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SPECIAL ISSUE: EXTRACELLULAR RNA COMMUNICATION CONSORTIUM

# Extracellular RNAs: development as biomarkers of human disease

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Ten ongoing studies designed to test the possibility that extracellular RNAs may serve as biomarkers in human disease are described. These studies, funded by the NIH Common Fund Extracellular RNA Communication Program, examine diverse extracellular body fluids, including plasma, serum, urine and cerebrospinal fluid. The disorders studied include hepatic and gastric cancer, cardiovascular disease, chronic kidney disease, neurodegenerative disease, brain tumours, intracranial haemorrhage, multiple sclerosis and placental disorders. Progress to date and the plans for future studies are outlined.

Keywords: *ERCC*; *exRNA*; *extracellular RNA*; *biomarkers*

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Clinical research across the spectrum of human disease is challenged by the immense cost of testing experimental therapeutics in human subjects. In every subspecialty of medicine, there is a need for surrogate markers of disease for the purposes of identifying subjects at risk, documenting "target engagement" and objectifying treatment outcomes in a manner that will permit "proof of concept" trials with feasible numbers of subjects and a reasonable duration of treatment. A targeted approach to identification and validation of such biomarkers has been successful in some arenas, such as the use of glycosylated haemoglobin for monitoring diabetes mellitus or use of quantitative viral load for monitoring HIV therapy. Non-targeted empirical approaches using proteomics and metabolomics have also seen some success, so it follows logically that microRNAs (miRNAs) are now being evaluated as biomarkers in a variety of human diseases. It remains to be seen whether miRNAs will be

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more informative or more useful than protein biomarkers, particularly as new methodologies make it possible to focus on exosomal proteins rather than total protein, and on exosomes derived from specific organs (1). A discussion of the utility of exosomal protein biomarkers is beyond the scope of this review, but the emphasis here on miRNA should not be interpreted as a conclusion that we have no more to learn from protein biomarkers. The focus on miRNAs is simply a reflection of the nature of the consortium being reviewed here. As described elsewhere in this issue, the NIH Common Fund Extracellular RNA Communication Program has funded 10 UH2/UH3 grants to identify extracellular RNA (exRNA) biomarkers in conditions ranging from gastric cancer to Alzheimer's disease (AD), relying on body fluids ranging from saliva to cerebrospinal fluid (CSF). This funding mechanism is relatively unique within the NIH, funding "high risk," "discovery" work over the first 2 years in a "UH2" phase, with each project advancing to more traditional NIH-funded hypothesis testing in years 3–5 of "UH3" funding, dependent on the attainment of predefined milestones. Each of these projects is currently in a "discovery" phase after about 1 year of activity. We review here the rationale, methods and progress of the UH2 projects, summarized in Table I. Both the table and the following text are listed according to organ system studied and the relevant body fluid analyzed, as depicted in Fig. 1. Since clinical phenotyping of the samples is so critical to the success of the projects, the clinical cohort for each is also indicated.

## Biomarkers of cancer

Even laypeople are familiar with the use of serum prostate specific antigen as a useful biomarker for both detection and response to therapy of prostate cancer, and several other protein biomarkers of cancer are currently employed, so it follows that miRNA biomarkers might be used productively in oncology. Two of the funded grants focus on identification of miRNA biomarkers of cancer, in hepatocellular and gastric cancer, respectively.

### *Biomarkers of hepatocellular cancer*

In "Extracellular Non-Coding RNA Biomarkers of Hepatocellular Cancer" (TR000884), Dr. Patel and colleagues at Mayo Clinic Jacksonville Florida are studying plasma biomarkers of hepatocellular carcinoma (HCC), making the point that HCC is the second most common cancer world-wide, with a global incidence of $\sim$750,000 new patients per year. The incidence of these cancers has been increasing in the United States as a consequence of the epidemics of chronic hepatitis C virus infection, obesity and non-alcoholic steatohepatitis increasing the prevalence of cirrhosis, which is the most important risk factor for HCC. Survival from this cancer remains poor, despite the fact that the population at risk (cirrhosis patients) is readily identified. Early diagnosis can reduce mortality of HCC, as resection and liver transplantation can be curative if disease is sufficiently localized when diagnosed. For example, the 5-year recurrence-free survival in patients with small tumours without macroscopic vascular invasion is over 80%. However, most patients with HCC are diagnosed at an advanced stage when these surgical procedures cannot be performed, and the survival of patients with HCC continues to be grim with 1- and 3-year survival rates of 29 and 8%. Screening and surveillance for HCC in patients at risk can consequently reduce mortality from HCC by early diagnosis, but the currently available biomarkers are not adequate. For example, the currently used tumour marker, alpha-fetoprotein (AFP), is elevated in less than 50% of HCC patients. Other blood-based biomarkers such as des-gamma-carboxy prothrombin (DCP) and lectin-reactive alpha-fetoprotein (AFP-L3) also lack the sensitivity to sufficiently detect early stage cancer amenable to surgical cure. Preliminary studies have demonstrated the feasibility of developing miRNA biomarkers of HCC by documenting miRNA dysregulation in these cancers (2,3), and by demonstrating exRNA in the circulation of persons with HCC (4).

#### *Biomarkers of hepatocellular cancer: approach*

The overall goals of this project are to identify and qualify circulating exRNA-based biomarkers in order to detect early stage HCC in patients at risk and to monitor response to therapy. A two-phase biomarker development approach is being undertaken. The goal of the first UH2 phase is to identify and develop assays for exRNA candidates with potential for further study as biomarkers for HCC, whereas the goals of the second UH3 phase will be to validate the clinical utility of promising exRNA candidates identified from the first phase.

#### *Biomarkers of hepatocellular cancer: progress to date*

A systematic search to identify, characterize and quantify long intergenic non-coding RNAs (lincRNAs) that are expressed in HCC, and non-coding exRNA that are selectively associated with HCC cells has been performed. To identify lincRNA associated with HCC, whole transcriptome deep-sequencing data from HCC samples obtained through The Cancer Genome Atlas were analyzed using a custom-derived bioinformatics pipeline. Candidate lincRNAs were further examined for evidence of transcriptional activity using the H3K4me3-H3K36me3 domains generated from the ENCODE project. Studies to date have identified candidate exRNA that are released by HCC cells in culture and can be detected in the circulation in patients with HCC. The expression of both miRNA and a selected panel of long ncRNA has been examined in a panel of both non-malignant (HH and THLE-2 cells) and malignant (Hep3B, HepG2, PLC/PRF/5, SNU-182 and SNU-398) hepatocytes, and in extracellular vesicles (EVs) released from these cells in culture. These screening profiling studies were done using quantitative polymerase chain reaction


