Published in final edited form as:

Wiley Interdiscip Rev RNA. 2017 March ; 8(2): . doi:10.1002/wrna.1385.

Extracellular RNA in Aging

Douglas F. Dluzen¹, Nicole Noren Hooten¹, and Michele K. Evans^{1,*}

¹Laboratory of Epidemiology and Population Sciences; National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA

Abstract

Since the discovery of extracellular RNA (exRNA) in circulation and other bodily fluids, there has been considerable effort to catalog and assess whether exRNAs can be used as markers for health and disease. A variety of exRNA species have been identified including mRNA, and non-coding RNA (ncRNA) such as microRNA (miRNA), small nucleolar RNA (snRNA), transfer RNA (tRNA), and long non-coding RNA (lncRNA). Age-related changes in exRNA abundance have been observed, and it is likely some of these transcripts play a role in aging. In this review, we summarize the current state of exRNA profiling in various body fluids and discuss age-related changes in exRNA abundance that have been identified in humans and other model organisms. MiRNAs, in particular, are a major focus of current research and we will highlight and discuss the potential role specific miRNAs might play in age-related phenotypes and disease. We will also review challenges facing this emerging field and various strategies that can be used for the validation and future use of exRNAs as markers of aging and age-related disease.

Graphical Abstract

Extracellular RNA is found in most body fluids.
Extracellular RNA profiles and abundance change with age.

Keywords

extracellular RNA; exRNA; circulating; aging; microRNA; tRNA; mRNA; serum; plasma; chronic disease; circulation; RNA-seq; humans; mice

*Address for Correspondence: Michele K. Evans, M.D., National Institute on Aging, National Institutes of Health, Laboratory of Epidemiology and Population Sciences, Biomedical Research Center, Room 04C222, 251 Bayview Boulevard, Suite 100, Baltimore, Maryland 21224-6825, Tel. 410-558-8573, Fax 410-558-8268, me42v@nih.gov.

The authors declare no conflicts of interest.

Introduction

Understanding the phenomena of aging, both in the context of healthy longevity and in the manifestation of age-related disease, will aid in identifying additional biological factors important for increasing human lifespan and health. Age remains a major risk factor for a myriad of human conditions, including cardiovascular disorders¹, cancer², Alzheimer's³, sarcopenia⁴, and cognitive decline⁵. Extracellular factors in the blood and other tissue-specific biofluids play a vital role in maintaining physiological homeostasis, immune response, and intercellular communication. Dysregulation and changes in nutrient sensing and intercellular communication are hallmarks of aging. Current research strategies have focused on profiling extracellular factors in order to isolate biomarkers of healthy aging and age-related disease⁶. For example, introduction of blood from young mice into older mice restored the regenerative capacity of skeletal muscle stem cells⁷ and improved cognitive function and synaptic plasticity⁸. Individual extracellular factors that either improve age-related phenotypes and/or change during the aging process have been identified, including CCL11⁹ and GDF11/8^{10, 11}, but more studies are needed to further verify their roles in this process.

Blood and other biofluids also contain numerous types of extracellular nucleic acids including circulating cell-free DNA^{12, 13}, mitochondrial DNA (mtDNA)¹⁴, and RNAs^{15–18}. The advent of next-generation sequencing (NGS) technology has deepened our understanding of the different subtypes of extracellular RNAs (exRNAs) in human biofluids and identified disease-related changes in exRNA abundance¹⁶. Identifying, cataloging, and investigating the molecular functionality of exRNAs in the context of normal and disease-associated aging will provide important insight into the development of physiological aging and ideally identify novel targets for therapeutic intervention for age-associated diseases. This review will summarize the current state of exRNA profiles and abundance during the aging process across numerous biofluids including blood, saliva, urine, cerebrospinal fluid (CSF), and others. Our primary discussion will focus on exRNAs identified in human biofluids, but we will also discuss relevant observations in other animal models. In addition, we present a reanalysis of previously published and unpublished data in order to identify important exRNAs within the context of aging. The goal of this analysis is to highlight those transcripts that change in abundance during aging and discuss potential roles of these individual transcripts in the context of aging phenotypes and age-related disease processes.

exRNAs: The What and The Where

In this review, exRNAs are defined as any RNA transcript found outside of the cell and either in circulation or in a local, tissue-specific fluid. Box 1 is provided as a brief summary of the major classes of RNA subtypes, their size, and function as well as references for more comprehensive reviews of the different subtypes of RNAs transcribed in humans^{19, 20}. mRNA transcripts and fragments have been found in extracellular fluid as well as non-coding RNAs (ncRNA), including microRNAs (miRNA), piwi-RNAs (piRNA), long non-coding RNAs (lncRNA), small nuclear RNAs (snRNA), small nucleolar RNAs (snoRNA), ribosomal RNAs (rRNA), transfer RNAs (tRNA), 5'-tRNA halves, Y-RNAs, circular RNAs (circRNA), and microbial RNAs^{15, 16, 21–27}. miRNAs represent the most abundant type of

small RNA species in sequencing of exRNA, ranging from ~40–75% of mapped small RNA reads in human plasma and serum^{15, 16, 27}. miRNAs were the most abundance transcript subtype (27%) of RNA transcripts ranging in size from 15–40 nt in human seminal fluid exosomes²⁴. However, miRNA transcript abundance can be tissue-dependent. miRNAs and piRNAs are the majority of human small RNAs sequenced using small RNA-seq in saliva, but are only 6% and 7.5% of all mapped reads, respectively, and a majority of RNA reads (~60%) in saliva map to microbial RNA sequences²¹. This may reflect the greater presence of microbes in the oral cavity or in other fluids that contain exogenous RNA, such as in serum or the gut^{25, 26}.

Box 1

Brief Overview of RNA Subtypes

This brief overview of the different types of RNAs is meant only as an introduction to the variety of RNAs found in circulation and other bodily fluids. As next-generation sequencing is further refined, we anticipate that even more subtypes of RNAs and their derivatives will be identified and classified in the extracellular environment.

There are a large variety of different RNAs that are actively transcribed from the human genome²⁰ apart from **protein-coding mRNA** transcripts. These transcripts, termed **non-coding RNAs (ncRNAs)**, include **ribosomal RNA (rRNA)**, which are the RNA component of ribosomes, as well as **transfer RNA (tRNA)**, which mediate protein synthesis and translation when covalently attached to an amino acid. Under conditions of oxidative stress, the enzyme angiogenin can cleave tRNAs within the anti-codon loop or tRNA to form **5' tRNA and 3' tRNA fragments**¹¹⁸. tRNA fragments can bind with Argonaute proteins and this association may play a role in cellular proliferation, however the physiological roles of tRNA fragment signaling is still unclear at this time¹⁴⁰.

The smallest of the ncRNAs are **microRNAs (miRNA)**. Mature miRNAs are single-stranded RNAs that are approximately 22 nucleotides (nts) in length. They guide the RNA-induced silencing complex (RISC) to bind with the 3' untranslated region (UTR) of target mRNAs and inhibit protein translation and cause mRNA degradation¹⁴¹. Mature miRNAs are processed from a precursor miRNA (pre-miRNA) that contains a canonical stem-loop and hairpin structure. Mature miRNAs can derive from either the 5' - or 3' -arm of the hairpin and the nomenclature of the mature miRNA transcript should reflect its origin with either a -5p or -3p designation in its mature name, i.e. miR-223-5p or miR-223-3p. For further clarification on the preferred miRNA nomenclature, the miRNA database miRBase has published previously extensively on this topic¹³⁷. **Piwi-interacting RNAs (piRNAs)** are single-stranded RNAs between 24–31 nts in length and these RNAs interact with PIWI proteins to maintain genome integrity in germ-line cells by regulating transposon activity. Recent evidence suggests that piRNAs can also regulate protein-coding gene expression via genomic imprinting¹⁴². **Small nucleolar RNAs (snoRNAs)** are nuclear-bound RNAs that play essential roles in rRNA modification (i.e. 2'-O-methylation), cleavage of precursor mRNAs, and chromatin remodeling. snoRNAs range in size from 60–300 nts and associate with small nucleolar ribonucleoprotein (snoRNP) complexes to guide rRNA modification¹⁴³. **Small nuclear RNAs (snRNAs)**

are essential components of the RNA spliceosome and participate in processing of precursor mRNA transcripts in the nucleus. They range in size from 100–300 nucleotides in length^{19, 144}.

The majority of ncRNAs are termed **long non-coding RNAs (lncRNAs)**, which are any ncRNA longer than 200 nts. lncRNAs have a variety of functions, including binding with chromatin to regulate chromatin structure and thereby influencing gene expression, mRNA transcript modification, modulation of miRNA abundance via a ‘sponge’ mechanism, and regulation of other ncRNAs^{145–147}. The complete functionality of lncRNAs is still being elucidated, with various classes within this subtype with a variety of different roles. **Circular RNAs (circRNAs)** are covalently closed loops of RNA that are formed from exons and introns during precursor mRNA splicing and processing. circRNAs vary in size and can have multiple isoforms processed from the same parental transcript they are derived from. The function of circRNAs is still relatively unknown but there is evidence to suggest that they have functional roles in miRNA regulation and gene expression, as well as contributing to the regulation of tissue-specific development, particularly in the brain^{148, 149}.

There are four **Y-RNA** genes that code for RNA transcripts between 80–112 nts in length. The physiological roles of Y-RNAs are still poorly understood but they have been associated with RNA processing and the initiation of DNA replication¹⁵⁰.

exRNAs in circulation and other bodily fluids are commonly bound with or contained within carrier molecules such as extracellular vesicles (EVs), including exosomes, microvesicles, and apoptotic bodies^{28–30}. EVs range in size from ~30 nm to 400 nm, depending on the type, and through fusion of multivesicular bodies with the plasma membrane (exosomes), budding from the plasma membrane of cells (microvesicles), or release from dying cells (apoptotic bodies), they enter the circulation or interact with other cells in the local microenvironment^{29, 31} (Figure 1). EVs can also travel to distal tissues in the body. EVs contain mRNAs, ncRNAs, proteins, and lipids^{28, 29} which can act as signaling molecules and can have both physiological and pathological roles in immune response³², cell-to-cell signaling^{33, 34}, and age-related disease, including neurodegenerative^{35, 36} and cardiovascular diseases³⁷ and cancer³¹ (for a detailed review of EV function and targeting to recipient cells, see Yanez-Mo et. al.³⁸). The mechanisms controlling the loading of RNAs into EVs is still being elucidated. Heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) regulates miRNA sorting into exosomes via recognition of sequence-specific motifs³⁹. However it is currently unknown if only a subset of miRNAs are regulated by hnRNA2B1 and/or if other proteins sort other miRNAs into EVs. The regulation of sorting and packaging of other ncRNAs into exosomes and EVs is not well understood at this time.

