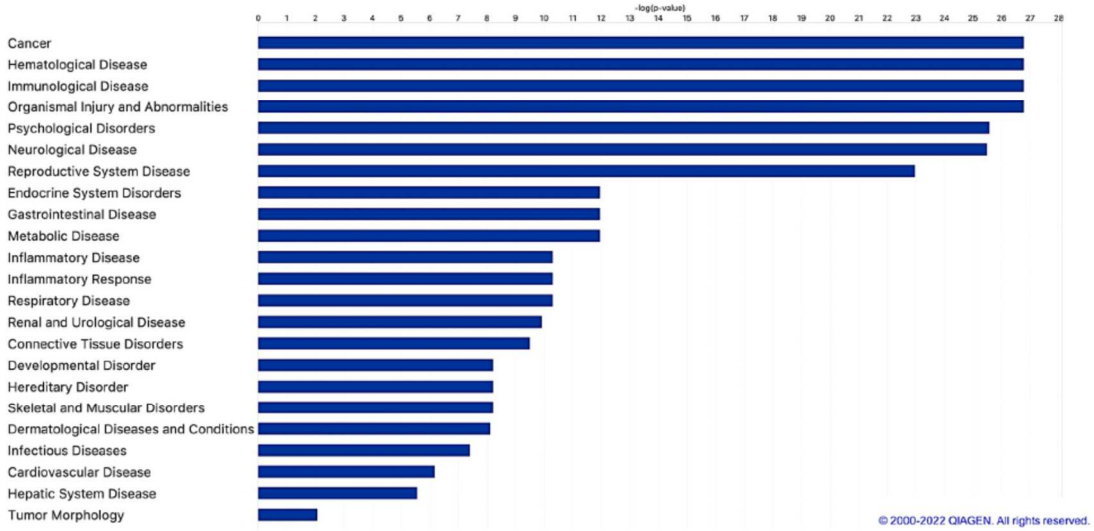

Figure 5. Most relevant human diseases associated with molecular pathways targeted by putative NF-kB responsive miRNAs. The diseases and functions associated with molecular pathways targeted by putative NF-kB responsive miRNAs are shown by the bar chart, sorted by their −log *p*-value (Fisher's Exact test *p*-value). A total of 23 relevant human diseases are listed. © 2000–2023 QIAGEN.

Cancers, immunological diseases, neurological diseases, and metabolic diseases, all well-recognized as inflammatory-based diseases, are among the diseases associated with the highest probability with NF-kB responsive miRNAs. Focusing on metabolic diseases, the most affected diseases are the non-insulin dependent diabetes mellitus (−log *p*-value 11.955), Alzheimer disease (−log *p*-value 9.532), and diabetes mellitus (−log *p*-value 7.680).

To better explain the association of identified NF-kB putative responsive miRNAs with these human diseases, we depicted miRNAs-diseases relationship in Figure 6. Figure 6A depicts NF-KB putative responsive miRNAs associated with metabolic diseases, whereas Figure 6B–D, show the association between identified NF-kB responsive miRNAs and cardiovascular diseases, neurological diseases, and cancer, respectively.

2.5. The 68 Putative NF-kB Responsive miRNAs and Previously Identified Inflammamirs

To test whether the 68 putative NF-kB responsive miRNAs could have a biological value in the context of the previous evidence, we compared our results with those already present in the literature. Among these 68 miRNAs, 21 have been experimentally validated to be transcribed by NF-kB1: miR-16-2 [75], miR-10a [76], miR-140-3p, miR-140-5p [77], miR-148b [78], miR-15b [79], miR-186 [80], miR-146a, miR-155, miR-19b, miR-20a, miR-19a, miR-17, miR-221, miR-222, miR-18a, miR-92a, miR-101, miR-23a, miR-27a, and miR-30c [21].

In addition, we have chosen as a reference all available data on the miRNAs relevant to aging, inflammation, and immunity that can be referred as inflammamiRs [81]. A detailed comparison table has been provided in Table S5. Figure 7A shows the "word cloud" with the 68 "high confidence" expressed miRNAs. The more features a specific miRNA holds (such as: the number of promoter types, the number of miRNA precursors, if it is expressed in more than one tissue, and, finally, if it is known to target NF-kB), the bigger and bolder it appears in the figure. Figure 7B depicts a Venn diagram modified from [81], displaying the miRNAs related to inflammation, immunity, and aging based on their circulating shuttles.