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*Table I.* ERCC biomarker projects

| PI(s)/site | Grant title | Organ system/biofluid | Clinical cohort |
|---|---|---|---|
| Patel/Mayo Clinic Jacksonville | Extracellular Non-Coding RNA Biomarkers of Hepatocellular Cancer | Hepatobiliary/serum and plasma | Mayo Hepatobiliary Neoplasia Biorepository |
| Wong/UCLA | Clinical Utility of Salivary ExRNA Biomarkers of Gastric Cancer Detection | Gastrointestinal/saliva | UCLA PRoBE |
| Das/Beth Israel Deaconess | Plasma miRNA Predictors of Adverse Mechanical and Electrical Modeling after MI | Cardiac/plasma | PROSPECT-CMR Cohort; Thrombolysis in Myocardial Infarction (TIMI) |
| Freedman/University of Massachusetts | Extracellular RNAs – Biomarkers for Cardiovascular Risk and Disease | Cardiac/plasma | Framingham Heart Study |
| Laurent/University of California San Diego | ExRNAs for Early Identification of Pregnancies at Risk for Placental Dysfunction | Placenta/serum | UCSD Placental Dysfunction Study; PAPR Cohort from Sera Prognostics |
| Saugstad, Quinn/Oregon Health & Science University | Clinical Utility of MicroRNAs As Diagnostic Biomarkers of Alzheimer's Disease | Central nervous system/ CSF | Oregon ADC Biorepository; Alzheimer's Disease Neuroimaging Initiative |
| Carter, Hochberg/University of California San Diego | ExRNA Biomarkers for Human Glioma | Central nervous system/ CSF and plasma | UCSD Biorepository |
| Van Keuren-Jensen, Huentelman, Adelson, Kalani/Translational Genomics Research Institute | ExRNA Signatures Predict Outcomes after Brain Injury | Central nervous system/ CSF and plasma | Barrow Neurological Institute Biorepository; Biologic Materials Availability Program at Phoenix Children's Hospital |
| Weiner, Gandhi/Brigham and Women's Hospital | Circulating MicroRNAs As Disease Biomarkers in Multiple Sclerosis | Central nervous system/ plasma | CLIMB MS Cohort |
| Tuschl, Suthanthiran/Rockefeller | Clinical Utility of Extracellular RNA As Marker of Kidney Disease Progression | Renal/urine | Multiple Longitudinal Cohorts |

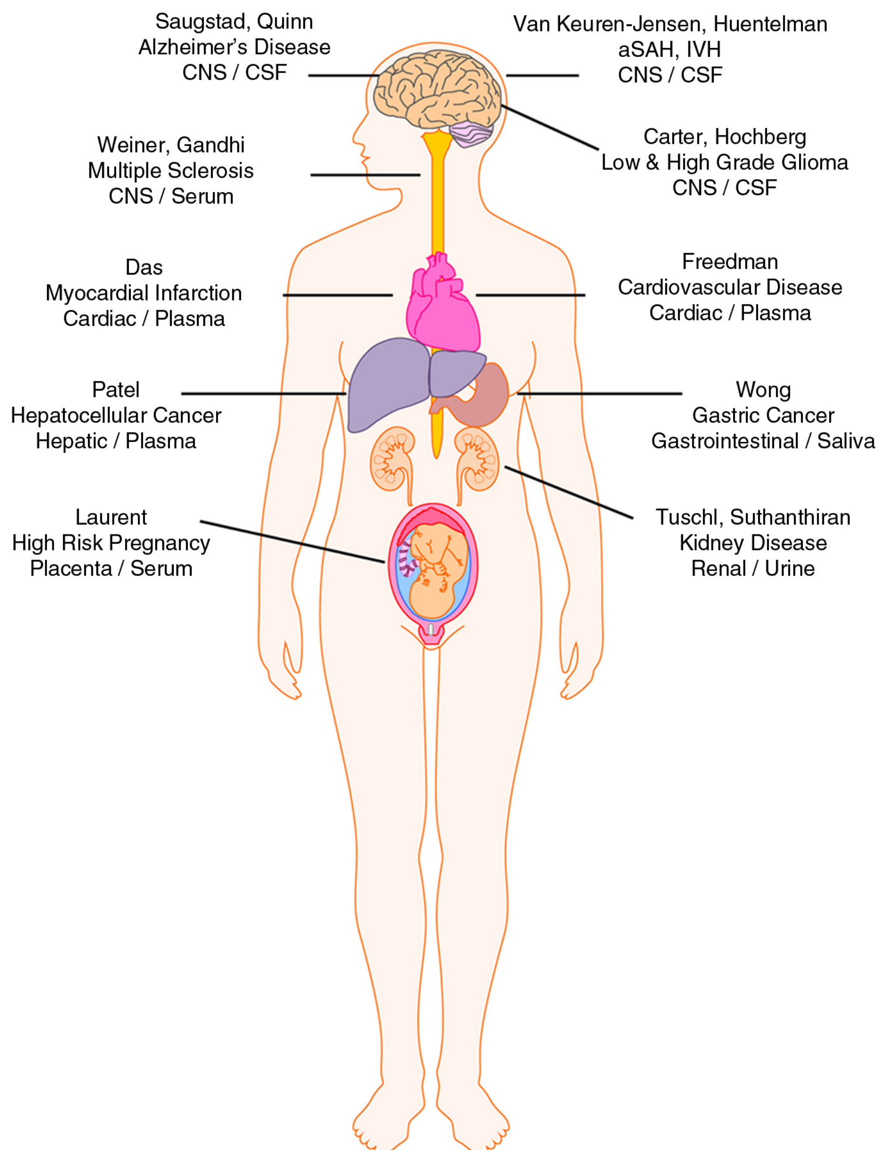


*Fig. 1.* ERCC biomarker groups.

(PCR) based assays. On average, HCC cells released a larger amount of exRNA compared to normal hepatocytes (average of 1.23% vs. 0.55% of total donor cell RNA). For selected samples, expression of 800 miRNAs and 33 lncRNAs was also analyzed using NanoString. These analyses identified 14 lncRNAs and 33 miRNAs that were selectively increased in HCC cells compared to normal hepatocytes, and enriched within exRNA preparations from these cells. The expression of selected non-coding RNA was then examined using different assay platforms, namely quantitative PCR (qPCR), NanoString, or droplet digital PCR (ddPCR) to directly compare these assays for the detection of extracellular non-coding RNA. Three miRNA (miR-25, miR-302d and miR-612) were not detected by qPCR assays but were identified in exRNA preparations from all HCC cells, as well as from serum obtained from 6 patients with HCC by NanoString and ddPCR. Sensitive assays based on quantitative and digital PCR for detection of exRNA are now being developed and validated for use in the UH3 validation studies.