Extracellular miRNAs also bind to members of the Argonaute (AGO) protein family, AGO1⁴⁰ and AGO2^{40–42}. Within the cell cytoplasm, miRNAs are loaded into an AGO2-associated protein complex called the RNA-induced silencing complex (RISC). Circulating ribonucleoprotein complexes in human samples that contain AGO2 are bound with mature miRNAs and provide the bound miRNA protection from circulating RNases^{41, 42}. miRNAs are associated with subviral surface antigen particles secreted by Hepatitis B virus-infected

hepatocytes⁴³. miRNAs in the circulation are also associated with lipid complexes including high-density lipoproteins (HDL; Figure 1)^{44, 45}. miR-223 is bound to HDL and transferred into recipient endothelial cells, which do not endogenously express miR-223. Transferred miR-223 exhibits an anti-inflammatory response in recipient endothelial cells by inhibiting endogenous protein expression of intercellular adhesion molecular 1 (ICAM-1)⁴⁶. As of yet, other ncRNAs have not been associated with vesicle-free ribonucleoprotein complexes or lipoproteins.

exRNAs are predominantly studied within circulation as biomarkers for overall health and disease^{16, 47, 48}, including therapeutic response^{49, 50}, inflammation⁵¹, and disease prognosis^{52–54}. This is presumably due to the relative ease of acquiring blood and serum from patients and study participants. Additionally, some ncRNAs such as miRNAs are very stable in patient serum, whole blood, and tissues, making them ideal for longitudinal studies^{41, 55} or for retrospective analyses of disease, phenotypes or mortality. Circulating RNAs have been identified in a variety of other human fluids (Figure 2). Extracellular mRNAs have been identified in serum^{16, 54}, CSF⁵⁶, amniotic fluid^{57, 58}, seminal fluid²⁴, and saliva⁵⁹. Fragments of *GAPDH* and *VEGF* mRNA have been identified in urine⁶⁰ and fragments of *ACTB* and *DDX4* mRNA are in seminal fluid⁶¹. miRNAs have been identified in plasma and serum^{15, 47, 62}, CSF^{56, 63}, tears⁴⁸, saliva²¹, breast milk⁶⁴, bile⁶⁵, seminal²⁴ and peritoneal⁶⁶ fluids, and urine^{17, 22, 48}. lncRNAs have been sequenced in plasma/serum^{15, 16, 67} and saliva⁵⁹. piRNAs have been sequenced in saliva²¹, plasma^{16, 47}, and seminal fluids²⁴. snRNA fragments have been identified in bile⁶⁸, urine²², and serum/plasma^{16, 52} and rRNAs are found in seminal fluid^{24, 61}, plasma^{15, 16}, and urine²². snoRNA have been identified in saliva⁵⁹, plasma⁴⁷, and urine²² and tRNAs, 5' tRNAs, and Y-RNAs have been sequenced in seminal fluid²⁴ and serum and plasma^{16, 23, 27}. circRNAs have been sequenced in saliva²¹ and in whole blood, but this analysis did not separate circulating blood cells and it is unclear if the identified circRNAs are extracellular or from blood cells⁶⁹. It is also important to note that many of these studies have focused on the changes in abundance of different subtypes of exRNAs in the presence of infection or chronic disease and compared to healthy controls. A complete catalog of all exRNAs, under one protocol examining both small and large ncRNAs, in a large cohort has yet to be performed.

exRNAs have not been identified in human sweat, pleural effusion, vaginal or synovial fluids, or in colostrum (Figure 2). One study identified exosomes in human nasal secretions which contained RNA profiles ranging from 25–2000 nucleotides, but an analysis of RNA subtypes within those exosomes was not performed⁷⁰. Given the distribution of exRNAs in both local and systemic human bodily fluids, it is plausible that most, if not all, human bodily fluids and excretions contain exRNAs.

exRNA in the Context of Aging

Age-related changes in RNA expression are well documented in tissue and cells in multiple animal models^{6, 71, 72} and these studies have offered glimpses into gene-specific mechanisms that contribute to the aging process. Expression of miRNAs and their mRNA targets change with age in circulating peripheral blood mononuclear cells (PBMCs)^{73–76} and are key regulators of cellular senescence and the senescence-associated secretory phenotype

(SASP)⁷⁷. Other ncRNAs change with age within a cellular context as well. LncRNAs regulate several pathways involved with aging and senescence, including DNA methylation, telomere extension, cycle division, and inflammation signaling⁷⁸. In monkey skeletal muscle, circRNAs exhibit age-dependent changes in expression but the functional consequence of these change is not well understood⁷⁹. Undoubtedly, continued study of ncRNAs within cells will shed light on their functional role in the context of aging and perhaps provide clues to potential roles in the context of the extracellular environments. We will now review in greater detail those studies specifically examining exRNA in aging and age-related disease in mice and in primates and we will speculate on potential functional roles.

Extracellular miRNAs and Aging in Mice—A vast majority of studies investigating changes in exRNA abundance during aging have focused on miRNAs in the circulation (Summarized in Table 1). The first study examining miRNA abundance throughout life were performed in mice. Mouse plasma levels of circulating miR-34a were found to increase with age and can be used as a biomarker for miR-34a expression levels in PBMCs and in brain tissue⁸⁰. Circulating miR-34a was also reciprocally associated with decreased levels of silence information regulator 1 (Sirt1), a target of miR-34a regulation. Sirt1 levels also declined in the brain, suggesting circulating miR-34a may be a biomarker for the interaction between miR-34a and Sirt1 in age-related tissue decline⁸⁰.

Dhabi et al. identified 73 miRNAs in mouse serum that significantly increased with age and 47 miRNAs which decrease with age (see Table 1). Calorie restriction (CR), a dietary intervention that extends lifespan⁸¹, alleviated the changes in levels of 72 of the miRNAs influenced by age on a normal diet (see Table 1, brackets)⁸². CR alone caused 18 miRNAs in serum to increase, while two miRNAs (mmu-miR-486-3p and mmu-miR-3107-3p) were significantly decreased. Pathway analysis of altered serum miRNAs with age and CR treatment identified potential pathways altered with aging, including positive regulation of macromolecule biosynthetic process, negative regulation of apoptosis, and the Wnt signaling pathway⁸². However, it should be noted that deep sequencing was only performed on three mice in each group and RT-qPCR validation of the sequencing reads was not performed. A replication study with increased sample size and target-specific PCR can help validate the sequencing results and identify those transcripts most likely to be changed during aging and CR-treatment.

Ames dwarf mice harbor a mutation in the prophet of pituitary factor 1 (Prop1) a transcription factor that controls development of the pituitary gland and results in depressed levels of growth hormone, thyroid stimulating hormone and insulin-like growth factor 1 (Igf-1), with the mice living an extended lifespan^{83, 84}. A recent study performed deep sequencing to identify circulating serum miRNAs that change with normal aging or in long-lived, age-matched Ames dwarf mice⁸⁴. 17 miRNAs were decreased in abundance during normal aging, while miRs 5107-5p, 146a-5p, and 342-5p were significantly increased (Table 1). In the Ames mice, there were only four miRNAs that increased in abundance with age. Interestingly, three of the increased miRNAs in the Ames mice (miR-34c-5p, miR-34b-5p, and miR-344d-2-5p) are decreased in normal, wild-type mice⁸⁴. Gene ontology analysis of the putative targets for each of these three miRNAs, with reciprocal changes in serum

abundance in the different mouse lineages, identified pathways associated with potential targets for each miRNA. Enrichment for neuron projection development, neurotransmitter transport, and positive regulation of transcription suggest altered regulation of these pathways during the aging process in normal vs. Ames mice⁸⁴. KEGG pathway analysis of putative targets from the above mentioned gene ontology analysis identified putative targets in MAPK signaling, pathways in cancer, pathways with adheren and gap junctions, melanogenesis, and axon guidance⁸⁴. Together, this analysis suggests that miRs 5107-5p, 146a-5p, and 342-5p may be important in the regulation of neuronal activity during mouse aging, including neuronal signaling and growth, or even plasticity. Future experiments should confirm the role of these miRNA:mRNA networks and how their interaction contributes to age-associated cognitive decline and/or neurodegeneration in rodent models.

Similarities and Differences in Mouse Extracellular miRNA Aging Studies—

Victoria and colleagues examined several miRNA gene families with differential expression in mouse aging common between their study and those affected by normal aging and CR^{82, 84}. In this review, we have extended this analysis by comparing the individual mature miRNAs identified in both studies. We found that two miRNAs, miR-195a-5p and miR-34c-5p, were commonly decreased in abundance in serum of aged mice (Figure 3A). Similarly, we performed target prediction and KEGG pathway analysis to identify the most significant pathways associated with mRNA targets for each mRNA using the DIANA-miRPath algorithm⁸⁵ (Figure 3B). mmu-miR-195a-5p is predicted to target genes in the PI₃K-AKT signaling pathway, pathways in cancer, pathways involved in the regulation of pluripotent stem cells, and HTLV-1 infection. Putative genes in these pathways including B-Cell CLL/Lymphoma 2 (Bcl-2), Vascular endothelial growth factor A (Vegfa), Mitogen-activated protein kinase kinase 1 (Map2k1), Bone morphogenic protein receptor, type IA (Bmpr1a), and Wingless-type MMTV integration site family, member 4 (Wnt4) (Figure 3B). Targets for mmuR-miR-34-5p include E2F transcription factor 3 (E2f3), Cyclin E2 (Ccne2), Notch1, Notch4, and Gamma-aminobutyric acid A receptor, alpha 3 (Gabra3) in pathways related to cancer, GABAergic response, and Notch signaling (Figure 3B). miR-34c regulates Notch1 protein expression and bone development in mice⁸⁶. In human endothelial cells, NOTCH1 expression regulates vascular endothelial cellular senescence by inhibiting a p16-dependent signaling cascade⁸⁷. It is interesting to speculate that lower levels of circulating miR-34c may be protective of vascular aging through a Notch-dependent mechanism. Additional studies will be needed to assess this relationship and confirm circulating miR-34c can regulate endothelial Notch signaling.

Our pathway analysis identified that both miR-146a-5p and miR-5107-5p were significantly increased in serum in both studies^{82, 84} (Figure 3A). These miRNAs putatively target genes involved in thyroid hormone synthesis and thyroid neoplasia. In humans, thyroid dysfunction increases in prevalence with age and may be associated with cardiovascular disease as well as cognitive impairment. Serum levels of thyroid-stimulating hormone and thyroxine often change with age, but the direction of that change and the effect on lifespan and health is still unclear^{88, 89}. miR-146a-5p is predicted to target thyroid peroxidase (Tpo), a glycoprotein involved with thyroid gland function and thyroid hormone synthesis⁹⁰. Thyroglobulin (Tg), which acts as a substrate for the production of thyroxine and other

hormones produced in the thyroid⁹¹. Altered regulation of thyroid hormone production via miR-146a-5p may contribute to known changes in circulating thyroid hormones during aging. miR-146a negatively regulates IL-6 and IL-8 expression and secretion in senescent human fibroblasts and is elevated in response to inflammatory cytokines⁹². Elevated serum miR-146a may be a biomarker for or response to systemic inflammation in aging, however the precise mechanism and cell types governing release of miR-146a is unknown.

The other miRNA affecting thyroid function, miR-5107-5p, is predicted to target Glutathione peroxidase 3 (Gpx3), a selenoprotein that protects the thyroid from excessive amounts of hydrogen peroxide damage⁹³. Over-expression of miR-5107-5p in mouse serum may influence local Gpx3 expression and affect or influence thyroid function over the course of aging. Additionally, miR-5107-5p does not appear to have a related, orthologous counterpart in the human genome and may represent a mouse-specific mechanism of aging in the thyroid gland. Both miRNAs are also predicted to target Collagen, type 11, alpha 1 (Col11a1; Figure 3B). In mice, Col11A1 regulates bone microarchitecture during development⁹⁴ and in humans COL11A1 has been linked with osteoarthritis⁹⁵ and cancer⁹⁶

While miR-34-5p is decreased in serum of wild-type mice in both studies, this effect is mitigated in aged mice on a CR diet⁸². This miRNA was also significantly increased in abundance in the Ames dwarf mice⁸⁴. Consistent with this data, metformin-treatment increased mouse life span and also increased liver expression of miR-34⁹⁷. Furthermore, overexpression of miR-34 prolongs *Drosophila* life span⁹⁸.