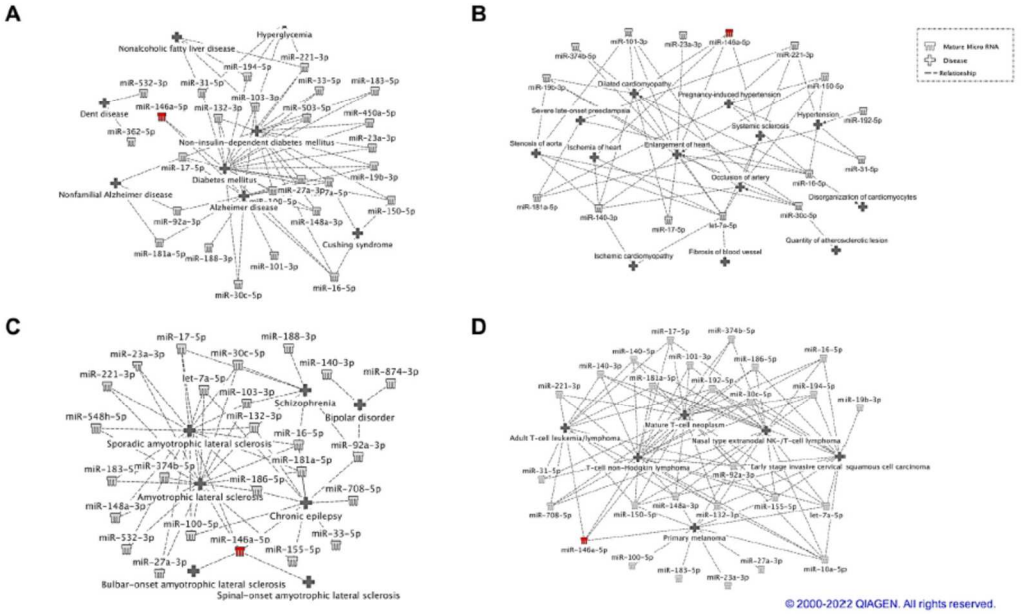

Figure 6. IPA diseases networks. Diseases networks targeted by NF-kB responsive miRNAs. Metabolic disease (panel (A)), cardiovascular diseases (panel (B)), neurological diseases (panel (C)), and cancer (panel (D)) © 2000–2023 QIAGEN.

In the inner circles are grouped exosome-associated miRNAs, while, in the outer circles, the circulating miRNAs associated with Ago-2, HDL, or other microparticles are grouped. In this version, it is important to note that bold characters indicate miRNAs overlapping among the two groups. Most of the 68 high-confidence NF-kB responsive miRNAs (reported in panel A) were previously identified as circulating miRNAs associated with aging, immunological functions, and inflammation, i.e., inflammaging [81]. Only three miRNAs, such as miR-154, miR-377, and miR-885-5p, were not retrieved in previous analysis [81]. However, based on recent literature, all of them are related to NF-kB/inflammation pathways [82–84]. All of the 68 NF-kB responsive miRNAs are therefore included in the Venn diagram reported in panel B, highlighting that these miRNAs identified as tissues expressed miRNAs are also detectable in blood, and most of them were identified inside extracellular vesicles, i.e., exosomes (miRNAs depicted in inner circles Figure 7B).

2.6. mRNAs Targeted by the 68 Putative NF-kB Responsive miRNAs Belonging to Pathways Involved in Aging Process and/or Age-Related Diseases

By further analyzing the IPA Target Filter Analysis results, we finally identified the mRNAs, either experimentally validated or highly predicted, to be targeted by the 68 putative NF-kB responsive miRNAs, belonging to pathways related to aging or to the most common ARDs. Among the 9613 mRNAs predicted to be targeted by such NF-kB responsive miRNAs, 189 mRNAs targeted by 46 out of 68 miRNAs were associated to "cellular senescence pathway" (Table S6). In addition, out of the 9613, 8599 mRNAs were related to diseases reported in Figure 6, such as metabolic diseases, cardiovascular diseases, neurological diseases, and cancer. All these conditions share an inflammatory etiopathogenesis and are prototypical ARD.