## Biomarkers of gastric cancer

In "Clinical Utility of Salivary ExRNA Biomarkers for Gastric Cancer Detection" (TR000923), Dr. Wong and colleagues at the University of California Los Angeles take a similar approach to developing biomarkers for early detection in gastric cancer. A key difference in this study is the focus on saliva as the source of exRNA biomarkers. Dr. Wong and colleagues are particularly suited to this effort, since their research group discovered the presence of a transcriptome in cell-free saliva (CFS) in 2004 (5). More than 3,000 different types of mRNAs were discovered by gene expression-based microarrays. Of the 3,000

mRNAs, 185 mRNAs were consistently detected in healthy subjects, forming what is now called the normal salivary core transcriptome (NSCT) (6). Since then, saliva has become an emerging biofluid poised for translational and clinical applications. Dr. Wong's group has established a robust platform for studying salivary mRNA that involves extraction, purification, amplification, high-throughput (HT) microarray screening and most recently a direct assay for salivary transcriptome analysis without the need for mRNA extraction (7). They have also developed statistical and informatics capabilities that are customized for salivary biomarker discovery and validation (8). The nature, origin and characterization of salivary mRNAs have been studied extensively. These include salivary exRNA characterizations (9) including cDNA library analysis (10), direct salivary transcriptome analysis (7), with the most recent comprehensive sequencing platform revealing 3 major class of exRNA in saliva: mRNA, miRNA (11) and snoRNA (12). These accomplishments provide the scientific foundation for utilizing saliva for clinical applications, specifically for early detection and diagnosis. Salivary extracellular transcriptomic biomarkers have been successfully applied for detection of oral squamous cell carcinoma (OSCC) (5,13), Sjögren syndrome (14), pancreatic cancer (15), breast cancer (16), lung cancer (17) and ovarian cancer (18), strengthening the plausibility of using this approach to biomarkers for gastric cancer.

The key rationale for selecting gastric cancer in this study is the existence of a biorepository of saliva samples from 500 cases of gastric cancer and non-gastric cancer in collaboration between Dr. Wong's group and the Samsung Medical Center. These are carefully annotated clinical samples that are procured based on the prospective-specimen-collection and retrospective-blinded-evaluation (PRoBE) design using a standard operating procedure (SOP). This existing clinical resource allowed for the immediate exploration of biomarker discovery in the UH2 phase, followed by a definitive validation of discovered biomarker candidates using independent clinical samples that will permit individual marker validation, panel configuration and then validation of the biomarker panel using additional independent clinical samples.

### *Biomarkers of gastric cancer: approach*

In the UH2 phase, comprehensive RNA sequencing (RNA-Seq) will be performed on 100 randomly selected gastric cancer saliva samples and 100 randomly selected non-gastric cancer matched control subjects. Candidate salivary exRNA biomarkers will be selected and ranked, and then verified using qPCR in the discovery set of clinical samples (100/100). Only verified candidates will be advanced to UH3 phase for validation. In the UH3 phase, additional saliva samples will be accrued from 750 gastric cancer patients and 750 non-gastric cancer matched controls. Individual biomarkers will then be validated by association (odds ratio) with gastric cancer, and a multimarker prediction model will be constructed for diagnosis of gastric cancer using saliva from 500 randomly selected gastric cancer and 500 randomly selected non-gastric cancer matched control subjects. Validated individual salivary RNA biomarkers will be configured to the most discriminatory panel by logistic regression analysis. Finally, the newly configured salivary exRNA biomarker panel for gastric cancer detection will be validated in an independent set of 250 gastric cancer and 250 non-gastric cancer matched control subjects.

### *Biomarkers of gastric cancer: progress to date*

RNA isolation techniques were optimized for salivary RNA isolation efficiency by systematically comparing 6 commercially available kits with optimized protocols: (a) organic extraction method (Trizol LS); (b) spin filter based method [QIAamp Viral (Qiagen), NucleoSpin (Clontech) and miRVana (Life Technologies)]; and (c) combined method of organic extraction and spin filter clean up (miRNeasy Micro Kit (Qiagen), Quick-RNA MicroPrep (Zymo Research). The quantity and size distributions of the resulting RNA samples were assessed using RiboGreen reagent and Bioanalyzer, respectively, with the best yields from NucleoSpin and miRNeasy Micro kits. qPCR and ddPCR were used to determine the efficiency of long and small RNA isolation from each kit; the studies revealed that the miRNeasy micro Kit and NucleoSpin are the best kits in yielding small RNAs at the same time as long RNAs.

Library construction kits were also compared to evaluate the performance of alternative methods for library construction, using multiple commercially available kits targeting different types of RNA. The results showed that the New England Biolabs (NEB) small RNA-Seq kit and the NEB directional RNA-Seq kit generated the most reproducible, sensitive and affordable profiling of respective types of mRNAs and ncRNAs in CFS. As of today, a total of 200 small RNA libraries and 200 long RNA libraries have been constructed with extracted salivary exRNA samples from 100 gastric cancer patients and 100 non-gastric cancer matched controls. All libraries were sequenced using Illumina HiSeq 2,000 sequencers at the UCLA core facility.

Comprehensive profiling of exRNAs in human CFS from healthy individuals was optimized according to previous methods (19). A total of 127–418 miRNAs and 32–109 piwi-interacting RNAs (piRNAs) were detected in CFS, and the abundance of CFS miRNAs was highly similar to that in other body fluids based on a comparative analysis of public datasets of different origins. Intriguingly, piRNAs are more abundant than those in most intracellular or extracellular samples but similar to those in embryonic stem cells and skin cells. Furthermore,

a customized bioinformatics method identified 400 circular RNA (circRNAs) in CFS, representing the first global characterization and experimental validation of circRNAs in any type of extracellular body fluid (19).

## Biomarkers of myocardial risk and injury

Cardiology is another medical subspecialty that already employs protein biomarkers in clinical practice, with the use of serum creatine phosphokinase (CPK) and troponin in the diagnosis of myocardial infarction (MI) as the best-established examples. Two of the grants in this program seek to expand the search for useful biomarkers of cardiovascular disease (CVD) to include exRNAs.

### *Biomarkers of post-MI risk*

In "Plasma MiRNA Predictors of Adverse Mechanical and Electrical Remodeling after Myocardial Infarction" (TR000901), Drs. Das, Shah, Danielson, Kwong, Rosenzweig and Sabatine at Beth Israel Deaconess and Brigham and Women's Hospital proposed to identify plasma RNA markers which distinguish "good" from "bad" cardiac remodellers after MI, with the goal of using such markers to stratify risk of adverse outcomes and thereby target intervention to individuals at highest risk. Dramatic advances in the management of acute coronary syndromes (ACS) have led to a significant decline in acute mortality from MI. However, there continues to be significant morbidity and mortality from the sequelae of MI. The adverse mechanical and electrical remodelling that occurs subsequent to an MI underlies the development of heart failure (HF) and sudden cardiac arrest (SCA), respectively. With 300,000 cases of SCA and 550,000 cases of HF diagnosed annually (20), these adverse outcomes in post-MI patients represent a leading cause of mortality in the United States and a significant economic burden. Although prophylactic therapy for SCA with implantable defibrillators and early treatment of HF with medications or devices improves clinical outcomes, these therapies are deployed based on markers that are inadequately robust to identify all patients at risk (21–23) (e.g. overall left ventricular function) or require invasive study (electrophysiology study). In addition, only a minority (2–5% per year) of patients who qualify for defibrillator implantation have ventricular arrhythmias resulting in SCA. Therefore, development of novel markers of adverse remodelling may improve the sensitivity and specificity for predicting adverse outcomes post-MI and may help reduce morbidity and mortality.