Extracellular miRNAs and Aging in Humans—The first study to evaluate age-related changes of circulating cell-free miRNAs in plasma from humans used microarrays to identify miRNA abundance in 20-, 80-, and 100-year old healthy human subjects⁹⁹. Exploratory factor analysis divided the results from the microarray screen into sub-groups of circulating miRNAs that changed in abundance between the three groups (see Table 1). A majority of the miRNAs that significantly changed with age increased between 20 yr olds and either 80- or 100-yr olds. Five miRNAs (miR-200b, miR-200c, miR-212, miR-425, and miR-579) were higher in centenarians compared with 80-year olds. miR-21 also increased with age and was validated in a larger cohort using RT-qPCR; however, the age ranges for the validation study had larger degrees of deviation than the discovery cohort. miR-21 significantly increased in abundance in 66–96 yr olds vs. 20–65 yr olds but levels in individuals >95 yrs old had levels similar to the younger cohort⁹⁹. Circulating miR-21 is also higher in patients with cardiovascular disease (CVD) compared with age-matched controls and lower in the offspring of centenarians⁹⁹, suggesting that age-related increases in circulating miR-21 may play a role in cardiovascular risk. To address this, the authors analyzed leukocyte expression levels of miR-21 and *TGFBR2* and observed that as miR-21 increases with age, its mRNA target *TGFBR2* is significantly decreased in leukocytes. However, older individuals (>98 yrs) had comparable expression levels of miR-21 and *TGFBR2* when compared to younger individuals (20–30 yrs)⁹⁹. Healthy centenarians exhibit expression levels in miR-21 in several tissues that are comparable to healthy, younger adults. Recently, elevated plasma levels of miR-21 are suggested to serve as diagnostic biomarkers for several cancers, including lung, colorectal, and breast¹⁰⁰. In this study, subjects were

age-matched at best as possible, however, age was not considered as a confounding variable in the analysis.

Previously our laboratory performed small RNA next-generation sequencing on serum from young (~30 yrs) and old (~64 yrs), African American and white individuals. Three miRNAs were significantly decreased with age after RT-qPCR validation, miR-151a-3p, miR-181a-5p, and miR-1248⁶². Reanalysis of our sequencing data also identified significant age-related decreases in miR-25-3p, miR-101-3p, miR-92a-3p, and miR-142-5p abundance (GEO #:GSE53439). miR-151a-5p and miR-1248 were also significantly decreased in serum from rhesus monkeys, indicated that changes in serum abundance with age for these miRNAs may be a conserved process among primates. miR-181a-5p, miR-181a-3p, and miR-151a-3p were also decreased in serum in monkeys but did not reach statistical significance⁶². Interestingly, we also found that miR-181a-3p, miR-181a-5p, and miR-151a-5p serum abundance was higher in extremely long-lived monkeys (39.7±0.39 yrs; n=3) compared to older monkeys (21.1±0.12 yr; n=3) (Noren Hooten, unpublished data). Given that rhesus monkeys age approximately three times the rate of humans, these ages correspond to ~63 yrs and ~120 yrs in humans.

Members of the miR-181 miRNA family are involved in regulation of inflammatory cytokines such as IL-6, TNF- α , and IL-8 in astrocytes, where repression of endogenous miR-181 members results in an increase in cytokine production¹⁰¹. miR-181a expression in monocytes reduces reactive oxygen species (ROS) production and expression of inflammatory IL-1a, IL-1b, and IL-6¹⁰². miR-181a family members appear to have an integral role in regulation of inflammatory response. The decline in serum levels found in the elderly may contribute to increase levels of chronic inflammation. We found that miR-1248 can bind and regulate expression of *IL6* and *IL8* mRNA and that endogenous expression levels decrease with age in PBMCs⁶². Over-expression of miR-1248 also significantly repressed mRNAs related to the NF- κ B pathway, cytokine pathway, IL1R pathway, and natural killer cell mediated cytotoxicity⁶². Systemic loss of circulating miR-1248 in the serum during aging may alter gene expression in these inflammation-related pathways. Future investigations may determine whether circulating miR-1248 is functional.

Recently, miR-126-3p plasma abundance was examined by RT-qPCR in a cohort of patients to study the age-related changes in healthy controls and age-matched Type-II diabetics¹⁰³. Plasma miR-126-3p levels were significantly elevated in healthy adults 75 yrs compared with adults 45 yrs of age. miR-126-3p, which can signal from endothelial cells to the underlying smooth-muscle layer¹⁰⁴, increases in expression in HUVECs undergoing cellular senescence¹⁰³. miR-126-3p was also one of several miRNAs increased in plasma abundance with age in another independent study in over 360 individuals between the ages of 22–79 yrs¹⁰⁵. In this study, miR-126-3p, miRs 30c-5p, 30b-5p, 26a-5p, 21-5p, 151a-5p, and let7a-5p were significantly elevated in the plasma of older individuals, after adjustment for blood cell parameters that are thought to influence miRNA levels, including hematocrit, platelet count, and mean platelet volume¹⁰⁵. Three miRNAs, miR-93-5p, miR-25-3p, and miR-101-3p, decreased in abundance with age. Increased levels of miR-21-5p in the circulation with age were also found in another study, as previously mentioned above⁹⁹. In

this report, miR-151a-5p was found to change with age however, in our study we did not find significant differences in this miRNA between young and old⁶². This discrepancy could be due the populations studied, as the individuals sequenced and validated in our study are younger then this cohort.

Another study sequenced serum from 50 individuals across four average age groups (22, 40, 60, and 70 yrs) and screened for changes in miRNA expression across age. Candidate miRNAs were assessed using RT-qPCR in an expanded cohort for each age group and several miRNAs were validated¹⁰⁶. miRs -29b, -106b, -130b, and 142-5p are decreased in serum abundance with age, whereas miRs -92a, -222, and -375 are increased. While each miRNA had significant differences between one or more of the age groups, only miR-92a and miR-29b were significantly different among all four age groups studied¹⁰⁶. In mice, miR-29b regulates the differentiation of T cells by regulating levels of IFN γ and T-box transcription factors and loss of miR-29b may contribute to Th1-induced chronic inflammation. Additionally, CD4+ T cells in patients with multiple sclerosis have decreased levels of miR-29b¹⁰⁷. This suggests that loss of circulating miR-29b may be a marker for age-related inflammation states in immune cells or perhaps decreased miR-29b extracellular signaling contributes to age-related systemic inflammatory states.

miR-92a is often upregulated during tumorigenesis and regulates expression of PTEN in response to IL-6 signaling and STAT3 transcriptional activation¹⁰⁸. It is a part of the miR-17-92 gene cluster that codes for six individual miRNAs from the same primary transcript. These transcripts are essential for normal B cell development and all of which are over-expressed in B cell lymphomas¹⁰⁹. Interestingly, miR-92a can regulate inflammatory cytokine production via targeting of MKK4 in the JNK/c-Jun pathway, where overexpression of miR-92a in macrophages attenuated TLR-induced production of TNF α and IL-6¹¹⁰. It appears these two regulatory roles of miR-92a are juxtaposed, but it is not unusual for miRNAs to act as mediators of the delicate balance between opposing regulatory systems that are perturbed in disease^{111–113}. Continued investigation of miRNA network mediators in the context of aging may help identify key extracellular miRNAs that change with age and predispose older individuals to disease. Another member of the miR-17-92 gene cluster, miR-20a-3p, is decreased in cell-free blood taken from young (~41 yrs) and old (~78 yrs) breast cancer patients (Table 1), among other miRNAs¹¹⁴, but these miRNAs should be examined in a healthy cohort.

A recent large study has identified small extracellular RNAs, including 669 miRNAs with at least one read per million fragments, by small RNA sequencing of exRNA in plasma from 40 participants. miR-451a and miR-223-3p were the most abundant reads, accounting for ~47% of all miRNA sequencing reads⁴⁷. Three hundred and thirty-one of the most abundant miRNA transcripts were validated with RT-qPCR in a larger cohort of 2,763 participants from the Framingham Heart Study. There were 159 miRNAs significantly decreased with age in old (>66.5 yrs) vs. young (<66.5) individuals (see Table 1), including both miR-451a and miR-223-3p, which were decreased in plasma abundance by 1.23 and 1.27-fold, respectively⁴⁷ (see Freedman et. al., Supp. Data 2⁴⁷). Both of these transcripts, including others significantly decreased such as miR-92a-3p, miR-30a-5p, miR-126-3p, miR-342-3p, miR-126-5p, miR-486-5p, and miR-16-5p were sequenced in at least 96% of the validation

cohort, representing over 2600 individuals. miR-543, also significantly decreased with age, was found in the fewest individuals (n=368, 13.3%). It should be noted that this study is representative of a general aging population as opposed to solely healthy aging individuals. Almost one quarter of the validation cohort (n=622) has been diagnosed with CVD and another 383 participants have diabetes. Additionally, roughly half of the participants are on either antihypertensive or lipid lowering medications, or both. Extracellular miRNAs can change in abundance in the circulation with respect to cancer⁴⁷ and diabetes^{115, 116} and may confound the results when looking at abundance changes from an aging perspective.

Similarities and Differences in Human Extracellular miRNA Aging Studies—

For this review, we combined the datasets of all seven human studies to assess changes in miRNA abundance in human serum/plasma (Figure 4). Together, there have been 176 unique miRNAs that investigators report are differentially expressed during aging. 19 miRNAs are increased in abundance in circulation and 157 are decreased (Figure 4A). These data are consistent with our findings that the majority of miRNAs decrease with age in PBMCs. Data from our laboratory suggests that this reduced expression with age may be due to lower levels of DICER1 with human age^{73, 97}. Eighteen miRNAs have been reported in two or more of the studies discussed here (Table 1). Of these transcripts, the most common is miR-126-3p, which has been sequenced in four studies; ten miRNAs (miR-101-3p, miR-106b-5p, miR-142-3p, miR-142-5p, miR-21-5p, miR-25-3p, miR-30b-5p, miR-30c-5p, miR-92a-30, and miR-93-5p) are present in three studies. Eight of these ten miRNAs (miR-151a-3p, miR-181a-5p, miR-93-5p, miR-25-3p, miR-101-3p, miR-142-5p, miR-92a-3p, and miR-106b-5p) are downregulated in all studies reporting on these transcripts. Interestingly, 10 of the 18 common miRNAs exhibit conflicting age-associated changes (Figure 4A). Pathway analysis of predicted miRNA targets for these 10 miRNAs identified commonly associated pathways including axon guidance and long-term potentiation, indicating that this pool of miRNA in the blood may have relevance to neuronal function and may have a role in cognitive health (Figure 4B).

Surprisingly, few pathways were commonly associated with all eight miRNAs that are commonly decreased in abundance with aging. Several of these miRNAs are predicted to target genes involved in extracellular matrix-receptor interaction, phosphatidylinositol signal, cell adhesion, and fanconi anemia (Figure 4C). These results suggest that the interaction between cells and the ECM, or with other cells, may be an area of interest in aging, particularly between circulating cells in the blood and the vasculature. However, considering there is little overlap among the pathways targeted by these miRNAs, more studies are needed to ascertain direct effects on age-related phenotypes for each miRNA – particularly in the case of cell to cell signaling, as these miRNAs are present in circulation. It may also indicate that these miRNAs target different pathways to modulate age-related decline.

Aging-related changes in exRNA in other body fluids—Extracellular miRNAs that change in abundance with age have also been identified in two other fluids other than blood. Microarray analysis has been performed on exosomes isolated from saliva from young (~21 yrs) and old (~66 yrs) individuals. Six miRNAs significantly decreased in abundance with

age (miR-24-3p, miR-371a-5p, miR-3175, miR-3162-5p, miR-671-5p, and miR-4667-5p) but only miR-24-3p was validated using RT-qPCR¹¹⁷. In aged individuals, miR-24-3p levels were also correlated with bleeding in the mouth during gentle probing while undergoing an oral examination. It is possible that miR-24-3p levels are influenced by intrusion of blood cells or other cells from fluids outside of the oral cavity and additional studies are needed to limit the outside influence of circulating cells in the oral cavity.

