**Human and Nonhuman Extracellular RNAs are Widely Expressed in the Circulation**

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**ABSTRACT**

Although much is known about the expression of miRNAs, other forms of bioactive small RNAs have yet to be identified in human populations. Also unknown is whether non-human forms of small RNAs are commonly expressed in the circulation. Limitations in bioinformatic analyses of RNA sequencing have precluded broad assessment of human and non-human RNAs from large numbers of samples. Using a pipeline developed by the NIH Common Fund Extracellular RNA Consortium, we analyzed sequencing data from 40 cohort participants and identified over 1,400 human and non-human RNAs. Using these data, we measured 644 transcripts (473 human and 171 non-human) in an additional 2,822 study participants. We found that many piRNAs, snoRNAs, viral RNAs and plant RNAs are expressed in large numbers of people in variable amounts. We present the first data to demonstrate the broad expression of diverse classes of circulating human and non-human small RNAs in a large population.

**INTRODUCTION**

The discovery of small ribonucleic acids (RNAs), including micro RNAs (miRNAs) has dramatically altered our understanding of gene expression regulation.[1](#_ENREF_1) Extracellular miRNAs are present in a variety of bodily fluids including plasma, urine, and saliva, and these molecules are notably stable and resist degradation despite the presence of RNase.[2](#_ENREF_2),[3](#_ENREF_3) The discovery of stable RNA outside of cells has transformed our understanding of the role RNA may play in cell-to-cell communication and other complex processes.  Additionally, expression of miRNAs has also been associated with a wide variety of diseases.[4-6](#_ENREF_4) Although miRNAs are observed in the extracellular space, little is known about the expression of other common varieties of small human RNAs such as piwi-interacting RNA (piRNA) and small nucleolar RNAs (snoRNAs), known to be key components of molecular interactions and gene regulation in eukaryotes. While viral RNAs have been sporadically identified in the circulation, it is unknown if small noncoding RNAs from plants or viruses are widely expressed in humans.

Based on limited RNA sequencing (RNAseq) data, it has been recently suggested that human extracellular spaces may contain a far broader array of RNAs,[7](#_ENREF_7), [8](#_ENREF_8) however, rapid simultaneous identification of human and nonhuman RNAs has been hampered by bioinformatic limitations. In response to this rapidly evolving area, the National Institute of Health (NIH) Common Fund strategic planning process identified and developed a new program to study extracellular RNAs (exRNAs). A portion of this program was to develop a catalog of exRNAs in human body fluids so as to allow investigation of their role in mechanism, health and disease. A crucial portion of this program was to develop bioinformatic tools and pipelines to assist in the broad identification of exRNAs from RNAseq. As part of the Extracellular RNA Consortium, using these recently developed tools, we were able to broadly identify a wide range of small RNAs using human plasma from 40 participants of a well-characterized cohort study. After identification of established and novel human and nonhuman small RNAs, we performed a secondary study in 2,822 cohort participants revealing the range of expression of these RNAs in the human circulation.

This is the first description demonstrating broad human expression of non-miRNA small RNAs beyond a limited sample size. Previous studies of exRNAs have studied small numbers or pooled samples for the purpose of identifying a class of small RNAs.[9](#_ENREF_9) Human studies have shown distribution of exRNAs, primarily miRNAs. However, many previous studies have been limited and possibly biased by measuring only targeted miRNAs with a limited numbers of patients or participants. Beyond the observation that these human exRNAs are present, our data will complement the current study of small RNAs known to be involved in many molecular interactions, including the regulation of gene expression. The observation that many plant and viral exRNAs are present in the human circulation will require further exploration to understand whether these RNAs are functional or a marker of exposure.

**Gene Expression in Human Plasma by RNAseq**

To determine the broadest number of exRNAs in human plasma, we performed RNAseq on 40 previously stored samples from Framingham Heart Study (FHS) participants (Offspring Cohort Exam 8). The FHS is a community-based, prospective study with cohorts undergoing an examination every ~4-8 years. These participants have been densely phenotyped over multiple prior examinations (Additional study details in Methods). RNA was isolated from the 40 plasma samples and RNAseq performed so as to determine uniquely expressed human and non-human small RNAs including miRNAs and other small exRNAs. The 40 participants of the Offspring Cohort had a mean age of 68, half were female, and had a range of disease-based risk factors (Table 1). Sequencing was performed using an Ion Proton platform (see Methods). Sequencing data was processed in the Genboree Sequencing pipeline and comparative analyses were performed.

