Telomere Research Network Annual Meeting 2023

A Workshop Co-sponsored by the National Institute of Environmental Health Sciences (NIEHS) and the National Institute on Aging (NIA)

March 30-31, 2023

NIEHS Building 101
Rodbell Auditorium
Research Triangle Park, NC

Workshop Summary

Revised March 13, 2024

This workshop summary was prepared by BANGL Lab Associates under contract to Stacy Drury and the Tulane University School of Medicine. The views expressed in this document reflect both individual and collective opinions of the workshop participants and not necessarily those of the National Institute on Aging, the National Institute of Environmental Health Sciences, or any organization represented by the workshop participants.

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Acronym Definitions

ACE = Adverse Childhood Experience

aTL = Absolute Telomere Length

ATP = Adenosine Triphosphate

BPA = Bisphenol A

Cas = CRISPR Associated Enzyme

CMV = Cytomegalovirus

CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats

DBS = Dried Blood Spot

DIN = DNA Integrity Number

DNAmTL = DNA methylation-estimated Telomere Length

DOHaD = Developmental Origins of Health and Disease

EPA = Environmental Protection Agency

EV = Extracellular Vesicle

GWAS = Genome Wide Association Study

HCT = Hematopoietic Cell Transplant

FISH = Fluorescence In-Situ Hybridization

ICC = Intraclass Correlation Coefficient

ISR = Integrated Stress Response

KB = Kilobase

mitoNUITs = Mitochondria Nutrient Uptake Inhibitors

MMqPCR = Monochrome Multiplex Polymerase Chain Reaction

NCI = National Cancer Institute

NIA = National Institute on Aging

NIEHS = National Institute of Environmental Health Sciences

NIH = National Institutes of Health

NTC = No Template Control

PBMC = Peripheral Blood Mononuclear Cell

PCB = Polychlorinated Biphenyl

PFA = Polyfluoroalkyls

PFC = Perfluorochemical

PNS = Prenatal Stress

qPCR = Quantitative Polymerase Chain Reaction

ROS = Reactive Oxygen Species

SASP = Senescence Associated Secretory Phenotype

SD = Standard Deviation

STELA = Single Telomere Length Assay

TeSLA = Telomere Shortest Length Assay

TL = Telomere Length

TRF = Terminal Restriction Fragment

TRN = Telomere Research Network

Executive Summary

On March 30 and 31, 2023 the Telomere Research Network (TRN), funded by the National Institute of Environmental Health Sciences (NIEHS) and the National Institute on Aging (NIA)), held their annual open meeting designed to present the collaborative results of the U24 (PI, Drury) and the U01s (PIs Aviv, Lin, Shalev, and Zhang) over the first three years of the TRN network. In addition, the annual meeting provided an open forum for the discussion of both the results of the collaborative and the presentation of new data, novel methods, methodologic challenges, and progress from TRN pilot recipients. The overarching goal of this meeting was to ensure transparency, multi-disciplinary engagement, and rigorous discussion about next critical directions in the exploration of telomeres as indicators of psychosocial and environmental exposures and predictors of health in human population studies. Four sessions and two keynote presentations from experts in basic telomere biology, medicine, biopsychology, and epidemiology. Throughout the workshop, participants discussed methodological factors influencing the precision and reproducibility of telomere length (TL) measurements, novel methods, new models for telomere measurement and characterization, and critical considerations regarding the role of telomeres as sentinels of environmental and psychosocial stress exposure.

The Telomere Research Network 2023 Annual Meeting was held in Durham, North Carolina at the National Institute of Environmental Health Sciences. The meeting was a hybrid event with 33 in-person attendees on Day 1, 30 attendees on Day 2, and 199 total online registrants. Attendees joined from all over the world including India, Israel, Pakistan, the Philippines, and the USA. There was also a wide range of fields represented at the meeting from professionals in Early Development to Aging and Molecular Genetics to Health Disparities. A majority of survey respondents (86.4%) agreed that the information covered in the meeting was relevant and important for methodology and study design for their research. Furthermore, the meeting was able to inspire and promote future research, with 81.8% of survey respondents stating that the information learned at the TRN meeting will inform their research studies.

Presentation recordings from the meeting can be found here.