EVs were also isolated from CSF and small RNA content was profiled for age-dependent changes in young (<2 yrs) and old (>70 yrs) individuals⁵⁶. Small RNA sequencing identified miRNAs and several small ncRNAs in EVs isolated from both age groups and reported no differences in miRNA abundance with age among the 41 most highly expressed miRNAs (see Table 1). Of the differentially expressed miRNAs by age, 2.2% were significantly decreased with age and 2.34% were increased. There were also 42 miRNAs unique to young individuals only, and 32 unique miRNAs in old individuals⁵⁶. However, other than a table listing some of the miRNA reads in EVs from young individuals, the authors do not provide a list of miRNAs significantly changed with age or those miRNAs unique to each age group. It could be useful to perform pathway analysis on these particular sets of identified miRNAs with respect to age and to identify common pathways that may be associated with disease or age-related phenotypes, particularly in older individuals. Since this study represents exRNA sequencing from the youngest population (<2 yrs) and in the CSF, it's speculated that the unique miRNA profile to this young age group may be related to development of the central nervous system. A more detailed analysis may help identify unique miRNAs and offer clues to pathways turned on/off during the course of aging in the CSF. Furthermore, detailed analysis of exRNAs in the CSF can also give possible clues to the diagnosis and response to treatment of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease.

Other exRNAs and Aging in Mice—Sequencing of RNA isolated from mouse serum has identified 5' tRNA fragments that are associated with vesicle-free complexes that are sensitive to EDTA ion chelation²³. 5' tRNA fragments derived from the Histidine tRNA were significantly increased in serum abundance in aged mice (27 ms vs. 7 ms) and Arginine, Cysteine, Glycine, Lysine, and Valine tRNA fragments were significantly decreased in abundance with age (Table 2). Caloric restriction partially and significantly mitigated the age-related changes for each of these 5' tRNA fragments²³. The tissue expression was also investigated for the 5' halves and it was reported that cells of the blood and lymphatic system have the highest concentration of 5' tRNA halves. It is currently unknown if blood cells take up 5' tRNA halves already in the blood or produce the 5' tRNA halves and shed them into circulation²³.

A follow-up study by the same laboratory compared changes in 5' tRNA fragments with normal aging and in age-matched Ames dwarf mice⁸⁴. tRNA fragments from Cysteine and Lysine decreased with age in normal mice, whereas tRNA fragments from Histidine and Aspartic acid increased with age. None of these tRNA fragments changed in the Ames dwarf mice. tRNA-derived fragments from Proline were decreased in serum abundance in the Ames mice (Table 2). In both studies of 5' tRNA fragments the authors report several isoforms for each tRNA, dependent upon where in the genome the parent RNA was transcribed from. It is not yet know if there are relevant differences in function in tRNA

fragments from different genomic loci. However, the role of tRNA fragments in aging is still unknown. tRNAs are known to be cleaved in response to stress, including nutrient deprivation and hypoxia¹¹⁸, and in *Drosophila* methylation of Aspartic acid, Valine, and Glycine tRNAs by Dnmt2 can protect them from cleavage during cellular stress¹¹⁹. The interplay between these pathways may be a point of interest in aging research in the future. Additionally, methylation of G, C, and A nucleosides in tRNA can inhibit reverse transcription and subsequent library generation¹²⁰. Because of this, tRNA reads may be underrepresented of what is actually present in the extracellular environment. Approaches to strip away base-pair modifications on tRNAs, such as methylation, and then perform RNA sequencing have identified novel tRNA-related fragments¹²⁰. The incorporation of these methods into sequencing projects in mouse and humans may provide a more accurate assessment of age-related changes in tRNA fragment abundance.

Other exRNAs and Aging in Humans—A recent study cataloged the exRNA profile within plasma EVs isolated from 50 healthy controls and 142 cancer patients using small RNA sequencing. While the primary objective of the study was to identify changes in circulating RNA profiles that may be useful biomarkers for cancer and disease, RNA transcript abundance analysis was performed within the 50 healthy controls (median age 54 yrs; range 25–79 yrs) and 15 transcripts were found to be positively associated with increased age¹⁶, including 7 mRNAs (*CSF2RA*, *DLD*, *RRP1B*, *RAB3GAP2*, *SLC35B4*, *LAMB2*, and *XM_005258610*), 3 lncRNAs (EHHADH-AS1, RP11-696N14.1, and AC022311.1), 1 pseudogene (*HSD3BP4*), and 4 unclassified transcripts (see Table 2). Surprisingly, there were no changes in miRNA abundance with age, even though ~40% of all mapped RNA reads were from miRNA sequences. This is the only study that has identified age-related changes in extracellular mRNA, lncRNA, and pseudogenes in any human bodily fluid.

CSF2RA encodes colony stimulating factor 2 receptor, alpha, a subunit of the receptor for the cytokine colony stimulating factor 2 (CSF2, also known as GM-CSF). CSF2 is an essential regulator of granulocyte and macrophage maturation¹²¹ and CSF2-activated monocytes can develop a pathogenic inflammatory phenotype, leading to tissue damage, particularly in the nervous system¹²². *RRP1B* encodes ribosomal RNA processing 1 homolog B, a nuclear protein involved in mRNA alternative splicing and has a role in suppressing metastasis via its interaction with splicing factor SRSF1¹²³. Dihydrolipamide dehydrogenase (DLD) is an enzyme that serves as a subunit of pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase, and other enzymatic complexes involved in metabolism and energy homeostasis. Mutations in DLD may alter its function and promote the increased production of ROS¹²⁴. Solute carrier family 35 member B4 (SLC35B4) transports nucleotide sugars in and out of the Golgi and has been linked with obesity^{125, 126}. RAB3 GTPase Activating Protein Subunit 2 (RAB3GAP2), a member of the RAB3 protein family, modulates exocytosis of hormones and neurotransmitters and mutations in this protein lead to abnormal neurodevelopment and Warburg Micro syndrome or Martsolf syndrome¹²⁷. Laminin Beta 2 (LAMB2) is part of a family of extracellular glycoproteins and LAMB2 is necessary for normal function of the kidney¹²⁸. The pseudogene Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta, Pseudogene 4 (HSD3BP4) is transcribed from the 3 beta-

hydroxysteroid dehydrogenase gene cluster¹²⁹ and may have a functional role in the activity of the 3 β -HSD isoenzymes, involved in the formation of steroid hormones¹³⁰. As of yet, no specific role of HSD3BP4 has been identified. The three lncRNAs identified in this study have not been functionally characterized at this time.

Freedman and colleagues identified 305 tRNAs, 144 piRNAs, and 74 snoRNAs using RNA sequencing of human plasma in 40 participants. After RT-qPCR validation using gene-specific primers and probes, the authors reported 20 piRNAs and 12 snoRNAs significantly decreased in plasma abundance with age⁴⁷ (see Freedman et. al., Supp. Data 2⁴⁷). While nearly all of these extracellular piRNAs and snoRNAs do not have a known function, this study represents the largest to date (>2700 participants) of a comprehensive small RNA profile that has identified age-related changes. It will be important to identify mechanisms and pathways associated with each of these transcripts, whether individually or as a profile biomarker for age and age-related phenotypes. Interestingly, the authors did not observe age-related changes in tRNA abundance.

Challenges, Conclusions, and Future Directions

Transcript profiling and NGS has offered researchers an unprecedented look into the abundance and subtypes of RNAs in circulation and in other bodily fluids. However, significant challenges remain that will need to be addressed by the research community as we move forward and try to characterize the meaning and importance of this data. Currently there are numerous ways that RNA and RNA-containing EVs can be isolated from biofluids. Traditional protocols and kits using phenol/chloroform or column-based isolation can influence downstream sequencing and PCR results and the potential bias each method needs to be carefully considered^{63, 131–133}. Strategies for normalization of sequencing reads or gene-specific expression are varied and each can influence which transcripts are significantly-associated with particular endpoints or introduce bias into the analysis by overcompensating read count^{134–136}. A reliable stratagem for normalization of extracellular abundance has not been identified and given the widespread (and sometimes opposing) results reported already (Tables 1 and 2), it may be challenging to pinpoint a specific reference gene or strategy that can be reproducible in multiple cohorts or across institutes. Concurrently, future studies should delineate the source of RNA in each biofluid assessed, i.e. RNA from enriched EVs vs. soluble RNA, RNA from EV-free and lipoprotein-free fluids, and/or RNA isolated only from EVs. These distinct methodologies can help assess the amount of RNA that is soluble vs. protected in any given sample, as well as provide insight into whether individual exRNAs are only found bound within vesicles, bound to proteins, or contained within some other fraction of the biofluid.

This problem will be exacerbated by the fact that aging-related cohorts will undoubtedly be confounded by additional demographic variables including disease, race, geographic location, gender, and most importantly - age range. The age ranges defined within each analysis discussed in this review vary in how old and young are distinguished from one another. Several have used cut offs above and below one specific age⁴⁷, used time points within a defined age range^{16, 56, 80, 103, 105}, or used specific age-ranges broken into two or more distinct groupings for analysis^{23, 62, 82, 84, 99, 106, 114, 117}. It is plausible that age-related

changes observed in these studies may lose significance dependent upon the way the analysis was performed or how the study was set-up. For example, in our analysis of the various studies we found that 10 miRNAs exhibit differing changes in abundance in the circulation depending on the study (Figure 4A). Freedman et al. reported that while there are many miRNAs that are present in nearly all individuals in their cohort (>2600), many miRNAs are also present in plasma in only hundreds of individuals⁴⁷. This indicates that some transcripts may exhibit age-related changes in only a subset of individuals. Future studies need to address if these observations are based on particular epidemiological factors and if the lack or presence of these transcripts have functional relevance. It will also be important to determine if technical issues with initial sample volume, library generation, or PCR validation are biasing these results. Additional challenges lie in nomenclature as the different fields evolve. It will be important in future studies to conform to common naming of small RNAs (i.e. using miRBase naming for miRNAs¹³⁷, see Box 1). However, comprehensive databases and uniform naming system is only beginning to be utilized for other classes of ncRNAs. For example, the naming of snoRNAs identified in the Freedman study do not conform to any class of snoRNAs in current databases.

Perhaps the greatest challenge, and the most important, is determining the functional relevance of the presence of exRNAs and the significance of observed age-related changes. It is widely established that RNAs packaged into EVs or bound with lipoproteins have functional importance, such as intercellular communication between cells and tissues^{31, 33, 34}. However, the specific mode of packaging and delivery is only known for a few individual RNAs and in specific contexts^{30, 31}. Additional studies are needed to identify if there are additional carrier molecule(s) for these transcripts other than vesicles, particularly for small RNAs such as snoRNAs, piRNAs, and tRNAs, where this is little data reported on this. Ideally these studies will shed light on the cell-of-origin of these transcripts as well as give insight into potential functional purpose. The questions of what tissues are purposefully releasing exRNAs, where they are going, and how are these perturbed in age-related diseases can help ascertain how much of the exRNA profile is functionally relevant. Are there specific transcripts indicative of healthy aging only? If so, how and why? It will also be important to identify if age-related changes in exRNA are important for normal development and physiology in infants and the young. Do changes in exRNA abundance in the young predispose individuals to specific disease? Are environmental factors influencing this phenomena, such as behavior or diet? For example, in young individuals smoking reduced the total levels of circulating plasma microvesicles and significantly lowered levels of miR-223 and increased levels of miR-29b in MVs¹³⁸.

In summary, the time is ripe for continued study of exRNAs and their role in normal physiology and aging. Future studies should also continue to incorporate underrepresented minority populations. As we continue to profile and understand the extracellular environments in the body, we may also discover global patterns that may be useful biomarkers for health and lifespan. For example, methylation patterns of nuclear DNA change with age in humans and constitute an 'epigenetic clock', predictive of chronological age across multiple tissues and which changes in disease states¹³⁹. The exRNA profile, particularly in blood, may also be representative of similar age-related changes during the course of the human lifespan, a potential '*exRNA clock*' of aging, perturbed by disease or

environmental stress, and one that is easily accessible from individuals. Assessing the predictive and information value of RNA profiles and identifying the functional mechanisms of individual transcripts may provide new avenues for therapeutic approach in combating age-related disease as well as increase our understanding of RNA markers of healthy longevity.