Using the analyses of RNAseq data from 40 human plasma samples, we identified a total of 1,401 small RNAs above 1³ RPM. These small RNAs were comprised of 669 miRNAs, 144 piwi-interacting RNA (piRNA), 74 small nucleolar RNA (snoRNA), 305 transfer RNA (tRNA), 190 plant miRNAs, and 19 viral miRNAs. While many plant and viral species were represented by a single exRNA, many expressed multiple RNAs (Tables 2a, 2b, Supplemental Table 1). We noted a wide range of types of grasses as well as variable numbers of transcripts expressed per species. In addition to grasses, transcripts mapped to dietary sources (Table 2a) were identified and included 34 for rice, 22 for soybeans, 5 for papaya, 5 for barley, and 4 for apple, in addition to others.

As expected, miRNAs were widely and abundantly expressed. MiR-223-3p and miR-451a were the most abundant in plasma. While there was wide distribution in abundance of miRNAs, miR-223-3P and miR-451a contributed to 47% of all plasma sequencing readings and their dominance could have resulted in false negative findings for some low abundant targets. However, despite this, an extraordinary range of human, non-human and non-miRNA transcripts were identified. We identified 144 piRNAs. Although not previously known to be widely found in the circulation, piRNAs form RNA-protein complexes by interacting with piwi proteins and these complexes may regulate either epigenetic or post-transcriptional gene silencing. They differ from miRNAs in size (26–31 nt rather than 21–24 nt).[10](#_ENREF_10) We found 74 snoRNAs and, although limited information is available in regards to their expression in human tissue, they have been previously reported by RNAseq.[11](#_ENREF_11) Limited snoRNAs have been shown in lung tissue and plasma from patients with non-small cell lung cancer[12](#_ENREF_12) and in a small cohort of patients with multiple myeloma.[13](#_ENREF_13)

In addition to plant RNAs, we noted several viral miRNAs (Table 2b). Notably, all but one, were dsDNA viruses. The dsDNA viruses were of the Herpesviridae family except for the herpes B virus that was represented by three transcripts. This is consistent with previous plasma studies examining unmapped sequences from RNAseq that showed a fraction of the circulating RNA appear to originate from exogenous species[8](#_ENREF_8) including viruses.[14-16](#_ENREF_14) Levels of specific plasma EBV miRNAs have previously been identified in infected patients[17](#_ENREF_17) although our data does not allow for identification of actively infection in participants.

**Human/Non-Human exRNAs in 2,822 Individuals**

From the RNAseq data, we developed a target exRNA list (n=666) to complete plasma gene expression measurements using high-throughput RT-qPCR (Fluidigm BioMark system, Methods)[18](#_ENREF_18), [19](#_ENREF_19) in 2,822 remaining participants of the Offspring 8 cohort (Table 3). All piRNAs, snoRNAs, viral and plant miRNAs, and the most highly expressed human miRNAs transcripts from the 40 plasma samples were studied by RT-qPCR. This included 339 human miRNAs, 104 human piRNAs, 43 human snoRNAs, and 180 non-human RNAs (plant, viral).

Examining this diverse data together demonstrates variable expression in terms of number of exRNAs expressed in plasma in each participant as well as overall percent expressed (Figure 1). Of the 339 human miRNAs that were measured (Supplementary Table 2), expression ranged from a Ct value of 12 (abundant) to 21 (lesser abundant). Most Ct values were in the 18-20 range. More abundant miRNAs such as hsa-miR-486-5p (Ct 14.8) and has-miR-451a (Ct 12) were expressed in almost all participants. However, many lower abundant human miRNAs had broad expression as well. In addition to a wealth of data related to miRNA expression, we found other human exRNAs expressed broadly within this population. This is consistent with sequence analysis from a single human plasma samples that showed a diverse collection of the exosomal RNA species among which microRNAs (miRNAs) were the most abundant, making up over 42% of all raw reads.[11](#_ENREF_11)

Examination of other human exRNAs shows variable expression of piRNAs and snoRNAs (Table 4a). Of the 104 human piRNAs measured in 2822 participants, expression ranged from Ct values of 13 to 21, although several of the most abundant piRNAs were expressed in only a few individuals. Nine piRNAs were expressed in over 2000 participants. Compared to the human miRNA data, this is the first study to show both ubiquitous and targeted expression of a broad number of piRNAs in a human population. For the 43 snoRNAs measured in the population (Table 4a), expression was generally less as compared to the piRNAs (Ct values of 18-20). Numbers of participants expressing specific snoRNAs ranged from 56 to 2773. While several piRNAs were expressed in less than 50 individuals, this was not the case for snoRNAs.

In addition to novel human exRNAs, we measured non-human species in 2822 participants. These non-human exRNAs had widely ranging percent expression in this human population (Figure 1). While some plant miRNAs were highly abundant, in general, most were relatively low abundant with specific Ct values ranging from 8-21 (Table 4b). We measured 21 distinct miRNAs from rice. For rice, variable human expression was noted with one exRNA, osa-miR2919, highly abundant in plasma. A miRNA (pgi-miR6136a2) from ginseng was only found in 43 individuals but was highly abundant. Several miRNAs from the Loblolly pine (*Pinus taeda*), were found and one, pta-miR1310 was highly expressed in 217 individuals (Table 4b). *Brachypodium distachyon* (bdi-miR7713-5p), a common grass species related to the major grain species’, was both highly and ubiquitously expressed.