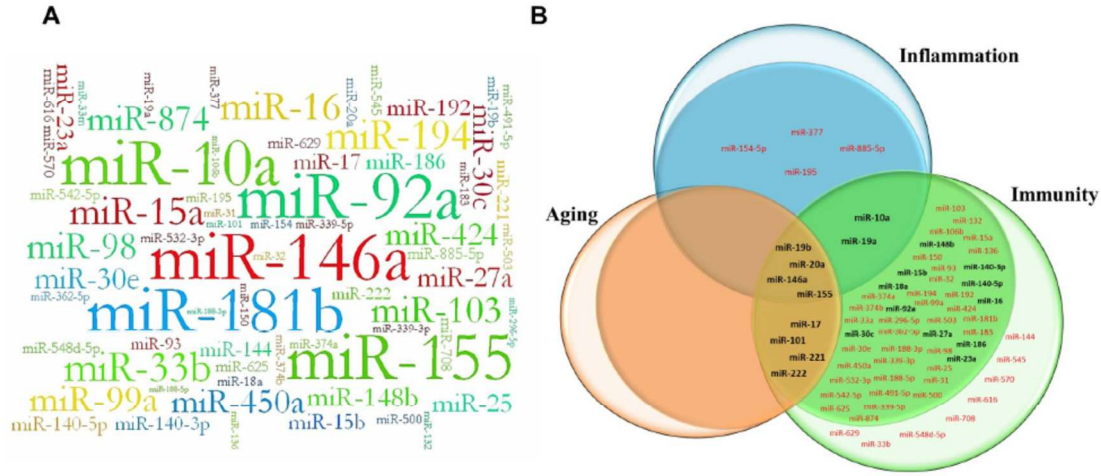

Figure 7. (**A**) Word cloud of the 68 putative NF-kB responsive miRNAs. The "word cloud" has been used to highlight the values of a miRNA based on its characteristics (such as: the number of promoter types, the number of miRNA precursors, if it is expressed in more than one tissue, and, finally, if it is known to target NF-kB). The more features a specific miRNA holds, the bigger and bolder it appears in the "word cloud". This word cloud has been drawn using Wordaizer version 6.0 APP Helmond (www.apphelmond.com, accessed on 10 January 2023). (**B**) Venn diagram showing the NF-kB and inflammamiRs research in context. Modified version of the Venn diagram from [81]. The Venn diagram displays the 68 NF-kB responsive miRNAs related to inflammation, immunity, and aging based on their circulating shuttles. In bold, the 21 experimentally validated miRNAs; in red, the 47 not yet experimentally validated miRNAs. In the inner circles are grouped exosome-associated miRNAs, while in the outer circles are the circulating miRNAs associated with Ago-2, HDL, or other microparticles.

3. Discussion

NF-kB is an ubiquitously and evolutionarily conserved TF activated by a plethora of external and internal proinflammatory stimuli [85–87]. The crucial role as a mediator of the inflammatory responses, together with the finding that the activation or inhibition of NF-kB can induce or reverse, respectively, the main features of aged organisms, has brought NF-kB under consideration as a key TF that drives the biological aging process [88]. In this framework, the identification of genes modulated by NF-kB can be considered a cutting-edge issue [89–91].

NF-kB-responsive genes were extensively investigated, whereas NF-kB-responsive genes for non-coding RNAs were only recently highlighted.

Here, we demonstrated that, by applying a data-mining approach, it is possible to select the most reliable NF-kB responsive miRNAs. Most notably, the availability of data on TFs binding sites on human miRNAs sequences constituted a starting point and the foundation for studying all human miRNAs with potential NF-kB binding sites in their promoter regions.

Some years ago, a general hypothesis was advanced that the aging process and the development of the most common ARDs could be fostered by a low-grade, chronic, systemic inflammatory process named "inflammaging" [92]. Inflammaging, which is principally sustained by the activation of the innate immune cells, is paralleled by the increased burden of senescent cells acquiring a senescence-associated secretory phenotype (SASP), which turns senescent cells into proinflammatory cells [86,93–96]. In immune cells and tissues obtained from patients affected by the most common ARDs, NF-kB is commonly constitutively activated [97]. Of note, NF-kB activation should be an inducible,

but transient, event in physiological conditions. However, despite the presence of multiple checks and balances that control NF-kB activation, in cellular and organismal aging, as well as in many ARDs, NF-kB activation becomes persistent [98,99].

In this study, using PROmiRNA software and a data-mining approach, we provide a list of 73 putative "high confidence" pre-miRNAs sequences corresponding to 68 NF-kB responsive mature miRNAs sequences.