Acknowledgments

This work was funded by the National Institute on Aging Intramural Research Program, National Institutes of Health. The authors thank Dr. Kevin Becker for critically reading the manuscript.

References

1. Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB, Flegal K, Ford E, Furie K, Go A, Greenlund K, et al. Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation*. 2009; 119:e21–181. [PubMed: 19075105]
2. Howlader, N.Noone, AM.Krapcho, M.Miller, D.Bishop, K.Altekruse, SF.Kosary, CL.Yu, M.Ruhl, J.Tatalovich, Z.Mariotto, A.Lewis, DR.Chen, HS.Feuer, EJ., Cronin, KA., editors. [Accessed 4/21/16] SEER Cancer Statistics Review, 1975–2013. Available at: http://seer.cancer.gov/csr/1975_2013/
3. Panpalli Ates M, Karaman Y, Guntekin S, Ergun MA. Analysis of genetics and risk factors of Alzheimer's Disease. *Neuroscience*. 2016; 325:124–131. [PubMed: 27026590]
4. Evans WJ, Campbell WW. Sarcopenia and age-related changes in body composition and functional capacity. *J Nutr*. 1993; 123:465–468. [PubMed: 8429405]
5. Hedden T, Gabrieli JD. Insights into the ageing mind: a view from cognitive neuroscience. *Nat Rev Neurosci*. 2004; 5:87–96. [PubMed: 14735112]
6. Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell*. 2013; 153:1194–1217. [PubMed: 23746838]
7. Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature*. 2005; 433:760–764. [PubMed: 15716955]
8. Villeda SA, Plambeck KE, Middeldorp J, Castellano JM, Mosher KI, Luo J, Smith LK, Bieri G, Lin K, Berdnik D, et al. Young blood reverses age-related impairments in cognitive function and synaptic plasticity in mice. *Nat Med*. 2014; 20:659–663. [PubMed: 24793238]
9. Villeda SA, Luo J, Mosher KI, Zou B, Britschgi M, Bieri G, Stan TM, Fainberg N, Ding Z, Eggel A, et al. The ageing systemic milieu negatively regulates neurogenesis and cognitive function. *Nature*. 2011; 477:90–94. [PubMed: 21886162]
10. Egerman MA, Cadena SM, Gilbert JA, Meyer A, Nelson HN, Swalley SE, Mallozzi C, Jacobi C, Jennings LL, Clay I, et al. GDF11 Increases with Age and Inhibits Skeletal Muscle Regeneration. *Cell Metab*. 2015; 22:164–174. [PubMed: 26001423]
11. Poggioli T, Vujic A, Yang P, Macias-Trevino C, Uygur A, Loffredo FS, Pancoast JR, Cho M, Goldstein J, Tandias RM, et al. Circulating Growth Differentiation Factor 11/8 Levels Decline With Age. *Circ Res*. 2016; 118:29–37. [PubMed: 26489925]
12. Anker P, Stroun M, Maurice PA. Spontaneous release of DNA by human blood lymphocytes as shown in an in vitro system. *Cancer Res*. 1975; 35:2375–2382. [PubMed: 1149042]
13. van der Vaart M, Pretorius PJ. Circulating DNA. Its origin and fluctuation. *Ann N Y Acad Sci*. 2008; 1137:18–26. [PubMed: 18837919]
14. Pinti M, Cevenini E, Nasi M, De Biasi S, Salvioli S, Monti D, Benatti S, Gibellini L, Cotichini R, Stazi MA, et al. Circulating mitochondrial DNA increases with age and is a familiar trait: Implications for “inflammaging”. *Eur J Immunol*. 2014; 44:1552–1562. [PubMed: 24470107]

15. Huang X, Yuan T, Tschannen M, Sun Z, Jacob H, Du M, Liang M, Dittmar RL, Liu Y, Liang M, et al. Characterization of human plasma-derived exosomal RNAs by deep sequencing. *BMC Genomics*. 2013; 14:319. [PubMed: 23663360]
16. Yuan T, Huang X, Woodcock M, Du M, Dittmar R, Wang Y, Tsai S, Kohli M, Boardman L, Patel T, et al. Plasma extracellular RNA profiles in healthy and cancer patients. *Sci Rep*. 2016; 6:19413. [PubMed: 26786760]
17. Cheng L, Sun X, Scicluna BJ, Coleman BM, Hill AF. Characterization and deep sequencing analysis of exosomal and non-exosomal miRNA in human urine. *Kidney Int*. 2014; 86:433–444. [PubMed: 24352158]
18. Liang H, Gong F, Zhang S, Zhang CY, Zen K, Chen X. The origin, function, and diagnostic potential of extracellular microRNAs in human body fluids. *Wiley Interdiscip Rev RNA*. 2014; 5:285–300. [PubMed: 24259376]
19. Cech TR, Steitz JA. The noncoding RNA revolution-trashing old rules to forge new ones. *Cell*. 2014; 157:77–94. [PubMed: 24679528]
20. Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, Tanzer A, Lagarde J, Lin W, Schlesinger F, et al. Landscape of transcription in human cells. *Nature*. 2012; 489:101–108. [PubMed: 22955620]
21. Bahn JH, Zhang Q, Li F, Chan TM, Lin X, Kim Y, Wong DT, Xiao X. The landscape of microRNA, Piwi-interacting RNA, and circular RNA in human saliva. *Clin Chem*. 2015; 61:221–230. [PubMed: 25376581]
22. Ben-Dov IZ, Whalen VM, Goilav B, Max KE, Tuschl T. Cell and Microvesicle Urine microRNA Deep Sequencing Profiles from Healthy Individuals: Observations with Potential Impact on Biomarker Studies. *PLoS One*. 2016; 11:e0147249. [PubMed: 26785265]
23. Dhahbi JM, Spindler SR, Atamna H, Yamakawa A, Boffelli D, Mote P, Martin DIK. 5' tRNA halves are present as abundant complexes in serum, concentrated in blood cells, and modulated by aging and calorie restriction. *Bmc Genomics*. 2013;14. [PubMed: 23324084]
24. Vojtech L, Woo S, Hughes S, Levy C, Ballweber L, Sauteraud RP, Strobl J, Westerberg K, Gottardo R, Tewari M, et al. Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions. *Nucleic Acids Res*. 2014; 42:7290–7304. [PubMed: 24838567]
25. Wang K, Li H, Yuan Y, Etheridge A, Zhou Y, Huang D, Wilmes P, Galas D. The complex exogenous RNA spectra in human plasma: an interface with human gut biota? *PLoS One*. 2012; 7:e51009. [PubMed: 23251414]
26. Leung RK, Wu YK. Circulating microbial RNA and health. *Sci Rep*. 2015; 5:16814. [PubMed: 26576508]
27. Dhahbi JM, Spindler SR, Atamna H, Boffelli D, Martin DI. Deep Sequencing of Serum Small RNAs Identifies Patterns of 5' tRNA Half and YRNA Fragment Expression Associated with Breast Cancer. *Biomark Cancer*. 2014; 6:37–47. [PubMed: 25520563]
28. Loyer X, Vion AC, Tedgui A, Boulanger CM. Microvesicles as cell-cell messengers in cardiovascular diseases. *Circ Res*. 2014; 114:345–353. [PubMed: 24436430]
29. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol*. 2014; 30:255–289. [PubMed: 25288114]
30. Zerneck A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, Denecke B, Hristov M, Koppel T, Jahantigh MN, Lutgens E, et al. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. *Sci Signal*. 2009; 2:ra81. [PubMed: 19996457]
31. Zhang L, Zhang S, Yao J, Lowery FJ, Zhang Q, Huang WC, Li P, Li M, Wang X, Zhang C, et al. Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. *Nature*. 2015; 527:100–104. [PubMed: 26479035]
32. Song J, Chen X, Wang M, Xing Y, Zheng Z, Hu S. Cardiac endothelial cell-derived exosomes induce specific regulatory B cells. *Sci Rep*. 2014; 4:7583. [PubMed: 25533220]
33. Hergenreider E, Heydt S, Treguer K, Boettger T, Horrevoets AJ, Zeiher AM, Scheffer MP, Frangakis AS, Yin X, Mayr M, et al. Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. *Nat Cell Biol*. 2012; 14:249–256. [PubMed: 22327366]

34. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol.* 2007; 9:654–659. [PubMed: 17486113]
35. Fiandaca MS, Kapogiannis D, Mapstone M, Boxer A, Eitan E, Schwartz JB, Abner EL, Petersen RC, Federoff HJ, Miller BL, et al. Identification of preclinical Alzheimer's disease by a profile of pathogenic proteins in neurally derived blood exosomes: A case-control study. *Alzheimers Dement.* 2015; 11:600–607. e601. [PubMed: 25130657]
36. Smith JA, Leonardi T, Huang B, Iraci N, Vega B, Pluchino S. Extracellular vesicles and their synthetic analogues in aging and age-associated brain diseases. *Biogerontology.* 2015; 16:147–185. [PubMed: 24973266]
37. Gaceb A, Martinez MC, Andriantsitohaina R. Extracellular vesicles: new players in cardiovascular diseases. *Int J Biochem Cell Biol.* 2014; 50:24–28. [PubMed: 24509128]
38. Yanez-Mo M, Siljander PR, Andreu Z, Zavec AB, Borrás FE, Buzas EI, Buzas K, Casal E, Cappello F, Carvalho J, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles.* 2015; 4:27066. [PubMed: 25979354]
39. Villarroya-Beltri C, Gutierrez-Vazquez C, Sanchez-Cabo F, Perez-Hernandez D, Vazquez J, Martin-Cofreces N, Martinez-Herrera DJ, Pascual-Montano A, Mittelbrunn M, Sanchez-Madrid F. Sumoylated hnRNP A2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. *Nat Commun.* 2013; 4:2980. [PubMed: 24356509]
40. Turchinovich A, Burwinkel B. Distinct AGO1 and AGO2 associated miRNA profiles in human cells and blood plasma. *RNA Biol.* 2012; 9:1066–1075. [PubMed: 22858679]
41. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A.* 2011; 108:5003–5008. [PubMed: 21383194]
42. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res.* 2011; 39:7223–7233. [PubMed: 21609964]
43. Novellino L, Rossi RL, Bonino F, Cavallone D, Abrignani S, Pagani M, Brunetto MR. Circulating hepatitis B surface antigen particles carry hepatocellular microRNAs. *PLoS One.* 2012; 7:e31952. [PubMed: 22470417]
44. Vickers KC, Landstreet SR, Levin MG, Shoucri BM, Toth CL, Taylor RC, Palmisano BT, Tabet F, Cui HL, Rye KA, et al. MicroRNA-223 coordinates cholesterol homeostasis. *Proc Natl Acad Sci U S A.* 2014; 111:14518–14523. [PubMed: 25246565]
45. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol.* 2011; 13:423–433. [PubMed: 21423178]
46. Tabet F, Vickers KC, Cuesta Torres LF, Wiese CB, Shoucri BM, Lambert G, Catherinet C, Prado-Lourenco L, Levin MG, Thacker S, et al. HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. *Nat Commun.* 2014; 5:3292. [PubMed: 24576947]
47. Freedman JE, Gerstein M, Mick E, Rozowsky J, Levy D, Kitchen R, Das S, Shah R, Danielson K, Beaulieu L, et al. Diverse human extracellular RNAs are widely detected in human plasma. *Nat Commun.* 2016; 7:11106. [PubMed: 27112789]
48. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, Galas DJ, Wang K. The microRNA spectrum in 12 body fluids. *Clin Chem.* 2010; 56:1733–1741. [PubMed: 20847327]
49. Jung EJ, Santarpia L, Kim J, Esteva FJ, Moretti E, Buzdar AU, Di Leo A, Le XF, Bast RC Jr, Park ST, et al. Plasma microRNA 210 levels correlate with sensitivity to trastuzumab and tumor presence in breast cancer patients. *Cancer.* 2012; 118:2603–2614. [PubMed: 22370716]
50. Melman YF, Shah R, Danielson K, Xiao J, Simonson B, Barth A, Chakir K, Lewis GD, Lavender Z, Truong QA, et al. Circulating MicroRNA-30d Is Associated With Response to Cardiac Resynchronization Therapy in Heart Failure and Regulates Cardiomyocyte Apoptosis: A Translational Pilot Study. *Circulation.* 2015; 131:2202–2216. [PubMed: 25995320]
51. Njock MS, Cheng HS, Dang LT, Nazari-Jahantigh M, Lau AC, Boudreau E, Roufaiel M, Cybulsky MI, Schober A, Fish JE. Endothelial cells suppress monocyte activation through secretion of