Viral miRNAs ranged in expression from Ct values of 15.9 to 21 with most having lower levels of expression (Table 4b). Percent expressed (Figure 1) ranged from 2 to 2809 participants. The most abundant, Marek's disease virus, the causative agent for avian herpesvirus was only expressed in 11 participants. A miRNA from infectious laryngotracheitis virus, also an avian virus, was expressed in nearly the entire cohort. Human dsDNA viruses including Epstein Barr virus, Herpes B virus and human cytomegalovirus were expressed in several hundred participants.

**Association of exRNAs with Clinical Variables (ERIC to provide additional data)**

Participants of the Offspring 8 Cohort were primarily older with a mean age of 67 and were 54% female (Table 3). There was a range of disease risk factors representative of this age range in the U.S. population. Less than 25% had documented heart disease. To determine if age, sex, body mass index or blood pressure were associated with expression of either human or non-human exRNAs, we used the normalized dCt and compared to age and sex. We also examined BMI adjusted for age and sex, and SBP and DBP using a regression model also adjusting for age and sex. Multiple comparisons corrections were performed within each predictor and within each type (Supplementary Table 3). Although the individual associations were not highly significant, patterns by species appear to emerge (Supplementary Table 3). Age appears to be ubiquitously associated with exRNAs, however, only snoRNAs are consistently associated with BMI. Several plant-derived exRNAs, as well as some piRNAs, appear to be associated with diastolic, but not systolic, blood pressure.

Although FHS is not a dietary study, a limited food frequency questionnaire was available from the 8th visit, concordant with when blood was drawn for the plasma samples. We evaluated the association of food intake with potentially dietary derived exRNAs. Intake data was available only for rice and apples and we detected no association with exRNA expression. Although 2 exRNA transcripts for tobacco plant were expressed (Figure 1), no association was noted with a history of smoking.

**DISCUSSION**

This is the first description demonstrating broad human expression of non-miRNA small RNAs beyond a limited sample size. Previous studies in plasma have focused on the expression of miRNAs, essential exRNAs that regulate the expression of messenger RNAs encoding factors.[20](#_ENREF_20), [21](#_ENREF_21) Their abundant expression and known bidirectional transport between cells and plasma is important as they are evolutionarily widespread, and participate in a wide range of genetic regulatory pathways.[1](#_ENREF_1) By regulating cell fate choices and transitions between pluripotency and differentiation, miRNAs help to orchestrate developmental events and to play a role in tissue homeostasis important for disease.[22-24](#_ENREF_22) Previous studies have shown that cellular circulating transcripts are associated with cardiovascular disease and its risk factors[4](#_ENREF_4), [5](#_ENREF_5), [18](#_ENREF_18), [25-29](#_ENREF_25) and future studies utilizing this data set can begin to explore the role of pRNAs, snoRNAs, and non-human miRNAs. Although the source of circulating exRNA remains unclear, the cellular secretion of unique miRNA suggests process specificity.[30](#_ENREF_30) [31-33](#_ENREF_31)

While our findings are consistent with previous studies demonstrating prevalent miRNAs in plasma, this is the first study to report widespread non-miRNAs in the human circulation. Despite extensive expression of piRNAs and snoRNA in the participants, their significance to homeostasis or disease is unknown. The observation that nonhuman exRNAs are widely, albeit sporadically, present in human plasma is intriguing. These data provide the first description of plant-based exRNA in a human population. While these data do not establish any form of direct transfer from food sources, the ubiquity of the expression is fascinating. Many of the plant sources have multiple miRNAs expressed (i.e. soy, rice) while others are sporadic and only at low levels. The presence of many grasses and non-edible plants, some known to be allergens, leads to speculation for possible pulmonary transfer. How the plant-based exRNAs arose in the circulation or if functional is not clear from these data and the limited data available addressing this issue is contentious. While, in worms, there is evidence for horizontal transfer,[34](#_ENREF_34) dietary transfer of RNA in humans is not established. Functional studies suggest that rice MIR168a could inhibit LDLRAP1 expression.[35](#_ENREF_35) However, subsequent studies have cast doubt about these findings demonstrating ineffective delivery of diet derived miRNAs to recipient animals.[36-39](#_ENREF_36)

The possibility of contamination when exogenous RNAs are found in human samples is always a concern but, in the current data set, contamination is unlikely as few plant or viral exRNAs are ubiquitously or uniformly expressed. Additionally, all RNA isolation, RNAseq and RT-qPCR were performed in a single laboratory using a specially designed clean room. Although this does not exclude the possibility of some contamination, the breadth of targets and number of samples make contamination highly unlikely as the cause of these findings. In addition, the concern of “sequence” contamination is mitigated by the use of RT-qPCR in a large number of samples. There are limitations to this study including an older population and poor ethnic diversity. However, future studies as part of this consortium will process samples from several ethnically and racially diverse groups as well as younger populations.