Likewise, we highlighted the presence of distinct types of promoters that can regulate NF-kB responsive miRNAs.

A total of 33 miRNAs of the 68 high confidence expressed miRNAs identified have an "intronic" promoter, and 5 of these have both an "intronic" and "host gene" promoter, whereas only one miRNA (miR-194) shares both "intergenic" and "host-gene" promoters. Alternative promoters are a common mechanism to create diversity in the transcriptional regulation of miRNA [38].

It has been demonstrated that "intronic" promoters convey an additional degree of freedom over intragenic miRNA transcriptional regulation by virtue of some peculiar characteristics, thus allowing the modulation of miRNA expression levels in a tissue- and condition-specific manner [23]. Besides the other features, in this context, it is important to stress that:

1. "Intronic" promoters can explain cases of poor correlation between host gene and miRNA expression, functioning as a real alternative promoter [23]. As shown in Figure S1, the expression levels of NF-kB-miRNAs modulated by both "host gene" and "intronic" promoters (i.e., miR-16, miR-103, miR-186, and miR-33b) or by both "host gene" and "intergenic promoters" (i.e., miR-194) are not correlated with the expression levels of their host gene, whereas most of the miRNAs that share the host gene promoters are characterized by directly (e.g., miR-15b) or inversely (e.g., miR-30c, miR-616, and miR-93) correlated transcription levels.
2. "Intronic" promoters are expressed in a tissue-specific manner, but "host gene" promoters are considered primarily for housekeeping gene regulation [23]. Housekeeping genes are required for the maintenance of essential functions of any cell type, so they are expected to be constitutively expressed in all cells and at any development stage [100]. Among the NF-kB-miRNA host genes, COPZ1, NFYC, and ZRANB2 have been cataloged as housekeeping genes (Table 2).
3. "Intronic" promoters are mainly triggered by tissue-specific master regulator TFs, instead of TFs of "host gene" promoters, which broadly overlap with those of protein coding genes and can be considered mainly for housekeeping ("intergenic" promoters are regulated by a combination of intronic-specific and host-gene specific TFs). This suggests a different evolutionary mechanism [23]. In this study, the expression levels of the three housekeeping host genes (COPZ1, ZRANB2, and NFYC) and their miRNAs (miR-148b-3p, miR-186-5p, and, lastly, miR-30c-5p and miR-30e-5p, respectively) are mainly inversely correlated or not showing clear correlation trends (Figure S1).
4. "Intronic" miRNA promoters are less evolutionarily conserved than either "intergenic" or "host gene" promoters [23].
5. Conversely, evolutionarily conserved miRNAs are more likely to be regulated by an "intronic" promoter [23].
6. Moreover, those intragenic miRNAs that share the promoters of the host gene interact with their own host genes (miR-16-2::MSC4; miR-106b::MCM7, miR-181b-2::NR6A1, miR-708::TENM4, miR-148b::COPZ1, and miR-10a::HOXB3), but also with the other functionally related host genes, creating a complex regulatory mechanism (Figure 2). NFKB1, REL, miR-16-5p, miR-103a-3p, and NR6A1 are the most important hub nodes in the network, whereas miR-10a-5p connects the hub nodes identified by NFKB1, NR6A1, and HOXB3, and miR-30e-5p connects REL, NR6A1, and ZRAMB2 hubs. Interestingly, in the network, it is possible to identify a clear TF-miRNA feed-forward loop involving DDIT3, miR-16-5p, and NFYC. In a TF-miRNA feed-forward loop, TF and miRNA co-regulate the target genes: in a "coherent" feed-forward loop, the

TF and miRNA have the same effects on their common targets, whereas, in an "incoherent" feed-forward loop, the TF and miRNA carry out opposing effects, which precisely fine-tune gene expressions to minimize noise and maintain stability [68,101]. TF-miRNA feed-forward loops have a specific function in noise buffering effects, which can minimize the response of stochastic signaling noise to maintain steady-state target levels [102,103]. Disruption of feed-forward loops could lead to serious dysregulations at the origin of diseases and cancers, e.g., interference in the NF-kB/miR-19/CYLD loop can induce T cell leukemogenesis [103,104]. Therefore, investigating the regulatory motifs among DDIT3, 16-5p, and NFYC could provide valuable insights to dissect the molecular mechanisms underlying biological processes and diseases triggered by NF-kB constitutive activation.