- extracellular vesicles containing antiinflammatory microRNAs. *Blood*. 2015; 125:3202–3212. [PubMed: 25838349]
52. Kohler J, Schuler M, Gauler TC, Nopel-Dunnebacke S, Ahrens M, Hoffmann AC, Kasper S, Nensa F, Gomez B, Hahnemann M, et al. Circulating U2 small nuclear RNA fragments as a diagnostic and prognostic biomarker in lung cancer patients. *J Cancer Res Clin Oncol*. 2016; 142:795–805. [PubMed: 26687686]
 53. Schwarzenbach H, Nishida N, Calin GA, Pantel K. Clinical relevance of circulating cell-free microRNAs in cancer. *Nat Rev Clin Oncol*. 2014; 11:145–156. [PubMed: 24492836]
 54. Stein U, Burock S, Herrmann P, Wendler I, Niederstrasser M, Wernecke KD, Schlag PM. Circulating MACC1 transcripts in colorectal cancer patient plasma predict metastasis and prognosis. *PLoS One*. 2012; 7:e49249. [PubMed: 23166620]
 55. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A*. 2008; 105:10513–10518. [PubMed: 18663219]
 56. Tietje A, Maron KN, Wei Y, Feliciano DM. Cerebrospinal fluid extracellular vesicles undergo age dependent declines and contain known and novel non-coding RNAs. *PLoS One*. 2014; 9:e113116. [PubMed: 25420022]
 57. Kamath-Rayne BD, Du Y, Hughes M, Wagner EA, Muglia LJ, DeFranco EA, Whitsett JA, Salomonis N, Xu Y. Systems biology evaluation of cell-free amniotic fluid transcriptome of term and preterm infants to detect fetal maturity. *BMC Med Genomics*. 2015; 8:67. [PubMed: 26493725]
 58. Zwemer LM, Hui L, Wick HC, Bianchi DW. RNA-Seq and expression microarray highlight different aspects of the fetal amniotic fluid transcriptome. *Prenat Diagn*. 2014; 34:1006–1014. [PubMed: 24852236]
 59. Spielmann N, Ilsley D, Gu J, Lea K, Brockman J, Heater S, Setterquist R, Wong DT. The human salivary RNA transcriptome revealed by massively parallel sequencing. *Clin Chem*. 2012; 58:1314–1321. [PubMed: 22773539]
 60. Zhao A, Peoc'h M, Cottier M, Genin C, Mottet N, Li G. Cell-free RNA content in urine as a possible molecular diagnostic tool for clear cell renal cell carcinoma. *Int J Cancer*. 2015; 136:2610–2615. [PubMed: 25379634]
 61. Huang S, Li H, Ding X, Xiong C. Presence and characterization of cell-free seminal RNA in healthy individuals: implications for noninvasive disease diagnosis and gene expression studies of the male reproductive system. *Clin Chem*. 2009; 55:1967–1976. [PubMed: 19745059]
 62. Noren Hooten N, Fitzpatrick M, Wood WH 3rd, De S, Ejiogu N, Zhang Y, Mattison JA, Becker KG, Zonderman AB, Evans MK. Age-related changes in microRNA levels in serum. *Aging (Albany NY)*. 2013; 5:725–740. [PubMed: 24088671]
 63. Burgos KL, Javaherian A, Bomprezzi R, Ghaffari L, Rhodes S, Courtright A, Tembe W, Kim S, Metpally R, Van Keuren-Jensen K. Identification of extracellular miRNA in human cerebrospinal fluid by next-generation sequencing. *RNA*. 2013; 19:712–722. [PubMed: 23525801]
 64. Munch EM, Harris RA, Mohammad M, Benham AL, Pejerrey SM, Showalter L, Hu M, Shope CD, Maningat PD, Gunaratne PH, et al. Transcriptome profiling of microRNA by Next-Gen deep sequencing reveals known and novel miRNA species in the lipid fraction of human breast milk. *PLoS One*. 2013; 8:e50564. [PubMed: 23418415]
 65. Li L, Masica D, Ishida M, Tomuleasa C, Umegaki S, Kalloo AN, Georgiades C, Singh VK, Khashab M, Amateau S, et al. Human bile contains microRNA-laden extracellular vesicles that can be used for cholangiocarcinoma diagnosis. *Hepatology*. 2014; 60:896–907. [PubMed: 24497320]
 66. Tokuhisa M, Ichikawa Y, Kosaka N, Ochiya T, Yashiro M, Hirakawa K, Kosaka T, Makino H, Akiyama H, Kunisaki C, et al. Exosomal miRNAs from Peritoneum Lavage Fluid as Potential Prognostic Biomarkers of Peritoneal Metastasis in Gastric Cancer. *PLoS One*. 2015; 10:e0130472. [PubMed: 26208314]
 67. Wang WT, Sun YM, Huang W, He B, Zhao YN, Chen YQ. Genome-wide Long Non-coding RNA Analysis Identified Circulating LncRNAs as Novel Non-invasive Diagnostic Biomarkers for Gynecological Disease. *Sci Rep*. 2016; 6:23343. [PubMed: 26987697]

68. Baraniskin A, Nopel-Dunnebacke S, Schumacher B, Gerges C, Bracht T, Sitek B, Meyer HE, Gerken G, Dechene A, Schlaak JF, et al. Analysis of U2 small nuclear RNA fragments in the bile differentiates cholangiocarcinoma from primary sclerosing cholangitis and other benign biliary disorders. *Dig Dis Sci*. 2014; 59:1436–1441. [PubMed: 24482036]
69. Memczak S, Papavasileiou P, Peters O, Rajewsky N. Identification and Characterization of Circular RNAs As a New Class of Putative Biomarkers in Human Blood. *PLoS One*. 2015; 10:e0141214. [PubMed: 26485708]
70. Lasser C, O'Neil SE, Ekerljung L, Ekstrom K, Sjostrand M, Lotvall J. RNA-containing exosomes in human nasal secretions. *Am J Rhinol Allergy*. 2011; 25:89–93. [PubMed: 21172122]
71. de Magalhaes DF, da Silva JA, Haddad JP, Moreira EC, Fonseca MI, de Ornelas ML, Borges BK, da Luz ZM. Dissemination of information on visceral leishmaniasis from schoolchildren to their families: a sustainable model for controlling the disease. *Cad Saude Publica*. 2009; 25:1642–1646. [PubMed: 19578588]
72. Fushan AA, Turanov AA, Lee SG, Kim EB, Lobanov AV, Yim SH, Buffenstein R, Lee SR, Chang KT, Rhee H, et al. Gene expression defines natural changes in mammalian lifespan. *Aging Cell*. 2015; 14:352–365. [PubMed: 25677554]
73. Noren Hooten N, Abdelmohsen K, Gorospe M, Ejiogu N, Zonderman AB, Evans MK. microRNA expression patterns reveal differential expression of target genes with age. *PLoS One*. 2010; 5:e10724. [PubMed: 20505758]
74. ElSharawy A, Keller A, Flachsart F, Wendschlag A, Jacobs G, Kefer N, Brefort T, Leidinger P, Backes C, Meese E, et al. Genome-wide miRNA signatures of human longevity. *Aging Cell*. 2012; 11:607–616. [PubMed: 22533606]
75. Lai CY, Wu YT, Yu SL, Yu YH, Lee SY, Liu CM, Hsieh WS, Hwu HG, Chen PC, Jeng SF, et al. Modulated expression of human peripheral blood microRNAs from infancy to adulthood and its role in aging. *Aging Cell*. 2014; 13:679–689. [PubMed: 24803090]
76. Serna E, Gambini J, Borrás C, Abdelaziz KM, Belenguer A, Sanchis P, Avellana JA, Rodriguez-Manas L, Vina J. Centenarians, but not octogenarians, up-regulate the expression of microRNAs. *Sci Rep*. 2012; 2:961. [PubMed: 23233880]
77. Abdelmohsen K, Gorospe M. Noncoding RNA control of cellular senescence. *Wiley Interdiscip Rev RNA*. 2015; 6:615–629. [PubMed: 26331977]
78. Grammatikakis I, Panda AC, Abdelmohsen K, Gorospe M. Long noncoding RNAs(lncRNAs) and the molecular hallmarks of aging. *Aging (Albany NY)*. 2014; 6:992–1009. [PubMed: 25543668]
79. Abdelmohsen K, Panda AC, De S, Grammatikakis I, Kim J, Ding J, Noh JH, Kim KM, Mattison JA, de Cabo R, et al. Circular RNAs in monkey muscle: age-dependent changes. *Aging (Albany NY)*. 2015; 7:903–910. [PubMed: 26546448]
80. Li XL, Khanna A, Li N, Wang E. Circulatory miR-34a as an RNA-based, noninvasive biomarker for brain aging. *Aging-Us*. 2011; 3:985–1002.
81. Anderson RM, Shanmuganayagam D, Weindruch R. Caloric restriction and aging: studies in mice and monkeys. *Toxicol Pathol*. 2009; 37:47–51. [PubMed: 19075044]
82. Dhahbi JM, Spindler SR, Atamna H, Yamakawa A, Guerrero N, Boffelli D, Mote P, Martin DIK. Deep sequencing identifies circulating mouse miRNAs that are functionally implicated in manifestations of aging and responsive to calorie restriction. *Aging-Us*. 2013; 5:130–141.
83. Bartke A, Wright JC, Mattison JA, Ingram DK, Miller RA, Roth GS. Extending the lifespan of long-lived mice. *Nature*. 2001; 414:412. [PubMed: 11719795]
84. Victoria B, Dhahbi JM, Nunez Lopez YO, Spinel L, Atamna H, Spindler SR, Masternak MM. Circulating microRNA signature of genotype-by-age interactions in the long-lived Ames dwarf mouse. *Aging Cell*. 2015; 14:1055–1066. [PubMed: 26176567]
85. Vlachos IS, Paraskevopoulou MD, Karagkouni D, Georgakilas G, Vergoulis T, Kanellos I, Anastasopoulos IL, Maniou S, Karathanou K, Kalfakakou D, et al. DIANA-TarBase v7. 0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucleic Acids Res*. 2015; 43:D153–159. [PubMed: 25416803]
86. Bae Y, Yang T, Zeng HC, Campeau PM, Chen Y, Bertin T, Dawson BC, Munivez E, Tao J, Lee BH. miRNA-34c regulates Notch signaling during bone development. *Hum Mol Genet*. 2012; 21:2991–3000. [PubMed: 22498974]