A limited number of studies have demonstrated by RNAseq, a wide variety of exRNAs in humans but the presence of nonhuman RNAs has been questioned. This expansive study demonstrates widespread, variable expression of novel human and nonhuman small exRNAs. Whether these are contributing to to cellular homeostasis or can be associated with disease pathobiology is not yet known. With broad patterns of expression, it is likely that these intriguing exRNAs are contributing, not in isolation, but in combination to regulate gene expression and influence cellular function.

**METHODS**

**Study Population:** The FHS Offspring Study is a community-based, prospective study of CVD and its risk factors. Cohorts undergo an examination at the FHS once every ~4-8 years and have been densely phenotyped over multiple prior examinations with a wide variety of noninvasive tests. Participants in the Offspring Study have been examined every 4-8 years since the 1970s, for 8 prior exams. The participants have an extraordinary wealth of antecedent clinical data available allowing us to examine the relation of disease and risk factors to gene expression.

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**RNA Isolation from Plasma:** RNAs samples were isolated from plasma using a miRCURY RNA Isolation Kit –Biofluids (Cat. No: 300112, Exiqon, Denmark). Isolation protocol was as follow;

Blood samples previously collected at Framingham Heart Study (Framingham, MA, USA) for Offspring exam 8 (March 2005 - Jan. 2008). Venipuncture was performed on study participants in a supine position after an overnight fast, using standard venipuncture techniques.  Blood was collected into blood collection tubes with a liquid buffered sodium citrate additive (0.105M). Blood collection tubes were centrifuged at 2500g for 22 minutes at 4 C.  Plasma was separated from the cells and frozen at -80 C within 90 minutes of draw.

An aliquot of 170 µl of plasma samples transferred to our laboratory (University of Massachusetts Medical School) in March 2014 and stored at -80°C. Freezers were generator and CO2 system were backed up.

RNA isolation:

1. After thawing, the plasma samples tubes were mixed by inverting. Plasma samples were centrifuged at 2,000 x g for 10 minutes. One hundred thirty µl of plasma samples from the top of the tubes were transferred into a new 2.0 mL DNA LoBind tubes (Cat. No: 022431048, Eppendorf, Germany) and 70 µl Nuclease-Free water added to each sample to bring the volume to 200 µl.
2. Sixty µl Lysis Solution BF were added to each sample.
3. Tubes were vortexed for 15 seconds.
4. Tubes were incubated for 5 minutes at room temperature.
5. Twenty µL Protein Precipitation Solution BF were added into each tube and tubes were vortexed for 15 seconds.
6. Vortexed tubes were incubated for 1 minute at room temperature.
7. Tubes were centrifuged for 3 minutes at 16,000 x g.
8. Clear supernatants were transferred into a clean 2.0 mL DNA LoBind tubes.
9. Two hundred seventy µL Isopropanol was added into each tube and tubes were vortexed for 5 seconds.
10. Tubes were centrifuged briefly (2-3 seconds) to collect all liquid at the bottom.
11. microRNA Mini Spin Columns BFs connected to vacuum manifold (QIAvac 24 Plus, Cat. No: 19413, Qiagen, Germany) by using VacValves (Cat. No: 19408, Qiagen, Germany).
12. Samples were transferred into microRNA Mini Spin Columns BFs using transfer pipettes.
13. Samples were incubated for 2 minutes at room temp on microRNA Mini Spin Column BFs with open lids.
14. Vacuum pump was turned on until all liquids passed.
15. Seven hundred µL Wash Solution 2 BF (80 mL Absolute ethanol was added to Wash Solution 2 BF bottle) was added on microRNA Mini Spin Column BFs and vacuum pump was turned on until all liquids passed.
16. Two hundred fifty µL Wash Solution 2 BF was added on microRNA Mini Spin Column BFs and vacuum pump was turned on until all liquids passed.
17. Hundred µL Wash Solution 1 BF was added on microRNA Mini Spin Column BFs and vacuum pump was turned on until all liquids passed.
18. Seven hundred µL Wash Solution 2 BF was added on microRNA Mini Spin Column BFs and vacuum pump was turned on until all liquids passed.
19. Two hundred fifty µL Wash Solution 2 BF was added on microRNA Mini Spin Column BFs and vacuum pump was turned on until all liquids passed.
20. microRNA Mini Spin Column BFs were transferred to collection tubes (2.0mL without a lid).
21. Tubes were centrifuged for 2 minutes at 11,000 x g at room temperature to dry the membranes completely.
22. Centrifuged microRNA Mini Spin Column BFs were transferred into 1.5mL DNA LoBind tubes (conical bottom tubes) and 30 µL Nuclease-Free water added directly onto membranes.
23. Tubes were incubated for 1 minute at room temp with lids open.
24. Lids were closed and centrifuged for 1 minute at 11,000 x g at room temperature.
25. RNA elutions were transferred into the V bottom, snap cap, 0.5 mL micronic tubes and capped. 2D barcodes on the tubes were recorded and RNA samples were kept at -80°C.