Meeting Summary

On March 30 and 31, 2023 the TRN, a U24 grant funded jointly by National Institute of Environmental Health Sciences (NIEHS) and the National Institute on Aging (NIA), convened a workshop that brought together experts in basic telomere biology, medicine, biopsychology, epidemiology, and related fields and sought to stimulate cross-disciplinary discussion. The objectives of the workshop were to:

1. Review methodological factors impacting the precision and reproducibility of telomere length measurements
2. Discuss current and future possibilities for the role of telomeres as sentinels of environmental and psychosocial stress exposure and predictors of human health and disease in human population studies
3. Explore novel methods and models for telomere measurement and characterization
4. Highlight the work of early career investigators supported by the pilot awards

Workshop participants were encouraged throughout to suggest next research questions for the TRN to address.

Progress to Date

This is the third annual in-person meeting held by the Telomere Research Network. During COVID webinars were held related to telomeres and human health. In total, 14 pilot awards have been supported by the TRN and multiple grants and postdoctoral fellow applications have also been sponsored. Additionally, the TRN has provided consultation to dozens of investigators considering telomere studies. The TRN has a freely accessible new investigator toolkit and collection of resources to enhance transparency and reproducibility in telomere research located on the TRN website (https://trn.tulane.edu/resources/). Studies and findings from the TRN have been presented at interdisciplinary scientific meetings including ISPNE, ISDP, PEE, AACAP, ABMR, EMBO, and SMFM. The TRN has supported two published meta-analyses (McLester Davis, et al 2023; Francis et al 2022) and multiple other methodologic papers. Lastly, the TRN recently completed the largest double blinded, multi-laboratory, multi-method international study of TL measurement that involved 7 different methods, 14 different laboratories and 6 different countries.

Session 1: Results from the Telomere Research Network U01 Collaboratory

The purpose of this session was to highlight collaborative work conducted by and across the U01 laboratories of the Telomere Research Network (TRN).

Telomere Research Network Progress Update: Cross Method and Cross Laboratory Effects on Measurement Precision

Simon Verhulst, PhD, University of Groningen

Dr. Simon Verhulst, an evolutionary biologist, serves as the statistician for the TRN studies. He presented the results, to date, from two multi-laboratory studies evaluating the reproducibility of TL measurement using different methods and different laboratories. As a first step in enhancing

TL measurement reproducibility the U01 PIs, U24 executive committee, scientific advisory board, and NIH partners facilitated the design and implementation of the first collaborative multi-method study of TL measurement repeatability. For this study the primary consideration was in a multi-lab study to (1) characterize the effect of DNA extraction methods on reproducibility and (2) to evaluate if laboratory or methodological differences were more relevant to reproducibility (Lin et al., under revision, *PLoSOne*). Results from this study highlighted strong effects of DNA extraction on the precision of TL measurements generated using quantitative polymerase chain reaction (qPCR). Specifically, measurement precision, quantified as the intraclass correlation coefficient (ICC), was on average 23% lower across repeated measurements of *different* DNA extractions of the same sample relative to repeated measurements made on DNA from the *same* extraction, suggesting processes of DNA extraction exert strong effects on downstream TL measurements. Although there were differences in precision between different extraction methodologies (e.g., salting out vs. spin column) these differences were similar in scale to differences in precision across laboratories, making it challenging to recommend a specific DNA extraction method. However, based on the results of the study, the method with the most consistent reproducibility was a spin column approach. In addition, results from this study provided empirical support for the recommendation that all studies report the ICCs of TL measurement from repeated extractions of the same sample, rather than ICCs of repeated measurement of the same DNA sample, as the most accurate estimate of reproducibility. **The TRN recommendation is that a minimum of 10% or >30 samples for every cohort are measured using repeated DNA extraction and reported with all results**. It was also clear that both between lab and between DNA extraction methods are relevant when considering repeatability.

Because, in the first TRN multi-site study, each lab extracted DNA independently for each extraction type, the experimental design was not able to disentangle the effects of DNA extraction from the effects of laboratory. To address this challenge, a second, larger TRN multi-laboratory study to compare TL measurement precision between methods and laboratories using DNA from PBMCS prepared by the National Institute of Aging (Nan-Ping Wang) extracted in one batch from a central source. A total of 16 laboratories participated in this study. Four laboratories conducted measurement using standard singleplex qPCR. Two laboratories conducted measurement using absolute telomere length (aTL), a qPCR-based approach providing output in kilobase (kb) units using oligomer standards with known TL. Three laboratories conducted measurements using monochrome-multiplex qPCR (MMqPCR), which measures telomere and single-copy gene content in the same reaction well. Three laboratories conducted measurements using southern blot (TRF, telomere restriction fragment). The remaining four laboratories conducted measurements using either the gel-based single telomere length assay (STELA) which is able to target telomeres of one specific chromosome, Nanopore sequencing, fluorescence in-situ hybridization (Flow-FISH), or DNA methylation estimated telomere length (DNAmTL).