Towards this end, Das and colleagues have conducted studies to identify, validate and investigate the pathobiology of specific exRNAs involved in myocardial remodelling in HF. In a registry of patients with HF undergoing biventricular pacemaker implantation (a therapy for HF) (24), they identified a miRNA signature of beneficial response to pacing therapy based on screening a panel of known miRNAs. Analysis of the data with a non-hierarchical clustering algorithm demonstrated that responders to coronary reperfusion therapy (CRT) could be segregated from non-responders and that 5 candidate miRNAs were significantly different between the two groups. Of these miRNAs, miR-30d was identified as dysregulated in canine models of HF (25) (with improvement after biventricular pacing). Furthermore, in a cohort of over 50 patients with HF undergoing biventricular pacing, miR-30d was independently associated with favourable response in cardiac function after biventricular pacing. These data suggested that the use of validated animal models in prioritizing biomarkers based on a mechanistic role in disease pathology may be useful in yielding robust candidates that validate prospectively. The overall goal of this proposal is to perform an unbiased profiling of circulating plasma exRNAs to develop a signature of adverse mechanical and electrical remodelling in post-MI patients, complemented by in-depth animal models to help identify the most robust markers for further investigation and validation. In addition, these signatures will then be developed into a rapid, multiplex digital PCR-based assay for clinical translation. The exRNA profiles will be readily available to the scientific community and may provide therapeutic targets that may be exploited in synergy with other investigators. These results will lay the foundation for a novel strategy of risk-stratification for post-MI patients that will allow for identification of patients at high risk of adverse electrical and mechanical remodelling, who may benefit from more aggressive monitoring and interventions with medications and defibrillators.

#### *Biomarkers of post-MI risk: approach*

To achieve the goal of identifying novel circulating plasma miRNAs that are markers for electrical and mechanical remodelling in post-MI patients, this study will utilize patients with blood collection and cardiac magnetic resonance imaging (for cardiac structure and function) 2–4 weeks post-MI and subsequently 6 months post-MI. Within this cohort, matched populations of individuals can be defined with beneficial and poor cardiac remodelling (by cardiac volumes and function; *mechanical remodelling*) and those who have died from suspected or known SCA (*electrical remodelling*). For biomarker discovery, next-generation sequencing approaches (RNA-Seq) will be used, in order to take advantage of the ability to detect novel exRNAs and low abundance transcripts relative to a traditional microarray platform (26). In the next phase, 20–40 candidate miRNAs will be carried forward to a validation phase, wherein (a) The biological/mechanistic significance of each candidate will be confirmed by use in ischemia-reperfusion models of murine MI; (b) Larger validation studies will be conducted in a cohort of more than 270 individuals (PROSPECT-CMR, PI Dr. Raymond Kwong) with concurrent cardiac magnetic resonance imaging and blood collection early and

late post-MI (to define roles in remodelling and sudden death susceptibility); and finally (c) The clinical significance of these miRNA biomarkers will be validated in a large cohort of over 4,000 patients in the Thrombolysis in Myocardial Infarction-36 study (TIMI).

*Biomarkers of post-MI risk: progress to date*

In concert with other NIH Extracellular RNA Communication Consortium (ERCC) members, Das and colleagues have optimized RNA isolation from plasma, assessed the influence of library preparation methods on RNA-Seq reads and explored pipelines for RNA-Seq mapping and analysis. Using these optimized techniques, RNA-Seq on a sample of patients with favourable and poor left ventricular remodelling post-MI has identified a significant number of initial potential targets for validation. Several miRNAs with known function in HF and CVD have been detected, including miR-30d (in addition to miR-423-5p; -92a; 451; 21; and 378). In addition, further mechanistic studies on miR-30d have confirmed its role in cardioprotection (against apoptosis and hypoxia-mediated cardiac stress). In the coming months, biomarker discovery will be completed, and the project will proceed to validation in animal models and then in patients carefully phenotyped by cardiac magnetic resonance early and late post-MI, and in a larger clinical study of patients in the TIMI group. This research occurs across the backdrop of a renaissance of exRNA research in CVD (27–33). Multiple groups have embarked on the task of identifying, validating and proving the biological significance of a host of exRNAs implicated in cardiac remodelling and risk. By using a discovery cohort that consists of patients with careful phenotyping of electrical and mechanical remodelling, and prioritizing candidates based on possible functional roles in cardiac remodelling, these studies are likely to identify novel prognostic biomarkers and provide additional groundwork for a better understanding of the role of non-coding RNAs in heart disease.

### Biomarkers of asymptomatic atherosclerosis and pre-MI risk

In "Extracellular RNAs: Biomarkers of Cardiovascular Risk and Disease" (TR000921), Drs. Freedman, Tanriverdi and colleagues at the University of Massachusetts Medical School extend the search for biomarkers of cardiovascular risk into the population of asymptomatic individuals. CVD and stroke are two of the leading causes of morbidity and mortality among US adults and are strongly determined by CVD risk factors including hypertension, diabetes, obesity, smoking and dyslipidemia. Specific exRNAs regulate key processes central to the pathogenesis of CVD. Several small human studies have associated exRNAs with CVD and several of its risk factors. This project postulates that circulating plasma levels of exRNAs (a) vary over time even in disease-free individuals, (b) are associated with the duration and burden of CVD risk factors, (c) relate to subclinical atherosclerosis and (d) are useful predictors of longitudinal changes in CVD risk factors and incident CVD events.

*Biomarkers of pre-MI risk: approach*

Using the Framingham Heart Study (FHS) cohort, this project will further examine and validate the diagnostic and prognostic utility of exRNAs associated with CVD and preclinical CVD phenotypes in the community and in a hospitalized acute coronary syndrome sample. The FHS is a community-based, prospective study of CVD and its risk factors. Beginning in 1971, the offspring study enrolled 5,124 participants who were the children of the original FHS cohort, and their spouses. All cohorts undergo an examination at the FHS once every ~4–8 years. Cohorts have been remarkably densely phenotyped over multiple prior examinations with a wide variety of non-invasive tests of cardiovascular structure and function.