**Library preparation for RNA Sequencing:** Ion Total RNAseq Kit v2 (Cat. No: 4479789, Life Technologies, USA) was used to create libraries for sequencing.

Hybridize and ligate the RNA

1. On ice, the hybridization master mix was prepared as follow;

Component Volume for 1 Reaction

Ion Adaptor Mix v2 2 μl

Hybridization Solution 3 μl

Total volume 5 μl

2. Five μl of hybridization master mix was added onto 3 μl small RNA sample.

3. Hybridization solution and master mix were pipetted up and down 5 times to mix, then centrifuged briefly to collect the liquid in the bottom of the tube.

4. Hybridization reaction was performed in a thermal cycler as below

Temperature Time

65°C 10 min

16°C 5min

5. Ligation master mix was prepared on ice as follow;

Component Volume for 1 Reaction

2X Ligation Buffer 10 μl

Ligation Enzyme Mix 2 μl

Total volume 12 μl

6. Twelve μl of ligation master mix was added to each 8 μl hybridization reaction, for a total of 20 μl per reaction.

7. Ligation solution and hybridization mixture were pipetted up and down 10 times to mix, then centrifuged briefly to collect the liquid in the bottom of the tube.

8. Ligation reactions was performed in a thermal cycler at 16°C for 2–16 hours.

Reverse Transcription

1. Reverse transcription master mix was prepared on ice as follow;

Component Volume for 1 Reaction

Nuclease-Free Water 2 μl

10X RT Buffer 4 μl

2.5 mM dNTP Mix 2 μl

Ion RT Primer v2 8 μl

Total volume 16 μl

2. Sixteen μl of RT master mix and 20 μl ligation reaction were mixed and incubated in a thermal cycler with a heated lid at 70°C for 10 minutes, then snap-cooled on ice.

3. Four μl of 10X SuperScript III Enzyme Mix was added to each ligated RNA sample and gently mixed.

4. Mixture were incubated in a thermal cycler with a heated lid at 42°C for 30 minutes.

Purification of cDNA Using MagMAX Beads

1. Five μl beads were added to one well of a 96-well plate for each sample.

2. Two hundred fifty μl Binding Solution Concentrate to each well containing beads, and mixed by pipetting up and down 10 times.

3. Sixty μl nuclease-free water were added to each of the 40 μl RT reactions and transferred to one of the wells of the 96-well plate.

4. Two hundred seventy five μl of 100% ethanol were added to each well and mixed by pipetting up and down.

5. Plate was placed on magnetic stand and hold for 5 minutes.

6. Supernatant was removed carefully without disturbing the beads.

7. One hundred fifty μl Wash Solution Concentrate added and incubated for 30 seconds.

8. While plate was on the magnetic stand Wash Solution Concentrate was removed.

9. Any leftover Wash Solution Concentrate was removed by using 10 μl pipet.

10. Beads were air dried for 2 minutes and plate was removed from magnetic stand.

11. Twelve μl pre-warmed (37°C) nuclease-free water was added on beads.

12. Beads were mixed by pipetting up and down for 10 times and incubated off the magnetic stand for 1 minute.

13. Plate was moved to magnetic stand and held for 1 minute. Purified cDNA removed carefully and transferred into a DNA LoBind 0.5 mL tube.

Amplification of cDNA

1. PCR mixture was prepared according to table below.

Component Volume for 1 Reaction

Platinum PCR SuperMix High Fidelity 45 μl

Ion Xpress RNA 3’ Barocde Primer 1 μl

Total volume 46 μl

2. One μl of selected Ion Xpress RNA-Seq Barcode was added into each sample tube.

3. Six μl of cDNA samples were added into each tube. Tubes were mixed and thermal cycling performed on ProFlex 3x32 block Thermal Cycler (Life Technologies, USA) according to the program described below.

Stage Temperature Time Cycle

Hold 94°C 2 min 1

Cycle 94°C 30 sec. 2

50°C 30 sec.

68°C 30 sec.

Cycle 94°C 30 sec. 16

62°C 30 sec.

68°C 30 sec.

Hold 68°C 5 min.

Purification of Amplified cDNA Using MagMAX Beads

1. Five μl beads were added to one well of a 96-well plate for each sample.

2. Two hundred eighty μl Binding Solution Concentrate to each well containing beads, and mixed by pipetting up and down 10 times.

3. Twenty seven μl nuclease-free water were added to each of the 52 μl Amplified cDNA reactions and transferred to one of the wells of the 96-well plate.