In this design, a set of 107 peripheral blood mononuclear cells (PBMCs) were shipped from Dr. Nan Ping's at NIA for central extraction in the lab of U24 PI Dr. Stacy Drury. These 107 samples

were gathered from 41 anonymous donors (ages 0-83; 51% female), such that each donor provided 2-3 samples, a fact not revealed to any laboratories throughout the course of the study, permitting a unique level of sample blinding and opportunity for analysis of repeatability. Throughout the course of the study only Dr. Verhulst, who had no connection to any of the participating laboratories, had the ability to unblind any samples. In the U24 laboratory the samples were blinded a second time before extraction. Following extraction, twice blinded sets of 107 DNAs were sent to each participating laboratory. Only after the first set of samples were analyzed and the data sent to Dr. Verhulst was the second set of samples, with different subject IDs, sent to each laboratory. Measurement and quality control (QC) data was sent to Dr. Verhulst for unblinding and data analysis. Measurements conducted on each set of 107 DNAs were z-scored for comparison across methods and laboratories. In this manner, the design allowed for tests of repeated extraction by calculating the ICC across measurements from different samples from the same donor within the same set of 107 DNAs (within sets, between extractions), tests for effects of assay by calculating the ICC of measurements across duplicate sets of 107 DNAs (between sets, within extractions), and the cumulative effects of assay and extraction by calculating the ICC across different samples from the same donor across different sets of 107 DNAs (between sets, between extractions).

As in the first collaborative study, there was wide inter-laboratory variation in the precision of telomere measurements, even for labs using the same assay. Even so, these estimates are helpful to establish an ‘upper bound’ of measurement precision for each method. For the ICC within sets between extractions, the singleplex qPCR method exhibited an upper bound near 0.90, while the aTL method exhibited an upper bound near 0.80. MMqPCR, Southern blot, and DNAmTL exhibited the highest precision, each producing and upper-bound measurement ICCs >0.95. Similar findings were observed for the ICC across different sets the same DNA sample (within sets, between extractions).

These findings have important implications for human population studies and provide empirical data to support clear guidance related to the use of specific methods for cross section and longitudinal studies. Given the effect of ICC on statistical power, and that that effect is amplified in longitudinal studies, the implication of the upped bound limits of aTL and single-plex qPCR suggest that these methods are not appropriate, regardless of sample size, for longitudinal studies. As Southern blot-based measurement and MMqPCR appear to have similar upper-bound limits of the ICCs these approaches, when appropriately powered and with enough time between sample collection, can be suitable for longitudinal studies. The relative importance of ICCs for each laboratory and each experiment is relevant for cross-sectional studies but has far stronger implications for longitudinal studies. Specifically, ICCs considered acceptable by other fields, and for cross-sectional studies of TL, are often insufficient to detect longitudinal change in TL. For example, a study with 400 individuals and an ICC of 0.90 has moderate power ($\beta \approx 0.58$) to detect a 33% difference in telomere change over an 8-year duration. However, measurement ICC of 0.80 nearly halves the power of the same study ($\beta \approx 0.30$). This is because the degree of telomere shortening is very small relative to the interindividual variation in TL on which ICC estimates are based. As a result, studies with sample size less than 500 individuals

would need to generate measurements with an ICC of 0.90 or greater to have sufficient power ($\beta > 0.90$) to detect a 33% differences in TL change over even a moderate follow-up interval.

Effects of Z-Transformation

Based on the empirical data from these studies, the TRN recommends z-scoring data for comparison of qPCR telomere data across studies, laboratories, and/or methods. This is an important consideration as differences in assay design, such as mastermix composition, single-copy gene selection, and DNA concentration result in differences in the scale of T/S ratio values. However, the z-transforming can be applied at different levels of analysis, for example z-scoring data within plates or z-scoring data across the entire batch. An important assumption of conducting a z-transformation is that the underlying mean and standard deviation (SD) of the data is the same between the two datasets being compared. Z-transforming the data across the entire batch of samples in the current study was justified because the mean and SD across different sets of 107 DNAs can be assumed to the same, because they are comprised of different aliquots of the same 107 DNA samples. However, it would not be justified to z-transform the data at the plate-level to correct for variation between plates, as you are unaware of the underlying TL variability of samples on those plates prior to assay measurement.