The UH2 phase will use an optimized non-commercial isolation method for high-yield plasma RNA extraction. Investigators conducted HT sequencing to identify known and as-yet undiscovered circulating exRNAs, thereby developing a broad panel of plasma-derived exRNA biomarkers (including miRNA, piRNA and other non-coding RNAs) in FHS participants. The specific goals are to: (a) isolate exRNA from human blood plasma samples using advanced fractionation methods (endosomes, proteins, lipids) and develop methods optimized for HT and robotic handling so as to transition to clinical populations, (b) perform HT RNA-Seq on previously stored plasma from FHS participants (Offspring Exam 8) including those with/without CVD so as to identify an initial working panel of ~588 plasma-expressed exRNA sequences, consisting of all those that appear to distinguish disease from disease-free states, as well as a set of invariant normalization controls, (c) use HT qRT-PCR assays to validate and refine the initial working panel of candidate exRNA biomarkers identified by RNA-Seq in Goal 2. In this study, exRNA levels (~588) will be quantified in samples of stored plasma from 2,914 participants of the FHS Offspring Cohort (8th visit). Data from these qRT-PCR profiles will be analyzed with other clinical data (risk factors and contemporaneously measured mRNA/protein expression) previously obtained and available for all participants and (d) using data from these goals, begin development of target-specific probes attached to commercially developed hydrogel particles and determine the feasibility of measuring specific panels of exRNA in clinical samples.

Because it is necessary to prioritize exRNAs for further examination, 40 samples from the FHS cohort ($N = 10$ each of males and females with and without CVD) were selected to be sequenced, and a large set of unbiased exRNAs has been identified. After RNA sequencing of

40 FHS participants and identification of targets, gene expression measurements will be performed using HT qRT-PCR. Plasma exRNA expression will be examined in almost 3,000 samples, and relationships will be examined between exRNA expression and cardiovascular risk factors, as well as measured subclinical atherosclerotic disease phenotypes (including coronary artery calcification; and carotid intimal medial thickness) and to prevalent CVD (n = ~435; including MI, unstable angina, sudden death, HF and stroke). The final portion of the UH2 project will use a method that utilizes unique post-hybridization ligation to fluorescently label bound miRNA targets. Based on findings from the efforts described above, the investigators will develop multiplexed assays to further evaluate clinical samples.

#### *Biomarkers of pre-MI Risk: progress to date*

The investigators have sequenced 20 CVD and 20 matched non-CVD plasma samples using an Ion Proton platform in-house in our laboratory. Sequencing data were processed in the Genboree Sequencing pipeline with the assistance of Drs. Gerstein, Rozowsky, Navarro and Kitchen from Yale University, and comparative analysis was performed. These data show that, (a) plasma RNA-Seq analysis shows that large numbers of miRNAs, small nucleolar RNA (snoRNAs), transfer RNA (tRNAs) and piRNAs exist in human plasma samples, (b) non-human RNAs are also detected by using the small exRNA-Seq pipeline on Genboree. Although only one miRNA was discriminatory between the groups, the data provide an expansive list for further study in the rest of the Offspring 8 cohort. Based on these data, the investigators are measuring close to 700 exRNAs in the full FHS Offspring 8 Cohort as described above. RNA isolations of these samples are completed and HT RT-PCR is being performed.

## Biomarkers of placental dysfunction

Placental dysfunction, most commonly manifested as preeclampsia or intrauterine growth restriction, is an important cause of maternal and foetal morbidity and mortality in both the developing and developed world. It is thought that placental dysfunction arises from abnormal trophoblast differentiation and/or invasion, events that occur in the first trimester of pregnancy, but become clinically apparent only in the late second and third trimesters. Optimal surveillance and management of placental dysfunction, as well as the development of effective therapies, have been hampered by the lack of methods for early and accurate identification of pregnancies at risk for this disorder.

### *Biomarkers of placental dysfunction*

In "ExRNAs for Early Identification of Pregnancies at Risk for Placental Dysfunction" (TR000906), Dr. Laurent and colleagues at UC San Diego aim to develop exRNA-based methods that can be used in early gestation to identify pregnancies that will develop clinically significant placental dysfunction.

#### *Biomarkers of placental dysfunction: approach*

Building upon knowledge that exRNAs of placental origin can be found in the maternal circulation, the first phase of the project is aimed at identification of candidate exRNA biomarkers from a prospectively collected high-risk cohort, the UCSD Placental Dysfunction Study. In the second phase, these candidate biomarkers will then be validated in a separate average-risk cohort, the 5,500 member Proteomic Assessment of Preterm Risk (PAPR) cohort from Sera Prognostics. Finally, a Clinical Laboratory Improvement Amendments (CLIA) certified assay will be developed and applied in a pilot randomized control trial to assess the clinical and cost impact of screening a general obstetric population for risk of placental dysfunction, and referral of screen-positive patients to a higher level of surveillance.

#### *Biomarkers of placental dysfunction: progress to date*

In initial experiments, the investigators have used serum samples from healthy non-pregnant and healthy pregnant women collected at different gestational ages to select the RNA isolation and small RNA sequencing method for use in samples from the UCSD Placental Dysfunction Study. Preliminary data show that differences between non-pregnant and pregnant samples become significantly more pronounced in the late second trimester, so biomarker discovery will be performed on samples collected at 24 weeks of gestation. Thus far, the research team has completed enrolment of 300 high-risk subjects in the UCSD Placental Dysfunction Study. A total of 160 of these subjects have delivered and had their clinical outcomes adjudicated. From these adjudicated subjects, small RNA sequencing has been performed for 20 cases and 32 controls. Analysis of this dataset is in process, while clinical outcome data from the remainder of the subjects is being collected for selection of a second set of samples for analysis.

## Biomarkers of central nervous system disease

Four of the grants probe the central nervous system, examining exRNA content of CSF and plasma. CSF has the advantage of being in direct contact with the central nervous system, so the blood brain barrier does not prevent the CSF from reflecting extracellular CNS biology. However, as CSF collection is an invasive procedure, patients prefer peripheral blood sampling consequently resulting in larger numbers of subjects included in a given study. One proposal studying neurodegenerative disease relies primarily on CSF, two other proposals examined both CSF and plasma in brain tumours and intracerebral haemorrhage, while the 4th study relies on plasma samples alone, since multiple sclerosis is known to be reflected in immune cells from the peripheral blood.