Protein–protein interaction analysis of protein-coding host genes revealed that most of them could be functionally related (Figure 3). Beyond the well-known functional association of NFKB1, REL (cREL), and RELA, some data have highlighted the association with endoplasmic reticulum stress, providing opportunities to fine-tune cellular stress responses [105]. In the framework of atherosclerosis, multiple links between NF-kB and ER stress were suggested. A disturbed flow can cause endoplasmic reticulum stress, leading to SREBF1 activation with nuclear localization and to DDIT3 expression triggered by endoplasmic reticulum stress response elements [106]. NFYC is a subunit of a trimeric complex (NFY) known to interact with several TFs to enable the synergistic activation of specific classes of promoters. NFY directly controls the expression of TF genes such as P53 (DNA-damage), XBP1, CHOP/DDIT3 (endoplasmic reticulum stress), and HSF1 (heat shock) [107,108]. Of note, experimental data have shown the upregulation of both SMC4 and MCM7 in mesenchymal stem cells after vitamin C treatment; the downregulation of DDIT3, SMC4, and TENM4 in replicative senescence of human fibroblasts; the upregulation of HOXB3 and TENM4 in Alzheimer's disease; and, finally, the deregulation of DDIT3 and SMC4 in COVID-19 disease (Table 3).

In this scenario, targeting NF-kB signaling is becoming a promising strategy for drug development and ARD treatments [91,109].

Almost all of the 68 miRNAs that we identified in our current analysis were previously associated with inflammaging processes and with the most common ARDs, such as metabolic diseases, cardiovascular diseases, neurodegenerative diseases, and cancers [110,111].

Out of the 9613 mRNAs targeted by the 68 NF-kB responsive miRNAs, 8599 mRNAs were related to such diseases. Of note, 189 mRNAs were associated with "cellular senescence pathway", which is recognized as the main culprit of the aging process.

Most of the NF-kB responsive miRNAs are involved in a negative feedback loop to restrain exacerbated inflammation [5,53,65–70].

Notably, the identified NF-kB responsive miRNAs are not able to directly modulate gene expression of NF-kB subunits but are able to target molecules belonging to NF-kB activation pathways (canonical and non-canonical pathway). Interestingly, among the NF-kB-responsive miRNAs genes identified with our approach, the most relevant examples of mRNAs that can target molecules belonging to the NF-kB canonical and non-canonical pathways, or related molecules, are miR-146a and miR-155. In fact, miR-146a and miR-155, control NF-kB activity during inflammation by a combinatory action without directly targeting NF-KB subunits [112]. miR-155 is rapidly upregulated by NF-kB during the early phase inflammatory response through a positive feedback loop necessary for signal amplification. miR-146a is rather gradually upregulated by NF-kB and forms a negative feedback loop attenuating NF-kB activity in the late phase of inflammation. The combined action of these two positive (NF-kB::miR-155) and negative (NF-kB::miR-146a) NF-kB-miRNA regulatory loops provides optimal NF-kB activity during inflammatory stimuli, and eventually lead to the resolution of the inflammatory response in physiological condition.

Another example is miR-16 that targets the IKKα/β complex of the NF-kB canonical pathway polarizing macrophages toward an M2 phenotype [113].These results are in line

with the known modulation of NF-kB biological activity, based on the activation and not on the expression of its subunits [5].

Interestingly, all the 68 NF-kB responsive miRNAs are detectable in blood, and most of them were identified inside extracellular vesicles, i.e., exosomes. Exosomes are currently considered to be a crucial intercellular cross-talk mechanism, acting at both the paracrine and systemic levels [114]. This result highlights the complexity of the feed-back loops between NF-kB activation in specific tissues, the expression of NF-kB responsive miRNAs, and their release in the bloodstream as a systemic intercellular communication mechanism. A further level of complexity can be envisaged considering that NF-kB is known to indirectly regulate miRNA expression through the modulation of other TFs. NF-kB can modulate AP-1 TF [115], which, in turn, is able to modulate different miRNAs genes, i.e., miR-21 [116].

Of note, among the 68 miRNAs, 21 were already experimentally identified as NF-kB responsive, reinforcing the reliability of our results. Our data also highlight the potential value of the 47 NF-kB putative responsive miRNAs (listed in Figure 7B) that are yet to be experimentally validated.