Accuracy vs. Precision

Methods assessed in the second collaborative study exhibited variation in precision. MMqPCR, Southern blot, and DNAmTL provided the most precise estimates of TL, with ICC values greater than 0.90 across repeated extractions and assays. However, qPCR and MMqPCR have unmeasurable accuracy, defined as how close a measurement is to the true value, because the T/S ratio output is a relative unitless metric. By contrast, the aTL method is less precise (lower ICC), but provides an output measurement in kb units that is interpretable in the context of the biological phenomena being measured, i.e., average TL in base pairs, meaning it has the potential to be accurate. Southern blot has long been considered a highly accurate method, but its estimates include the sub-telomere region which exhibits genetic variation between individuals. Moreover, the degree to which subtelomeres are included in southern blot measurements varies depending on which restriction enzymes are used in the procedure. In addition, there are unidentified factors affecting measurements, as highlighted by two of the three laboratories conducting Southern blot measurement, while both exhibiting high ICCs, generated estimates of TL that were systematically different- despite using the exact same protocols, including restriction enzymes, indicating other sources of variability beyond the polymorphic variation in sub-telomeres. These same facets also limit the accuracy of Flow-FISH and DNAmTL, which are calibrated based on measurements made using Southern blot. STELA may be highly accurate but is limited in application to only one arm of one chromosome and is not practical, due to the complexity of the methodologic protocol, for scaling to population studies at this time. Finally, Nanopore sequencing has the potential to be the most accurate, but the validation and evaluation of precision in different laboratories for this method has not been performed. Thus, without a true standard against which methods can be compared, we lack an answer as to the true length of human telomeres and the best assay for accuracy remains

unknown and there is no true “gold standard” of telomere measurement at this time. This critical finding was noted as an important next step for the TRN.

Final Considerations

Explicit recommendations for what method to use in different studies are difficult to generate, as many techniques can generate high ICCs when optimized through rigorous protocol development and optimization within labs, in combination with the number of replicate measurements required to reach target precision. However, these same techniques, if implemented in different laboratories, also generate substantially lower ICCs. Moreover, even less precise methods, e.g., singleplex qPCR, can generate sufficient statistical power provided a large enough sample size. There is sufficient data to recommend that, for each study, the generation of ICCs, on repeated extraction of the same DNA samples is performed on a minimum subset of samples to ensure (and report) consistent reproducibility even in the most experienced of laboratories.

Another consideration is whether the method provides absolute or relative estimates such that it can be *potentially* accurate. Singleplex qPCR can be sufficiently precise when optimized for cross-sectional studies with a larger sample size but is not suitable for longitudinal studies and provides a relative estimate of TL. Thus, the data does not support the use of singleplex qPCR for longitudinal analysis. The aTL method is similarly suitable for cross-sectional studies and provides an absolute estimate with ascertainable accuracy but lacks the precision for longitudinal studies. Moreover, the two labs measuring aTL differed considerably in the level of estimates, indicating a need for further refinement. MMqPCR stands out as a high-throughput method with a precision ceiling matching that of southern blot, making it suitable for cross-sectional and longitudinal studies. However, MMqPCR still provides a relative estimate with unascertainable accuracy. Southern blot provides a highly precise measurement in absolute units and remains the approach of choice for labs able to optimize the complexities of the method. Limitations to the Southern blot method center on moderate throughput, extensive set up requirements, and the need for intact DNA of sufficient quantity for analysis. Even with precise and identical protocols there remain differences in the absolute unit across laboratories, highlighting the need for continual methodologic advancement including future studies of DNAm repeatability and longitudinal relation to other TL measurements, refinement of the nanonpore methodology and establishment of its precision and accuracy, and the consideration of new method development.

Telomere Length Measurements and Opportunities for Clinical Applications

Sharon Savage, MD, National Cancer Institute (remote)
Shahinaz Gadalla, MD, PhD, National Cancer Institute (remote)
Abraham Aviv, MD, New Jersey Medical School, Rutgers (remote)

This session presentation was split into two parts, each reviewing distinct application of TL in the clinical sphere. The first half, presented by Dr. Shahinaz Gadalla, aimed to assess the utility of Southern Blot and qPCR measurements to capture TL dynamics and patient outcomes following hematopoietic cell transplant (HCT). The second half, presented by Dr. Sharon

Savage, reviewed applications of the Telomere Shortest Length Assay (TESLA) in patients with telomere biology disorders.