4. Two hundred thirty μl of 100% ethanol were added to each well and mixed by pipetting up and down.

5. Plate was placed on magnetic stand and held for 5 minutes.

6. Supernatant was removed carefully without disturbing the beads.

7. One hundred fifty μl Wash Solution Concentrate added and incubated for 30 seconds

8. While plate was on the magnetic stand Wash Solution Concentrate was removed.

9. Any leftover Wash Solution Concentrate was removed by using 10 μl pipet.

10. Beads were air dried for 2 minutes and plate was removed from magnetic stand.

11. Ten μl pre-warmed (37°C) nuclease-free water was added onto beads.

12. Beads were mixed by pipetting up and down for 10 times and incubated off the magnetic stand for 1 minute.

13. Plate was moved to magnetic stand and hold for 1 minute. Purified Amplified cDNA removed carefully and transferred into a DNA LoBind 0.5 mL tube.

**Template Preparation for RNA Sequencing:** Ion Chef System and Ion PI IC 200 kits were used for template preparation. The entire procedure was automated by the Ion Chef System. At the end of the template preparation loaded PI Chips (Life Technologies, USA) were ready.

**RNA Sequencing on Ion Proton System:** Sequencing reactions were performed on Ion PI Chip Kit v2 BC and the Ion Proton System (Life Technologies, USA) by using Ion PI Sequencing 200 Kit v2 (Life Technologies, USA). Sequencing reads were maximum 200 nucleotides.

**Sequencing Data Analysis Using the Genboree Sequencing Pipeline:**

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**Comparative Analyses of RNAseq Data from the FHS Offspring 8 Participants:**

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**RT-qPCR Analyses:**

RNA samples (2,822) were reverse transcribed by using miScript II RT Kit (Cat. No: 218161, Qiagen, Fredrick, MD, USA) for measuring human miRNAs and snoRNAs. For plant RNAs, virus RNAs as well as piRNAs miScript Plant RT Kit (Cat. No: 218762, Qiagen, Fredrick, MD, USA) was used.

Reverse Transcription for human miRNAs and snoRNAs

RNA samples (2,822) were reverse transcribed by using miScript II RT Kit (Cat. No: 218161, Qiagen, Fredrick, MD, USA) for measuring human miRNAs and snoRNAs. For plant RNAs, virus RNAs as well as piRNAs miScript Plant RT Kit (Cat. No: 218762, Qiagen, Fredrick, MD, USA) was used.

1. RNA samples thawed on ice. Reverse-transcription master mix was prepared on ice according to table below.

Component Volume

5x miScript HiSpec Buffer 950 μl

10x miScript Nucleics Mix 475 μl

Nuclease-free water 475 μl

miScript Reverse Transcriptase Mix 475 μl

Total volume 2,375 μl

This mixture was enough to prepare 4x96 samples.

2. Five μl of this mixture was dispensed into each well of a 96 well PCR plate (Cat. No: AB17500, Bioplastic, Netherland). Five μl of RNA samples were transferred into each well by using Vioflo384 Pipetting System (Integra Biosciences, Hudson, NH, USA) with 96 well head. Plates were sealed with EU optical Wide 8-Cap Strip Mats (Cat. No: B57651, Bioplastic, Netherland).

3. Plates were vortexed on plate shaker for 10 seconds and then centrifuged for 1 minute at 1,000 rpm on a small plate centrifuge.

4. Plates were incubated at 37ºC for 60 minutes and at 95ºC for 5 minutes at ProFlex 96 block Thermal Cycler (Life Technologies, USA).

5. When reverse transcriptase reactions were completed, plates were centrifuged for 1 minute and plate mats opened and 40 μl nuclease-free water were added into each well by using Vioflo384 Pipetting System (Integra Biosciences, Hudson, NH, USA) with 96 well head.

6. Plates were sealed with EU optical Wide 8-Cap Strip Mats (Cat. No: B57651, Bioplastic, Netherland) and vortexed at plate shaker for 10 seconds and then centrifuged for 1 minute at 1,000 rpm on a small plate centrifuge.

Ligation and Reverse Transcription for plant miRNAs, virus miRNAs and human piRNAs

1. RNA samples thawed on ice. Ligation master mix was prepared on ice according to table below.

Component Volume

10x miScript Ligation Buffer 950 μl

10x miScript Plant Adaptor 950 μl

Nuclease-free water 475 μl

Total volume 2,375 μl

This mixture was enough to prepare 4x96 samples.

2. Five μl of this mixture were dispensed into each well of a 96 well PCR plate (Cat. No: AB17500, Bioplastic, Netherland). Five μl of RNA samples transferred into each well by using Vioflo384 Pipetting System (Integra Biosciences, Hudson, NH, USA) with 96 well head.