87. Yoshida Y, Miki T, Ono S, Haneda T, Ito M, Okada N. Functional characterization of the type III secretion ATPase SsaN encoded by Salmonella pathogenicity island 2. *PLoS One*. 2014; 9:e94347. [PubMed: 24722491]
88. Boelaert K. Thyroid dysfunction in the elderly. *Nat Rev Endocrinol*. 2013; 9:194–204. [PubMed: 23438834]
89. Jones CM, Boelaert K. The Endocrinology of Ageing: A Mini-Review. *Gerontology*. 2015; 61:291–300. [PubMed: 25471682]
90. Ruf J, Carayon P. Structural and functional aspects of thyroid peroxidase. *Arch Biochem Biophys*. 2006; 445:269–277. [PubMed: 16098474]
91. Di Jeso B, Arvan P. Thyroglobulin From Molecular and Cellular Biology to Clinical Endocrinology. *Endocr Rev*. 2016; 37:2–36. [PubMed: 26595189]
92. Bhaumik D, Scott GK, Schokrpur S, Patil CK, Orjalo AV, Rodier F, Lithgow GJ, Campisi J. MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8. *Aging (Albany NY)*. 2009; 1:402–411. [PubMed: 20148189]
93. Schmutzler C, Mentrup B, Schomburg L, Hoang-Vu C, Herzog V, Kohrle J. Selenoproteins of the thyroid gland: expression, localization and possible function of glutathione peroxidase 3. *Biol Chem*. 2007; 388:1053–1059. [PubMed: 17937619]
94. Hafez A, Squires R, Pedracini A, Joshi A, Seegmiller RE, Oxford JT. Col11a1 Regulates Bone Microarchitecture during Embryonic Development. *J Dev Biol*. 2015; 3:158–176. [PubMed: 26779434]
95. Rodriguez-Fontenla C, Calaza M, Evangelou E, Valdes AM, Arden N, Blanco FJ, Carr A, Chapman K, Deloukas P, Doherty M, et al. Assessment of osteoarthritis candidate genes in a meta-analysis of nine genome-wide association studies. *Arthritis Rheumatol*. 2014; 66:940–949. [PubMed: 24757145]
96. Wu YH, Chang TH, Huang YF, Huang HD, Chou CY. COL11A1 promotes tumor progression and predicts poor clinical outcome in ovarian cancer. *Oncogene*. 2014; 33:3432–3440. [PubMed: 23934190]
97. Noren Hooten N, Martin-Montalvo A, Dluzen DF, Zhang Y, Bernier M, Zonderman AB, Becker KG, Gorospe M, de Cabo R, Evans MK. Metformin-mediated increase in DICER1 regulates microRNA expression and cellular senescence. *Aging Cell*. 2016; 15:572–581. [PubMed: 26990999]
98. Liu N, Landreh M, Cao K, Abe M, Hendriks GJ, Kennerdell JR, Zhu Y, Wang LS, Bonini NM. The microRNA miR-34 modulates ageing and neurodegeneration in Drosophila. *Nature*. 2012; 482:519–523. [PubMed: 22343898]
99. Olivieri F, Spazzafumo L, Santini G, Lazzarini R, Albertini MC, Rippo MR, Galeazzi R, Abbatecola AM, Marcheselli F, Monti D, et al. Age-related differences in the expression of circulating microRNAs: miR-21 as a new circulating marker of inflammaging. *Mech Ageing Dev*. 2012; 133:675–685. [PubMed: 23041385]
100. Chen H, Liu H, Zou H, Chen R, Dou Y, Sheng S, Dai S, Ai J, Melson J, Kittles RA, et al. Evaluation of Plasma miR-21 and miR-152 as Diagnostic Biomarkers for Common Types of Human Cancers. *J Cancer*. 2016; 7:490–499. [PubMed: 26958084]
101. Hutchison ER, Kawamoto EM, Taub DD, Lal A, Abdelmohsen K, Zhang Y, Wood WH 3rd, Lehrmann E, Camandola S, Becker KG, et al. Evidence for miR-181 involvement in neuroinflammatory responses of astrocytes. *Glia*. 2013; 61:1018–1028. [PubMed: 23650073]
102. Xie W, Li M, Xu N, Lv Q, Huang N, He J, Zhang Y. MiR-181a regulates inflammation responses in monocytes and macrophages. *PLoS One*. 2013; 8:e58639. [PubMed: 23516523]
103. Olivieri F, Bonafe M, Spazzafumo L, Gobbi M, Prattichizzo F, Recchioni R, Marcheselli F, La Sala L, Galeazzi R, Rippo MR, et al. Age- and glycemia-related miR-126-3p levels in plasma and endothelial cells. *Aging (Albany NY)*. 2014; 6:771–787. [PubMed: 25324472]
104. Zhou J, Li YS, Nguyen P, Wang KC, Weiss A, Kuo YC, Chiu JJ, Shyy JY, Chien S. Regulation of vascular smooth muscle cell turnover by endothelial cell-secreted microRNA-126: role of shear stress. *Circ Res*. 2013; 113:40–51. [PubMed: 23603512]
105. Ameling S, Kacprowski T, Chilukoti RK, Malsch C, Liebscher V, Suhre K, Pietzner M, Friedrich N, Homuth G, Hammer E, et al. Associations of circulating plasma microRNAs with age, body

- mass index and sex in a population-based study. *BMC Med Genomics*. 2015; 8:61. [PubMed: 26462558]
106. Zhang H, Yang H, Zhang C, Jing Y, Wang C, Liu C, Zhang R, Wang J, Zhang J, Zen K, et al. Investigation of MicroRNA Expression in Human Serum During the Aging Process. *J Gerontol A Biol Sci Med Sci*. 2014
 107. Smith KM, Guerau-de-Arellano M, Costinean S, Williams JL, Bottoni A, Mavrikis Cox G, Satoskar AR, Croce CM, Racke MK, Lovett-Racke AE, et al. miR-29ab1 deficiency identifies a negative feedback loop controlling Th1 bias that is dysregulated in multiple sclerosis. *J Immunol*. 2012; 189:1567–1576. [PubMed: 22772450]
 108. Zhu H, Han C, Lu D, Wu T. miR-17-92 cluster promotes cholangiocarcinoma growth: evidence for PTEN as downstream target and IL-6/Stat3 as upstream activator. *Am J Pathol*. 2014; 184:2828–2839. [PubMed: 25239565]
 109. Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkland SJ, Newman J, Bronson RT, Crowley D, Stone JR, et al. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell*. 2008; 132:875–886. [PubMed: 18329372]
 110. Lai L, Song Y, Liu Y, Chen Q, Han Q, Chen W, Pan T, Zhang Y, Cao X, Wang Q. MicroRNA-92a negatively regulates Toll-like receptor (TLR)-triggered inflammatory response in macrophages by targeting MKK4 kinase. *J Biol Chem*. 2013; 288:7956–7967. [PubMed: 23355465]
 111. Gurtan AM, Sharp PA. The role of miRNAs in regulating gene expression networks. *J Mol Biol*. 2013; 425:3582–3600. [PubMed: 23500488]
 112. Leung AK, Sharp PA. MicroRNA functions in stress responses. *Mol Cell*. 2010; 40:205–215. [PubMed: 20965416]
 113. Sundaram GM, Common JE, Gopal FE, Srikanta S, Lakshman K, Lunny DP, Lim TC, Tanavde V, Lane EB, Sampath P. ‘See-saw’ expression of microRNA-198 and FSTL1 from a single transcript in wound healing. *Nature*. 2013; 495:103–106. [PubMed: 23395958]
 114. Hatse S, Brouwers B, Dalmaso B, Laenen A, Kenis C, Schoffski P, Wildiers H. Circulating MicroRNAs as easy-to-measure aging biomarkers in older breast cancer patients: correlation with chronological age but not with fitness/frailty status. *PLoS One*. 2014; 9:e110644. [PubMed: 25333486]
 115. Jansen F, Wang H, Przybilla D, Franklin BS, Dolf A, Pfeifer P, Schmitz T, Flender A, Endl E, Nickenig G, et al. Vascular endothelial microparticles-incorporated microRNAs are altered in patients with diabetes mellitus. *Cardiovasc Diabetol*. 2016; 15:49. [PubMed: 27005938]
 116. Wang C, Wan S, Yang T, Niu D, Zhang A, Yang C, Cai J, Wu J, Song J, Zhang CY, et al. Increased serum microRNAs are closely associated with the presence of microvascular complications in type 2 diabetes mellitus. *Sci Rep*. 2016; 6:20032. [PubMed: 26831044]
 117. Machida T, Tomofuji T, Ekuni D, Maruyama T, Yoneda T, Kawabata Y, Mizuno H, Miyai H, Kunitomo M, Morita M. MicroRNAs in Salivary Exosome as Potential Biomarkers of Aging. *Int J Mol Sci*. 2015; 16:21294–21309. [PubMed: 26370963]
 118. Fu H, Feng J, Liu Q, Sun F, Tie Y, Zhu J, Xing R, Sun Z, Zheng X. Stress induces tRNA cleavage by angiogenin in mammalian cells. *FEBS Lett*. 2009; 583:437–442. [PubMed: 19114040]
 119. Schaefer M, Pollex T, Hanna K, Tuorto F, Meusbürger M, Helm M, Lyko F. RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage. *Genes Dev*. 2010; 24:1590–1595. [PubMed: 20679393]
 120. Cozen AE, Quartley E, Holmes AD, Hrabeta-Robinson E, Phizicky EM, Lowe TM. ARM-seq: AlkB-facilitated RNA methylation sequencing reveals a complex landscape of modified tRNA fragments. *Nat Methods*. 2015; 12:879–884. [PubMed: 26237225]
 121. Hamilton JA. GM-CSF in inflammation and autoimmunity. *Trends Immunol*. 2002; 23:403–408. [PubMed: 12133803]
 122. Croxford AL, Spath S, Becher B. GM-CSF in Neuroinflammation: Licensing Myeloid Cells for Tissue Damage. *Trends Immunol*. 2015; 36:651–662. [PubMed: 26431942]
 123. Lee M, Dworkin AM, Gildea D, Trivedi NS, Program NCS, Moorhead GB, Crawford NP. RRP1B is a metastasis modifier that regulates the expression of alternative mRNA isoforms through interactions with SRSF1. *Oncogene*. 2014; 33:1818–1827. [PubMed: 23604122]