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### Biomarkers of neurodegenerative disease

In "Clinical utility of MicroRNAs as Diagnostic Biomarkers of Alzheimer's Disease" (TR000903), Drs. Saugstad, Quinn and colleagues at Oregon Health and Science University examine the utility of exRNA in CSF from AD patients. AD, characterized by brain pathology including amyloid plaques and neurofibrillary tangles, is the most common neurodegenerative disease, affecting 10–15% of individuals between age 75 and 85, and up to 50% of those over age 85. As the population ages, AD is becoming more prevalent and represents a looming public health crisis, described by some as "the disease of the century." The need for improved biomarkers of AD is substantial, as the clinical diagnosis of AD is confirmed in only 90% of patients with overt dementia, meaning that clinical trials are confounded by mis-diagnosis in 10% of the population, even in the case of established dementia. The need is even more acute as interventions are applied in prodromal states like mild cognitive impairment (MCI) (34) or even pre-symptomatic or latent AD (35). Nuclear medicine imaging of cerebral amyloidosis has been an important and useful advance in this area, but these PET scans are prohibitively expensive and are limited to the single feature of amyloid deposition. CSF protein biomarkers of classical AD pathology, namely beta amyloid 1–42 (the chief component of plaques) and total and phosphorylated tau (the chief component of neurofibrillary tangles) have also been utilized to enrich study populations for clinical trials, but the sensitivity and specificity are not optimal, and variability across laboratories has also been problematic (34). The plausibility of developing exRNAs as AD biomarkers is supported by several exploratory clinical studies (36–40). However, variability in results reported to date illustrates the importance of attending to details of sample collection, RNA isolation and RNA quantification, in addition to careful phenotyping of the subject population. This project consequently uses CSF collected under standardized conditions, from carefully characterized subjects sampled in a uniform morning fasting condition, with specimens aliquoted in uniform fashion, snap frozen and stored at -80 degrees until use (41).

#### Biomarkers of neurodegenerative disease: approach

The UH2 phase will utilize CSF banked at the Oregon Alzheimer's Disease Center (OADC) Biorepository for the "discovery" phase of the project. The expression of all known human miRNAs will be determined using Taqman miRNA qRT-PCR arrays on existing CSF samples isolated from healthy subjects and AD patients in the OADC. Candidate biomarkers will be determined by statistical analysis and then verified using customized arrays with the discovery sample. The verified miRNA biomarkers will then be validated in a new set of CSF samples isolated from healthy subjects and AD patients obtained from the OADC. The UH3 phase will utilize CSF from the NIA-funded Alzheimer's Disease Neuroimaging Initiative (ADNI) to test the performance of candidate biomarkers in the ADNI CSF bank of approximately 100 asymptomatic elderly, 200 MCI and 100 AD subjects.

#### Biomarkers of neurodegenerative disease: progress to date

Preliminary studies were performed to optimize RNA isolation and to determine the effects of storage time and CSF gradient upon outcomes. Total RNA recovered from samples stored for 1 year, 5 years and 10 years was comparable, with no indication of loss of total RNA over time. Expression of miRNA was comparable in samples that were stored for different lengths of time, with ~206 miRNAs from an array of 756 probes detected in CSF at all three time points. These preliminary experiments were important for confirming that banked, frozen CSF samples stored for up to 10 years would be suitable for this work, optimizing the number of samples available for the discovery phase. After these preliminary methodological studies, CSF miRNA expression was compared in 47 AD and 47 healthy control subjects, matched for age and sex. A panel of 19 candidate miRNAs that are differentially expressed in the two groups has been identified for further analysis. Combinations of 3–4 miRNA distinguish AD from control subjects more effectively than individual miRNAs. Interestingly, most of the miRNAs distinguishing between the two groups are downregulated in AD compared to healthy control subjects. Confirmation of this AD signature is currently being performed using a panel of miRNAs in custom qRT-PCR assays. The UH3 validation phase in ADNI samples is scheduled to begin in fall 2015.

### Biomarkers of brain tumours

In "ExRNA Biomarkers for Human Glioma" (TR000931), Drs. Carter, Hochberg and colleagues at UC San Diego are testing the hypothesis that exRNA in CSF will include markers that will help diagnose and subtype primary brain tumours. Glioblastoma (GBM) is the most common and aggressive primary brain tumour, with a 2-year survival rate less than 20% (42). Several subtypes of GBM have been identified (43–46) and in some cases subtyping of glioma can guide treatment decisions. Since MRI imaging does not adequately distinguish GBM from other tumours, diagnosis and subtyping currently depend on biopsy and tissue diagnosis. However, brain biopsy is challenging in some cases due to inoperable localization of lesions, and the diffusely infiltrative nature of GBM makes it difficult to ensure that any biopsy will include representative tissue. Indeed in the United States, it is estimated that one in 5 brain tumour patients never undergoes a diagnostic operation and only 30% experience surgery that benefits their outcome. Biopsies do not improve morbidity or survival, inadequately predict histopathologic diagnosis and seldom take into account

topographic tumour heterogeneity (47–49). Current non-surgical diagnostic tests neither predict resistance to therapy nor provide metrics of molecular changes within the tumour and seldom distinguish tumour progression from tumour necrosis (50–53). Non-invasive GBM-specific biomarkers would provide the clinician and patient with a window into tumour diagnosis and molecular structure with a high degree of sensitivity and specificity, with the ultimate goal of developing targeted therapeutic agents based on these diagnostics and utilizing the "liquid biopsies" to assess drug efficacy.

The search for informative biomarkers in CSF has recently turned to exRNA, which is promising for several reasons. RNA in EVs is stable in biofluids where it is likely protected from RNases, can be isolated from serum and CSF, and can be easily detected from small copy numbers with high sensitivity using reverse transcription PCR (54). In addition, tumour derived EV RNAs (especially miRNA and mRNA) can be amplified up to 100-fold within target cells and are thus likely in the microenvironment. And finally, multiple glioma-specific EV RNA amplifications and mutations have been identified as biomarkers (54–58). These include epidermal growth factor receptor variant III (EGFRvIII, a highly specific deletion mutation found in 20–25% of GBMs) and isocitrate dehydrogenase 1 and 2 (IDH1/2) mutations (found in 10% of GBMs). Each is associated with GBM molecular subtypes, correlates with patient survival and can be targeted with specific therapies (45,46,55,59–61).

*Biomarkers of brain tumours: approach*

Key milestones of this project include (a) collection and banking of biospecimens to support pilot studies and then cohort studies, (b) determination of the optimal platform for isolation and analysis of EV RNA gene products and (c) identification and further assessment of the most promising EV RNA GBM biomarkers. A summary of this work to date is provided below.