3. Ten μl of 2x miScript Ligation Activator was pipetted onto each well very slowly by using Vioflo384 Pipetting System (Integra Biosciences, Hudson, NH, USA) with 96 well head. Entire volume were mixed by using Vioflo384 Pipetting System (Integra Biosciences, Hudson, NH, USA) with 96 well head ten times. Plates were sealed with EU optical Wide 8-Cap Strip Mats (Cat. No: B57651, Bioplastic, Netherland).

4. Plates were centrifuged for 1 minute at 1,000 rpm on a small plate centrifuge.

5. Plates were incubated at 16ºC for 60 minutes and at 65ºC for 20 minutes at ProFlex 96 block Thermal Cycler (Life Technologies, USA).

6. Reverse-transcription master mix was prepared on ice according to table below.

Component Volume

5x miScript Plant RT Buffer 1,900 μl

10x miScript Plant RT Nucleics 950 μl

Nuclease-free water 4,275 μl

miScript Plant Reverse Transcriptase 475 μl

Total volume 7,600 μl

This mixture was enough to prepare 4x96 samples

7. Sixteen μl of this mixture was dispensed into each well of a 96 well PCR plate (Cat. No: AB17500, Bioplastic, Netherland). Four μl of Ligation products were transferred into each well by using Vioflo384 Pipetting System (Integra Biosciences, Hudson, NH, USA) with 96 well head. Entire volume were mixed by using Vioflo384 Pipetting System (Integra Biosciences, Hudson, NH, USA) with 96 well head ten times.

8. Plates were sealed with EU optical Wide 8-Cap Strip Mats (Cat. No: B57651, Bioplastic, Netherland). Plates were centrifuged for 1 minute at 1,000 rpm on a small plate centrifuge.

9. Plates were incubated at 37ºC for 2 hours and at 95ºC for 5 minutes at ProFlex 96 block Thermal Cycler (Life Technologies, USA).

5. When reverse transcriptase reactions were completed plates were centrifuged for 1 minute and plate mats opened and 80 μl nuclease-free water were added into each well by using Vioflo384 Pipetting System (Integra Biosciences, Hudson, NH, USA) with 96 well head.

6. Plates were sealed with EU optical Wide 8-Cap Strip Mats (Cat. No: B57651, Bioplastic, Netherland) and vortexed at plate shaker for 10 seconds and then centrifuged for 1 minute at 1,000 rpm on a small plate centrifuge.

Preamplification of cDNAs (this step was same for both type cDNAs)

1. Preamplification master mix was prepared on ice according to table below.

Component Volume

5x miScript PreAMP Buffer 1,425 μl

HotStarTaq DNA Polymerase 570 μl

miScript PreAMP Primer Mix 1,425 μl

RNase-free water 1,995 μl

miScript PreAMP Universal Primer (10 μM) 285 μl

Total volume 5,700 μl

2. Twelve μl of this mixture were dispensed into each well of a 96 well PCR plate (Cat. No: AB17500, Bioplastic, Netherland). Three μl of diluted cDNA samples transferred into each well by using Vioflo384 Pipetting System (Integra Biosciences, Hudson, NH, USA) with 96 well head. Plates were sealed with EU optical Wide 8-Cap Strip Mats (Cat. No: B57651, Bioplastic, Netherland).

3. Plates were vortexed on plate shaker for 10 seconds and then centrifuged for 1 minute at 1,000 rpm on a small plate centrifuge.

4. Plates were cycled on at ProFlex 96 block Thermal Cycler (Life Technologies, USA) according the conditions below.

Stage TIME TEMP Cycle

PCR initial activation step 15 min 95°C 1

3-step cycling 2

Denaturation 30 sec. 94°C

Annealing 60 sec. 60°C

Extension 60 sec. 60°C

2-step cycling 10

Denaturation 30 sec. 94°C

Annealing/ Extension 3 min. 60°C

5. When preamplification reactions were completed plates were centrifuged for 1 minute and plate mats opened and 2 μl SR1 (side reaction reducer = Exonuclease I) added into each well by using Vioflo384 Pipetting System (Integra Biosciences, Hudson, NH, USA) with 96 well head.

6. Plates were sealed with EU optical Wide 8-Cap Strip Mats (Cat. No: B57651, Bioplastic, Netherland) and vortexed at plate shaker for 10 seconds and then centrifuged for 1 minute at 1,000 rpm on a small plate centrifuge.

7. Plates were cycled on at ProFlex 96 block Thermal Cycler (Life Technologies, USA) according the conditions below.

Temperature Time

37°C 15 min

95°C 5min

8. When SR1 reactions were completed plates were centrifuged for 1 minute and plate mats opened and 58 μl nuclease-free water added into each well by using Vioflo384 Pipetting System (Integra Biosciences, Hudson, NH, USA) with 96 well head.

9. Plates were sealed with EU optical Wide 8-Cap Strip Mats (Cat. No: B57651, Bioplastic, Netherland) and vortexed at plate shaker for 10 seconds and then centrifuged for 1 minute at 1,000 rpm on a small plate centrifuge.