124. Ambrus A, Adam-Vizi V. Molecular dynamics study of the structural basis of dysfunction and the modulation of reactive oxygen species generation by pathogenic mutants of human dihydrolipoamide dehydrogenase. *Arch Biochem Biophys*. 2013; 538:145–155. [PubMed: 24012808]
125. Ashikov A, Routier F, Fuhrlott J, Helmus Y, Wild M, Gerardy-Schahn R, Bakker H. The human solute carrier gene SLC35B4 encodes a bifunctional nucleotide sugar transporter with specificity for UDP-xylose and UDP-N-acetylglucosamine. *J Biol Chem*. 2005; 280:27230–27235. [PubMed: 15911612]
126. Chen J, Meng Y, Zhou J, Zhuo M, Ling F, Zhang Y, Du H, Wang X. Identifying candidate genes for Type 2 Diabetes Mellitus and obesity through gene expression profiling in multiple tissues or cells. *J Diabetes Res*. 2013; 2013:970435. [PubMed: 24455749]
127. Bem D, Yoshimura S, Nunes-Bastos R, Bond FC, Kurian MA, Rahman F, Handley MT, Hadzhiev Y, Masood I, Straatman-Iwanowska AA, et al. Loss-of-function mutations in RAB18 cause Warburg micro syndrome. *Am J Hum Genet*. 2011; 88:499–507. [PubMed: 21473985]
128. Schaeffer V, Hansen KM, Morris DR, LeBoeuf RC, Abrass CK. RNA-binding protein IGF2BP2/IMP2 is required for laminin-beta2 mRNA translation and is modulated by glucose concentration. *Am J Physiol Renal Physiol*. 2012; 303:F75–82. [PubMed: 22513850]
129. McBride MW, McVie AJ, BurrIDGE SM, Brintnell B, Craig N, Wallace AM, Wilson RH, Varley J, Sutcliffe RG. Cloning, expression, and physical mapping of the 3beta-hydroxysteroid dehydrogenase gene cluster (HSD3BP1-HSD3BP5) in human. *Genomics*. 1999; 61:277–284. [PubMed: 10552929]
130. Simard J, Moisan AM, Morel Y. Congenital adrenal hyperplasia due to 3beta-hydroxysteroid dehydrogenase/Delta(5)-Delta(4) isomerase deficiency. *Semin Reprod Med*. 2002; 20:255–276. [PubMed: 12428206]
131. El-Khoury V, Pierson S, Kaoma T, Bernardin F, Berchem G. Assessing cellular and circulating miRNA recovery: the impact of the RNA isolation method and the quantity of input material. *Sci Rep*. 2016; 6:19529. [PubMed: 26787294]
132. Rekker K, Saare M, Roost AM, Kubo AL, Zarovni N, Chiesi A, Salumets A, Peters M. Comparison of serum exosome isolation methods for microRNA profiling. *Clin Biochem*. 2014; 47:135–138. [PubMed: 24183884]
133. Tanriverdi K, Kucukural A, Mikhalev E, Tanriverdi SE, Lee R, Ambros VR, Freedman JE. Comparison of RNA isolation and associated methods for extracellular RNA detection by high-throughput quantitative polymerase chain reaction. *Anal Biochem*. 2016; 501:66–74. [PubMed: 26969789]
134. Li P, Piao Y, Shon HS, Ryu KH. Comparing the normalization methods for the differential analysis of Illumina high-throughput RNA-Seq data. *BMC Bioinformatics*. 2015; 16:347. [PubMed: 26511205]
135. Raabe CA, Tang TH, Brosius J, Rozhdestvensky TS. Biases in small RNA deep sequencing data. *Nucleic Acids Res*. 2014; 42:1414–1426. [PubMed: 24198247]
136. Risso D, Ngai J, Speed TP, Dudoit S. Normalization of RNA-seq data using factor analysis of control genes or samples. *Nat Biotechnol*. 2014; 32:896–902. [PubMed: 25150836]
137. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res*. 2014; 42:D68–73. [PubMed: 24275495]
138. Badrnya S, Baumgartner R, Assinger A. Smoking alters circulating plasma microvesicle pattern and microRNA signatures. *Thromb Haemost*. 2014; 112:128–136. [PubMed: 24573468]
139. Horvath S. DNA methylation age of human tissues and cell types. *Genome Biology*. 2013
140. Sobala A, Hutvagner G. Transfer RNA-derived fragments: origins, processing, and functions. *Wiley Interdiscip Rev RNA*. 2011; 2:853–862. [PubMed: 21976287]
141. Carthew RW, Sontheimer EJ. Origins and Mechanisms of miRNAs and siRNAs. *Cell*. 2009; 136:642–655. [PubMed: 19239886]
142. Iwasaki YW, Siomi MC, Siomi H. PIWI-Interacting RNA: Its Biogenesis and Functions. *Annu Rev Biochem*. 2015; 84:405–433. [PubMed: 25747396]

143. McMahon M, Contreras A, Ruggero D. Small RNAs with big implications: new insights into H/ACA snoRNA function and their role in human disease. *Wiley Interdiscip Rev RNA*. 2015; 6:173–189. [PubMed: 25363811]
144. Karijolich J, Yu YT. Spliceosomal snRNA modifications and their function. *RNA Biol*. 2010; 7:192–204. [PubMed: 20215871]
145. Kim J, Kim KM, Noh JH, Yoon JH, Abdelmohsen K, Gorospe M. Long noncoding RNAs in diseases of aging. *Biochim Biophys Acta*. 2016; 1859:209–221. [PubMed: 26141605]
146. Rinn JL, Chang HY. Genome regulation by long noncoding RNAs. *Annu Rev Biochem*. 2012; 81:145–166. [PubMed: 22663078]
147. Ulitsky I, Bartel DP. lincRNAs: genomics, evolution, and mechanisms. *Cell*. 2013; 154:26–46. [PubMed: 23827673]
148. Chen I, Chen CY, Chuang TJ. Biogenesis, identification, and function of exonic circular RNAs. *Wiley Interdiscip Rev RNA*. 2015; 6:563–579. [PubMed: 26230526]
149. Salzman J. Circular RNA Expression: Its Potential Regulation and Function. *Trends Genet*. 2016; 32:309–316. [PubMed: 27050930]
150. Kowalski MP, Krude T. Functional roles of non-coding Y RNAs. *Int J Biochem Cell Biol*. 2015; 66:20–29. [PubMed: 26159929]

Figure 1. Schematic of location and packaging of extracellular RNA in circulation
 miRNA maturation from a primary miRNA to a mature miRNA is indicated and its packaging into EVs, cholesterol or bound to AGO2 for release into the circulation. Potential avenues for release of other ncRNAs and mRNAs into the circulation are indicated, but little is known about this process. The main types of circulating cells in the blood are also indicated.

Figure 2. Summary of human bodily fluids containing extracellular RNA
The specific RNA reported and also the body fluid for each extracellular RNA is indicated.

Figure 3. Extracellular miRNA changes in mice with age
(A) Overlapping changes in miRNAs from the indicated studies are shown (B) KEGG pathway analysis was performed for the miRNAs that were changed with age in the various studies.

Figure 4. Extracellular miRNA changes in humans with age
(A) All miRNAs that were increased in the circulation with age from Table 1 were compared to miRNAs that were decreased in the circulation. Overlapping miRNAs are indicated. (B) KEGG pathway analyses were performed on the miRNAs differed in abundance with age in the different studies. (C) KEGG analyses were performed on the miRNAs that were found to be decreased with age in multiple studies.

Expression Change in Old	Study Population/Notes	Method	Reference
Up	Varied timepoints between Young (n=3; 2 dys) and Old (n=3; 25 ms)	RT-qPCR	Li, et al., 2011, <i>Aging</i>
Down	Young (n=3; 7 ms) vs. Old (n=3; 27 ms); [alleviated with CR]	NGS	Dhahbi, et al., 2013, <i>Aging</i>
Up	Young (n=3; 7 ms) vs. Old (n=3; 27 ms); [alleviated with CR]		
Down	Old (n=3; 27 ms) vs. Old CR (n=3; 27 ms)		
Up	Old (n=3; 27 ms) vs. Old CR (n=3; 27 ms)		
Down	Young (n=5; 5–6 ms) vs. Old (n=5; 21–22 ms)	NGS	Victoria, et al., 2015, <i>Aging Cell</i>
Up	Young (n=5; 5–6 ms) vs. Old (n=5; 21–22 ms)		
Down in Ames dwarf mice	Young Ames (n=5; 5–6 ms) vs. Old (n=5; 21–22 ms)		
Up in Ames dwarf mice	Young Ames (n=5; 5–6 ms) vs. Old (n=5; 21–22 ms)		
Down	Group 1 (n=4, 20 yrs) vs. Group 3 (n=3, 100 yrs)	Microarray; RT-qPCR validation of miR-21	Olivieri, et al., 2012, <i>Mech. Ageing Dev.</i>
Up	Group 1 vs. Group 2 (n=4; 80 yrs) or Group 3 (n=3, 100 yrs)		
Up	Group 1 and Group 2 (n=4; 80 yrs) vs. Group 3 (n=3, 100 yrs)		

Expression Change in Old	Study Population/Notes	Method	Reference
Down	Young (n=20; 31 yrs) vs. Old (n=20; 64 yrs)	NGS; RT-qPCR	Noren Hooten, et al., 2013, <i>Aging</i>
Down	Young (n=10; 7.7 yrs) vs. Old (n=10; 21.7 yrs)	RT-qPCR	Olivieri, et al., 2014, <i>Aging</i>
Up	Young (n=44; <45 yrs) vs. Old (n=35; 75 yrs)	RT-qPCR	Ameling, et al., 2015, <i>BMC Med. Genet.</i>
Down	Young vs. Old (Total n=367–370; 22–79 yrs)	RT-qPCR Array	Zhang, et al., 2015, <i>J. Gerontol A Biol. Sci. Med. Sci.</i>
Up	Group 1 (n=52; 22 yrs), Group 2 (n=41; 40 yrs), Group 3 (n=40; 60 yrs), Group 4 (n=40; 70 yrs) (N reflects size of validation cohorts)	NGS; RT-qPCR	Halse, et al., 2014, <i>PLoS One</i>
Down	Young (n=10; 41 yrs) vs. Old (n=20; 78 yrs) Breast Cancer Patients (N reflects validation cohort)	RT-qPCR Array; RT-qPCR Validation	Freedman, et al., 2016, <i>Nat. Comm.</i>
Up	N=2763; Young (<66.5 yrs) vs. Old (>66.5 yrs)	NGS; RT-qPCR	Machida, et al., 2015, <i>Int. J. Mol. Sci.</i>
Up	Young (n=15; 21 yrs) vs. Old (n=13; 66 yrs)	RT-qPCR Array; RT-qPCR Validation	Tietje, et al., 2014, <i>PLoS One</i>
No Change	Young (n=3–6; <2 yrs) vs. Old (n=3–6; >70 yrs)	NGS	

Table 2

Extracellular Small and Large RNAs that Change with Age

Type of RNA Transcript	Species	Biofluid	Gene (s)	Expression Change in Old	Study Population/Notes	Method	Reference
5' tRNA halves	Mouse	Serum	Arg-CCG, Cys-GCA, Gly-GCC, Lys-CTT, and Val-AAC Derived 5' Fragments	Down	Young (n=3; 7 ms) vs. Old (n=3; 27 ms)	NGS	Dhabhi, et al., 2013, <i>BMC Genomics</i>
	Mouse	Serum	His-GTG Derived Fragments	Up			
			Cys-GCA and Lys-CTT Derived 5' Fragments	Down	Young (n=5; 5–6 ms) vs. Old (n=5; 21–22 ms)	NGS	Victoria, et al., 2015, <i>Aging Cell</i>
			His-GTG and Asp-GTC Derived 5' Fragments	Up			
			Pro-AGG Derived 5' Fragments	Down in Ames dwarf mice	Young Ames (n=5; 5–6 ms) vs. Old (n=5; 21–22 ms)		
mRNA	Human	Plasma	None	Up in Ames dwarf mice			
			CSF2RA, DLD, RRP1B, RAB3GAP2, SLC35B4, LAMB2, XM_005258610	Up	Young and Old (n=50; 25–79 yrs, median age 54)	NGS	Yuan et al., 2016, <i>Sci Rep</i>
lncRNA	Human	Plasma	EHHADH-AS1, RP11-696N14.1, AC022311.1	Up	Young and Old (n=50; 25–79 yrs, median age 54)	NGS	Yuan et al., 2016, <i>Sci Rep</i>
PseudoGene	Human	Plasma	HSD3BP4	Up	Young and Old (n=50; 25–79 yrs, median age 54)	NGS	Yuan et al., 2016, <i>Sci Rep</i>
Unclassified	Human	Plasma	C-BRTHA2014909, GNOMON_232972365, GNOMON_232988955	Up	Young and Old (n=50; 25–79 yrs, median age 54)	NGS	Yuan et al., 2016, <i>Sci Rep</i>
piRNA	Human	Plasma	PIR12151,PIR198666,PIR20101,PIR2096,PIR2229,PIR2888,PIR2962,PIR40039,PIR40304,PIR43376,PIR51124,PIR54042,PIR54043,PIR54535,PIR57322,PIR57403,PIR57576,PIR57581,PIR58593,PIR58596	Down	N=2763; Young (<66.5 yrs) vs. Old (>66.5 yrs)	NGS; RT-qPCR	Freedman, et al., 2016, <i>Nat. Comm.</i>
snoRNA	Human	Plasma	SNO1209,SNO1210,SNO1277,SNO1407,SNO1408,SNO1409,SNO1413,SNO1417,SNO1426,SNO1457,SNO1458,SNO1502	Down	N=2763; Young (<66.5 yrs) vs. Old (>66.5 yrs)	NGS; RT-qPCR	Freedman, et al., 2016, <i>Nat. Comm.</i>