*Biomarkers of brain tumours: progress to date*

To date, specimen collection and banking has resulted in an established tissue-fluid biorepository which houses over 4,000 specimens from over 600 subjects, the majority with brain tumours (62). This facility employs strict SOPs in accordance with International Society of Extracellular Vesicles recommendations for sample collection and storage, and utilizes bar-coded specimen tracking technology adapted to multi-institutional collaborations. The optimal platform for sample processing and analysis has been determined, and a collaborative CSF group is conducting comparative trials of EV RNA isolates using multiple isolation kits, RNA assays and analytic techniques. These systems are being compared for EV RNA yield, purity and gene product detection. Identification of the most promising candidate biomarkers resulted in 2 categories: (a) GBM-specific mutations (e.g. EGFRvIII deletions and IDH1.132 point mutations) which may be scored and analyzed as "present" versus "absent" and (b) wild-type EV genes known to be quantitatively differentially expressed in GBM, such as EGFR, IDH1 and promoter methylated proteins. Thus, the expression of the mutant growth factor receptor EGFRvIII provides for differentiation of EVs from GBM and non-tumour tissue. EGFRvIII RNA can be isolated from the serum at a level approaching a sensitivity of 60% and specificity of 98% (63). Similarly, human blood and CSF samples could provide a predictive metric of treatment-induced changes (64). The detection of single amino acid mutations is more difficult given the vast background "noise" of wild-type sequences, a difficulty which has resulted in the use of high-resolution techniques such as BEAMing (beads, emulsion, amplification, magnetics) PCR and ddPCR. These techniques can detect mutations as rare as 0.01% or as few as 20 copies. These methods identified mutant IDH1 mRNA in CSF-derived EVs from patients with mutant IDH1 gliomas, and established that patients with tumours have higher levels of CSF and serum EV mutant IDH1 mRNA than healthy controls (65).

Expression changes in the EV RNA levels of wild-type genes obligates quantitative analyses as opposed to "present or absent" scoring. Methylated promoters of nucleotide repair enzymes, such as $O^6$-methylguanine methyl transferase (MGMT), are EV biomarkers of interest due to their link to temozolomide resistance (66). The study team has recently demonstrated that expression of 2 miRNAs (miR-603 and miR-181d) inversely correlated with MGMT expression and that a combined index of these molecules can serve as a proxy for MGMT expression (67). Extrapolating from these findings, EV RNA sampling of miR-603 and miR-181d, or similar regulatory molecules, has the potential to predict GBM temozolomide resistance. Expression levels of EV *wild-type* EGFR have also shown promise as a stand-alone or multipanel biomarker for GBM (64).

There are unique challenges of working with EV RNAs in a world without clear consensus. Yields of EV RNA vary based on the gene of interest and biofluid analyzed, and RNA from specific genes may be as rare as ~40 copies/mL in these fluids (65). As an additional challenge, reference transcripts or "housekeeping genes" commonly used for qPCR (including GAPDH and 18S rRNA) may be under-expressed in biofluids such as CSF and vitreous and have not been defined for EV-associated miRNA. As an alternative, these investigators normalize the absolute EV miRNA expression to input EV number (determined via Nanoparticle Tracking Analysis [NTA]) (58). Using this technique, they recently demonstrated that quantification of EV miR-21 (an miRNA highly over-expressed in GBM) was able to prospectively distinguish CSF derived from GBM and non-oncologic patients in an independent cohort analysis (58). Thus, EV RNA

biomarkers have clinical potential for GBM diagnosis, subtype assessment, prognostication and disease progression tracking. The establishment and continued growth of a biospecimen repository supported by the NIH plays a central role in efforts towards biomarker discovery and validation. These technologies will enable GBM treatments tailored to specific "liquid biopsy" tumour profiles and the collaboration of drug designers to implement novel therapeutics.

### Biomarkers of intracranial haemorrhage

In "ExRNA Signatures Predict Outcomes After Brain Injury" (TR000891), Drs. Van Keuren-Jensen and Huentelman at the Translational Genomics Research Institute, in collaboration with Drs. Kalani, Spetzler, Arango and Adelson at the Barrow Neurological Institute, St. Joseph's Hospital and Medical Center (Kalani, Spetzler) and the Barrow Neurological Institute at Phoenix Children's Hospital (Arango, Adelson), examined exRNA in 2 devastating cerebrovascular events in need of biomarkers for prediction of risk and targeting of therapy: subarachnoid haemorrhage in adults (aSAH) and intraventricular haemorrhage (IVH) in children. This study is in collaboration with Drs. Michael Lawton (UCSF) and Samuel Cheshier (Stanford). In adults, aSAH occurs in 10–15 people per 100,000 every year. The risk for aSAH rises with age and accounts for 1–7% of all strokes. Cerebral vasospasm is a recognized and poorly understood complication for many patients who have aSAH. Severe vasospasm can lead to ischemia, permanent neurological damage and death. Vasospasm develops approximately 2–4 days after initial aSAH, providing an opportunity for early diagnosis and potential intervention to blunt the cascades of secondary injury caused by this phenomenon. In children, IVH is a significant complication of premature birth. With improved preterm infant survival, there is increased incidence of severe IVH and the potential for lifelong neurodevelopmental deficits. Babies who develop hydrocephalus as a result of IVH often require a permanent ventriculoperitoneal (VP) shunt. Approximately one-third of babies with IVH develop post-haemorrhagic hydrocephalus, and 15% require a VP shunt. An improved understanding of the pathophysiological mechanisms at work in this patient population may prevent unnecessary interventions, decrease short- and long-term morbidity, and decrease the cost of care. Both aSAH and IVH have very specific temporal courses and represent a substantial opportunity to identify biomarkers and interventions that may impact patient health. In the case of brain injury, the timing of intervention is of the utmost importance to minimize the degree of secondary neurological insults and delayed neurological deficits. Aggressive treatments are contraindicated in patients who will recover with close monitoring and standard care. While aggressive treatments do exist for patients who develop complications of secondary injury cascades, these interventions are neither benign nor cheap and are frequently initiated only after the pathways of secondary injury are in full effect.

Although 98% of the human genome does not encode proteins, >90% of the genome is likely transcribed (68); therefore, evaluation of the full transcriptome is critical. ExRNAs are enriched for non-coding, novel, endogenous mediators of mRNA post-transcriptional regulation, usually acting through gene silencing (69). Non-coding RNAs (ncRNAs), including miRNAs, have been found to play a role in most cellular processes and can have very specific temporal, spatial and cell-specific expression (70,71). More ncRNAs are enriched or uniquely expressed in the central nervous system than anywhere else in the body, and many RNAs are temporally expressed during neurodevelopment and normal aging (72,73). More importantly, the abnormal expression of ncRNAs has been detected in cellular dysfunction and disease, including ischemia, stroke and traumatic brain injury (74–77). The use of exRNAs as a sensitive readout of changing pathophysiological conditions underlying temporally defined brain insults could save thousands of lives each year and significantly improve the quality of patient life following intracranial haemorrhage.

#### Biomarkers of intracranial haemorrhage: approach

In the UH2 phase, CSF and plasma samples from aSAH patients were collected on days 1–7 of their stay in the intensive care unit, while CSF and plasma from infants with IVH were collected at 1–2 weeks in the neonatal intensive care unit. The miRNA profile in these samples was described using small RNA sequencing approaches. The whole transcriptome was sequenced in a subset of these samples. The investigators have sequenced >500 small RNA samples and >250 whole transcriptome samples and are currently analyzing the data and validating the RNA targets using a second platform. In the UH3 phase, the research team will sequence the samples from an additional cohort to increase the numbers of subjects with varying degrees of haemorrhage and stroke severity. They will then test platforms for rapid detection of the RNA profile in order to return results to the clinician as quickly as possible. The goal is to achieve accurate and prognostic profiling as close to real time as achievable.