Overall, our results are of interest in the framework of the research on the biomarkers/drugs of aging and inflammation related diseases. If NF-kB responsive miRNAs are hyper-transcribed in tissues involved in the modulation of inflammatory responses, the hypothesis that circulating miRNAs could be useful tools to track the trajectories of healthy or un-healthy aging is reinforced [117–121] and possible therapeutic strategies based on the inhibition of those miRNAs could be further tested.

Limitation of the Study

The data-mining process frequently encompasses a further phase involving the extraction of implicit relational patterns through traditional statistics or machine learning, but the particularity of the research question and the type of data available have been a hindrance to this kind of analysis.

4. Methods

4.1. Data Mining Process

In the field of Knowledge Discovery in Databases (KDD), a data-mining approach is used to extract meaningful information and to develop significant relationships among variables stored in large data sets [122]. In this study, we have mined and integrated data from multiple databases to select NF-kB responsive miRNAs, and the process has been tailored based on the research question. Four main steps can be distinguished:

4.1.1. Database Selection

The following data sources have been investigated to retrieve the data and develop the study: PROmiRNA [23], FANTOM4 libraries [26], "High confidence human hairpins" in miRBase [27], and "Human Expression" dataset (microrna.org) [28].

- PROmiRNA provides an interesting approach for miRNA promoter annotation based on a semi-supervised statistical model trained on deepCAGE data and sequence features [23]. It was used to identify all human miRNAs potentially modulated by NF-kB, i.e., "NF-kappaB", "NFKB1", "REL", and "RELA".
- FANTOM4 libraries, generated by the FANTOM4 project [26], collects a wide range of genome-scale data from several tissues. The analysis of FANTOM4 libraries retrieved those miRNAs showing "expression at the promoter level" in different human tissues. The following libraries from healthy tissues were selected: "blood", "bone marrow", "immune system cells", "liver", "monocytic-cells", "T cells", and "T cells 2".
- miRBase database is the public repository for all published miRNA sequences and associated annotations [27,32,123–126]. The "High confidence human hairpins" dataset [27] was downloaded to identify all human miRBase entries with high confidence levels assessed using the deep sequencing data sets collated in miRbase (The original

dataset is provided in Supplementary File S1, and it has been downloaded from this link: https://www.mirbase.org/blog/2014/07/high-confidence-mirna-set-available-for-mirbase-21/, accessed on 10 January 2023).

- Finally, microRNA.org [28], a comprehensive resource of miRNA target predictions and expression profiles, was searched to extract the "Human Expression dataset", meaning the mature miRNA expression profiles in various tissues as presented by Landgraf et al. [127]. Expressed miRNAs from the following healthy tissues were selected (library names for each sample type are indicated in brackets): liver (hsa_Liver), pluripotent hematopoietic stem cell (hsa_HSC-CD34), B cells from peripheral blood (hsa_B-cell-CD19, hsa_B-cell-CD19-2, hsa_B-cell-CD19-pool), T-lymphocytes (hsa_T-cell-CD4, hsa_T-cell-CD4-2, hsa_T-cell-CD4-effector, hsa_T-cell-CD4-memory, hsa_T-cell-CD4-naïve, hsa_T-cell-CD8, hsa_T-cell-CD8-2→hsa_T-cell-CD8-naïve), NK cells (hsa_NK-CD56), monocytes (hsa_Monocytes-CD14), granulocytes (hsa_Granulocytes-CD15), and Dendritic cells (hsa_DC-unstim). Libraries from cell lines, tumor samples, genetic disorders, and so on, have been discharged. (The original dataset is provided in Supplementary File S2, and it has been downloaded from this link: http://www.microrna.org/microrna/getDownloads.do, accessed on 10 January 2023).

4.1.2. Data Extraction and Integration

This phase includes downloading, extracting, filtering, and combining the data from the databases previously identified. The integration of multiple datasets has been possible through the following steps.

4.1.3. Data Cleaning and Transformation

Because the data originates from multiple sources, the integration often involves converting data formats, cleaning, removal of incorrect data, generating new variables, resolving redundancy, and checking against miRNA nomenclature consistency, both between miRNAs names originating in different miRBase versions and between the names of pri-miRNAs and the mature forms. This issue has been manually curated by comparing miRNA names in miRBase database version 21.