Real-Time PCR miRNA Profiling Using miScript miRNA PCR Arrays

Assay plates preparation

1. Human miRNA Assays were purchased from Qiagen in dried down format. Assay plates were centrifuged at 2,000 rpm for 10 minutes.

2. Twenty seven add half μl nuclease-free water was added into each well and incubated at room temperature for 110 minutes. Plates were vortexed in a plate shaker for 3 minutes at room temperature at 1,400 rpm.

3. These assay were at 100 μM concentrations. Eighteen μl of these assays were collected in a reservoir (up to 384 assays) to make preamplification primer pool.

4. 14.25 μl nuclease-free water was added onto remaining 9.5 μl 100 μM assays. Total volume of these assays were 23.75 μl and concentrations were 40 μM. Equal volume of miScript Microfluidics Universal Primer (40 μM) added onto these assays and volume became 47.5 μl and concentrations were 20 μM.

5. Equal volume of Assay Loading Reagents (Cat. No: 100-7611, Fluidigm, South San Francisco, CA, USA) (47.5 μl) were added onto these assays. This concentration and condition assays were ready to load into Dynamic Arrays.

6. Twenty eight μl aliquots were made in 96 well Piko plates (Cat. No: SPL0960, Fisher Scientific, USA), sealed and stored at -20°C.

7. snoRNA assays, Plant miRNA assays, virus miRNA assays and piRNA assays were custom designed by Qiagen and synthized primers were delivered in 100 μM concentration in liquid form.

8. These assays were also prepared as described above.

Dynamic Array Preparations for qPCR

1. Control fluids were injected into specific positions on two Dynamic Array 96.96 GE and Dynamic Arrays were placed into IFC Controller HX and prime started. While priming was performed (took about 20 minutes) qPCR reagents prepared on ice according to table below.

Component Volume

Microfluidics qPCR Master Mix (Qiagen) 720.00 µl

20x DNA Binding Dye Sample Loading 72.00 µl

Reagent (Fluidigm, PN 85000746)

RNase-free water168.00 µl

TOTAL 960.00 µl

2. Four μl of this mixture were dispensed into each well of a 96 well PCR plate (Cat. No: AB17500, Bioplastic, Netherland). Two μl of diluted Preamplified cDNA samples transferred into each well by using Vioflo384 Pipetting System (Integra Biosciences, Hudson, NH, USA) with 96 well head. Plates were sealed with EU optical Wide 8-Cap Strip Mats (Cat. No: B57651, Bioplastic, Netherland).

3. Plates were vortexed on plate shaker for 10 seconds and then centrifuged for 1 minute at 1,000 rpm on a small plate centrifuge.

4. Five μl of this mixture was aspirated by using Viaflo 8 channel electronic pipet and dispensed into sample inlets of Dynamic Arrays as 4.75 μl in order to not to create any bubbles.

5. 4.75 μl assays were aspirated by using Viaflo 8 channel electronic pipet and dispensed into sample inlets of Dynamic Arrays as 4.50 μl in order to not to create any bubbles.

6. Dynamic Arrays were placed into IFC Controller HXs and standard Load script was started. This took 90 minutes.

7. When the Loading script finished Dynamic Arrays were loaded to BioMark Reader to perform cycling as described below.

8. ROX is used as a passive reference dye. Dissociation curve analysis was also performed.

Stage Temperature Time Cycle

Thermal Mix 2 min 50°C 1

30 min 70°C 1

10 min 25°C 1

PCR Initial activation step 10 min 95°C 1

3-step cycling 23

Denaturation 15 sec. 94°C

Annealing 30 sec. 55°C

Extension 30 sec. 70°C

Perform fluorescence data

Collection and dissociation curve analysis

Linear derivative and user global setting (0.002) were used as setting parameters and samples and assays names were entered and data analyses performed on BioMark Real Time PCR Analysis software. Then, Cq values were exported as csv files.

**Analysis of RT-qPCR Data:**

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**Acknowledgements**

This work was supported by N01-HC 25195, P01-HL085381 (to J.E. Freedman);  UH2TR000921 and U01HL126495 (to J.E. Freedman), are supported by the NIH Common Fund, through the Office of Strategic Coordination/Office of the NIH Director.

**Author Contributions**. Overall project development and coordination: J.E.F. K.T. coordinated the project’s laboratory studies and writing of laboratory methods. Project development and RNA isolation quality control: R.L. and V.A. Analyses for RNAseq and development of pipeline: M.G., J.R. and R.K. Analyses for RT-qPCR: E.M. Laboratory assistance, sample processing, and quality control: S.T. and E.M. Sample and FHS study: D.L. Writing: J.E.F. All authors commented on the interpretation of results, and reviewed and approved the final manuscript.

**Author Information**. The Authors declare no competing financial interests.

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