#### Biomarkers of intracranial haemorrhage: progress to date

Investigators have profiled the blood and CSF of patients with haemorrhagic strokes in search of biomarkers that are associated with robust activation of secondary injury pathways and poor outcomes. An effective exRNA biomarker would also be a means to quickly evaluate treatment and help modify therapeutic approaches. Total exRNA (whole transcriptome as well as miRNAs) is being examined in the blood and CSF of patients with aSAH and IVH. Whole transcriptome sequencing with the addition

of small RNA sequencing has permitted close examination of exRNA content in both biofluids and to monitor changes associated with patient outcome. For the aSAH patients, preliminary analysis has identified differentially expressed RNAs that cluster the patients into outcome categories 2 days prior to current clinical vasospasm detection methods. The analysis for the IVH patients is still ongoing.

### Biomarkers of multiple sclerosis

In "Circulating MicroRNAs as Disease Biomarkers in Multiple Sclerosis" (TR000890), Drs. Weiner, Gandhi and colleagues at Brigham and Women's Hospital address the need for biomarkers for multiple sclerosis disease activity to measure disease activity and for the purpose of predicting and monitoring response to disease-modifying therapies. Although there is a better understanding of immune mechanisms in MS and several FDA-approved immune modulating therapies are available, a major challenge is to develop biomarkers that will allow a better understanding of an individual MS patient, whether they are a responder or non-responder to therapy, which medicine is more effective, and the degree to which they may be entering the progressive phase of disease. MiRNAs provide a new avenue to understand immune regulation, and the ability to measure them in the plasma or serum makes them an ideal candidate as an immune biomarker.

#### *Biomarkers of multiple sclerosis: approach*

The UH2 phase of this project will take advantage of a unique resource termed CLIMB (Comprehensive Longitudinal Investigation of Multiple Sclerosis at Brigham and Women's Hospital). CLIMB is a cohort of over 2,000 MS patients that began enrolment 12 years ago and includes yearly blood samples, clinical exams and quantitative MRI imaging. This proposal was based upon preliminary data, suggesting that circulating miRNAs are measurable in this MS cohort and are related to disease stage, response to therapy and disability. The approach is organized under the following aims: (a) identify miRNAs that will act as diagnostic biomarkers by comparing MS patients to healthy controls, patients with other neurological diseases and patients with other autoimmune diseases, (b) identify disease stage miRNA biomarkers by comparing relapsing remitting (RRMS), secondary progressive (SPMS) and primary progressive (PPMS) patients, (c) identify prognostic miRNA biomarkers by determining the change in each miRNA over 2 years and assessing which miRNAs are sensitive to long-term change in disease status, (d) identify treatment response miRNA biomarkers by comparing the baseline miRNA expression in responders and non-responders to treatment and (e) identify disability miRNA biomarkers by correlating miRNAs with EDSS and MRI measures of disease status.

#### *Biomarkers of multiple sclerosis: progress to date*

During Year 1, analysis of groups of 80 MS patients, 20 healthy controls and 50 other disease controls suggested that miRNAs could provide potential biomarkers related all the five categories: diagnostic, disease stage, prognostic, treatment response and disability biomarker. During the second year and UH3 phase of the study, selected miRNAs will be validated on larger cohort of MS patients and controls.

### Biomarkers of renal disease

In "Clinical Utility of Extracellular RNA as Marker of Kidney Disease Progression" (TR000933), Drs. Tuschl, Suthanthiran, Bitzer, Putterman, Goilav and colleagues propose to examine exRNA biomarkers for progression of chronic kidney disease (CKD). CKD can result from high blood pressure, diabetes, disorders of the immune system, genetic defects and developmental disorders, and causes early death from heart disease, infections and cancer. Many CKD patients develop end-stage kidney disease and need dialysis or kidney transplants. Recipients of kidney transplants also are prone to CKD. Current tests cannot predict which patients will have CKD that worsens over time. Identifying CKD patients at risk for disease progression could allow clinicians to treat patients earlier and slow further decline in kidney function. It also could help scientists develop therapies that prevent decline in kidney function in patients at risk. This is the only project in this group to focus on urine as the extracellular fluid source of exRNA. The population studied includes adult and paediatric cohorts, both glomerular and non-glomerular kidney disease, and both native and transplanted kidneys. The research team will identify types of exRNA in the urine of CKD patients and will determine if this approach can identify patients at risk for worsening disease. They plan to use these findings to develop a urine test that clinicians can use to guide treatment of CKD patients.

#### *Biomarkers of renal disease: progress to date*

Renal involvement in systemic lupus erythematosus (SLE), or lupus nephritis (LN), is associated with significant morbidity and worse mortality than when this clinical manifestation is not present. Furthermore, LN is more common and severe when the onset of disease is during childhood (78,79). Since it is believed that quicker diagnosis can lead to better clinical outcomes in LN, there is tremendous interest in identifying early biomarkers for kidney involvement. Putterman and his group demonstrated that urinary miRNA deep-sequencing counts vary by LN disease activity, in a manner suggestive of biomarker potential (80). Specifically, they identified a set of urinary miRNA that correlated with active versus inactive LN in both adult and childhood LN, as well as urinary miRNA that were indicative of particular LN histologic classes. Interestingly, in a mouse model of immune complex nephritis induced by passively transferred pathogenic

antibodies, several dysregulated miRNAs present in the serum of nephritic mice (identified by deep sequencing) and which correlated with the peak of kidney involvement were identical to those previously found in the urine of lupus patients (81). Confirmation of the diagnostic utility of these and other urinary miRNA in human lupus, and investigating whether they have a pathogenic role in kidney disease, is currently under active study.

## Conclusions

The NIH Extracellular RNA Communication Consortium (ERCC) and the UH2/UH3 funding mechanism have allowed investigators from diverse medical subspecialties to advance methodologies for describing and quantifying exRNAs from a wide range of human body fluids with the goal of exploiting this relatively new biological phenomenon for the sake of improving clinical research and care in each of these fields. While each of the projects has spent a portion of the first year on method development, all the projects have also successfully identified candidate biomarkers for advancing to hypothesis testing in the next phase. The dissemination of methodological recommendations is in itself an important development in any new field and will promote continued success both within and beyond the ERCC.

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David Wong is co-founder of RNAmeTRIX Inc., a molecular diagnostic company. He holds equity in RNAmeTRIX, and serves as a company Director and Scientific Advisor. The University of California also holds equity in RNAmeTRIX. Intellectual property that David Wong invented and which was patented by the University of California has been licensed to RNAmeTRIX. All other authors have not received any funding or benefits from industry or elsewhere to conduct this study.

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