4.1.4. Assessment of the Results

This is the final stage of a KDD process, involving the translation of aggregated data into comprehensible knowledge. The validity and reliability of the data were tested by comparing the results obtained in the data-mining process with those already published in the literature.

The whole data-mining process is illustrated in the data flow diagram in Figure 1. Data obtained at each intermediate step are provided in Supplementary Tables S1–S3. The final miRNA-pool is reported in Table 1.

4.2. Bioinformatic Evaluations

4.2.1. Evaluation of miRNA-Host Gene-Transcription Factor Interactions

Host gene and intragenic miRNAs information (Table 2), as well as expression correlation data between miRNAs and their host genes (Figure S1), were retrieved from MiRIAD, a database integrating miRNA inter- and intragenic data (https://www.miriad-database.org/, accessed on 10 January 2023) [128]. In Table 2, host gene biological process were obtained from the UniProt database (Release 2022_05) (https://www.uniprot.org/, accessed on 10 January 2023) [129]; the Housekeeping and Reference Transcript Atlas (HRT Atlas v1.0) (https://housekeeping.unicamp.br/, accessed on 10 January 2023) [100] was investigated to discover those host genes cataloged as housekeeping genes.

Experimentally validated interactions shared among NFKB1, REL, RELA, the NF-kB-responsive miRNAs sharing the host gene promoter, and their host genes, were identified (Figure 2) by querying: (i) DIANA-TarBase v8 (http://www.microrna.gr/tarbase, accessed on 10 January 2023), retrieving experimentally supported miRNA-gene interactions [130];

(ii) TRRUST v2 (www.grnpedia.org/trrust, accessed on 10 January 2023), a manually curated database of transcriptional regulatory interactions [131]; and (iii) STRING v10, to highlight the protein–protein interactions, with the constraint to retrieve only experimental evidences [132]. The whole process, including the final network creation and visualization, was handled using miRNet (version 2.0), a miRNA-centric network visual analytics platform (https://www.mirnet.ca/, accessed on 10 January 2023) [68,70,133].

STRING database Version 11.5 (https://string-db.org/, accessed on 10 January 2023) was used to discover known and potential interactions among REL, RELA, NFKB1, and miRNA-host gene proteins (Figure 3). STRING is a database of predicted and known protein–protein interactions. The interactions include direct (physical) and indirect (functional) associations; these stem from knowledge transfer between organisms, from interactions aggregated from other (primary) databases, and from computational prediction [72–74]. The network was created by setting a minimum required interaction score of 0.15.

The RNA-seq datasets in Aging Atlas (https://ngdc.cncb.ac.cn/aging/index, accessed on 10 January 2023) were examined to explore age-related changes in host gene expression [134]. Table 3 shows differentially expressed host genes in strictly age-related conditions; only those genes showing $|\log2FC| > 1$ and q-value < 0.005 (or p-value < 0.005 if q-value was not provided) have been reported. Data relative to particular experimental conditions (e.g., gene knockdown) have not been reported. All websites and online tools were accessed in the period between January and February 2023.

4.2.2. Ingenuity Pathway Analysis

Bioinformatic evaluations (networks and disease analyses) were performed by the Ingenuity Pathway Analysis software (Qiagen, Hilden, Germany). The putative NF-kB responsive miRNAs identified through the data-mining process were analyzed to explore the experimentally observed or high predicted mRNA targets via the microRNA Target Filter Analysis.

Furthermore, an IPA Core Analysis was performed to define the associated diseases and functions. Direct and indirect relationships from the Ingenuity Knowledge Base (gene only) datasets were considered. We filtered only molecules and/or relationships experimentally observed in any tissue from human, rat, or mouse. Across the observations, 51 miRNAs were ready to be analyzed (Table S4) [135]. All the networks, diseases, and biological functions were assessed using IPA software (Qiagen, Hilden, Germany).

5. Conclusions

Here, we demonstrated that a well-settled data-mining approach may disclose the most reliable miRNAs having a key role in the regulation of specific pathways of interest. Deciphering the crosstalk between miRNAs and NF-kB is one of the major topics to be investigated to understand the complex derailment of several metabolic pathways in normal and pathological aging. Future studies are needed to confirm that the identification of such miRNAs is of diagnostic/prognostic/therapeutic relevance for the most common inflammatory- and age-related conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24065123/s1. Reference [136] is cited in the Supplementary Materials.

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