Southern Blot Assessment of TL Dynamics in HCT

TL dynamics are of relevance to HCT, a treatment for malignant and nonmalignant diseases of the hematopoietic system. In this procedure, donor hematopoietic stem cells are transplanted to into a patient to reconstitute white and red cell populations, to either replace stem cells destroyed from chemotherapy or radiation therapy in the case of malignant diseases, or to supplement insufficient bone marrow production of blood cells, as in the case of severe aplastic anemia. Donor cells undergo rapid cell division to refresh blood cell populations, resulting in rapid TL shortening of donor cells following transplantation, with overall single year TL attrition comparable to that observed in over a decade in healthy adults. In line with this, longer donor TL measured via Flow-FISH and qPCR is associated with lower mortality risk post HCT in severe aplastic anemia. It is unclear whether these results are generalizable to malignancy-driven instances of HCT, as qPCR-based measurements of donor TL were not predictive of patient outcomes in leukemia driven instances of HCT. However, this may be due to decreased precision of qPCR measurements to detect discrete differences in donor TL associated with survival.

This U01 study design involved TL measurement in 702 patient-donor pairs using Southern Blot and qPCR. Donor TL was measured prior to the HCT procedure and patient TL was measured 3 months following the HCT procedure. Telomere parameters of interest included donor TL and the magnitude of TL change in the three months following HCT. Patient outcomes of interest included survival and relapse in the two years following HCT. In addition, disease severity, defined as whether the leukemia was early stage (CR1) or intermediate/advanced (CR2+), was investigated as a potential moderator of the association between TL dynamics and patient outcomes.

Southern blot measurements effectively captured TL dynamics following HCT. TL in patients following HCT was approximately 450 bp shorter than TL from donors measured three months prior. This degree of shortening is approximately 15-fold higher than the 30 bp/year TL attrition rate reported in Southern Blot measurements from healthy adults in the general population. The relationship between TL dynamics and patient outcomes was variable between those in early stage versus intermediate or late stage leukemia. Specifically, there was no association between Southern Blot assessed TL and patient outcomes for those patients in intermediate/advanced stage of disease. Similarly, the degree of TL attrition was also not related to patient survival or relapse for those with intermediate/advanced stage of diseases. However, patients in early stage leukemia receiving transplants from donors with longer Southern Blot TL (>6.7 kb) exhibited significantly higher survival probability relative to patients receiving transplants from donors with TL shorter than 6.7 kb ($p=0.009$). Decreased TL attrition was also associated with increased survival in patients with early stage disease ($p=0.003$). Although explicit donor TL was not related to risk of relapse, the degree of TL attrition was, with patient-donor pairs characterized by less 3-month attrition (<230 bp) exhibiting lower odds of relapse.

qPCR Assessment of TL Dynamics in HCT

qPCR measurements were moderately correlated with measurements generated with Southern Blot ($r=0.6$, $p<0.001$). However, Cohen's kappa indicated less than reliable overlap between TL quartiles between measurements ($\kappa=0.36$, $p<0.001$). Quartile assignment overlap was the highest in the upper (56%) and lower (49%) quartiles but was notably lower for quartiles in the middle of the distribution (Q2=27%; Q3=32%). Survival curves for those in the lowest quartile of donor TL did not distinguish survival risk as was observed for Southern Blot measurements ($p=0.31$). Although qPCR measurements of donor TL was highly correlated to post HCT TL ($r=0.9$), measurements of TL attrition between donor and 3-month post HCT exhibited low agreement with Southern Blot assessments ($r=0.2$; $\kappa=0.11$). Post HCT-attrition TL and survival were not significant for qPCR measurements ($p=0.16$).

Summary and Implications

These results demonstrated the sensitivity of Southern Blot generated TL measurements to capture TL dynamics in leukemia driven HCT and the utility of Southern Blot generated TL measurements to predict patient outcomes for those undergoing HCT at an early disease stage. By contrast, qPCR TL measurements are suboptimal for prediction of patient outcomes and detection of longitudinal TL change in HCT clinical applications. Moreover, these results suggested the value in measuring donor TL prior to HCT. Specifically, these results offered initial evidence that avoiding the use of donor stem cells with TL <6.7 kb, irrespective of donor age, may improve outcomes in transplant patients and future studies measuring and monitoring TL post-transplant using Southern blot may be an important predictor of leukemia relapse.

Telomere Shortest Length Assay (TeSLA) in Telomere Biology Disorders

Telomere biology disorders (TBDs) are a cluster of diseases caused by mutations in genes related to telomere structure and function. Patients with TBDs are characterized by extremely short TL and are diagnosed on the basis of having lymphocyte TL falling below the 10th percentile of age-matched distributions. Current diagnosis utilizes fluorescence in-situ hybridization (Flow-FISH) to measure TL, which has superior sensitivity (80%) and specificity (85%) to detect telomeres in this percentile relative to qPCR measurements (Sensitivity=40%; Specificity= 63%). Even so, there remains a gap in the ability of measurement approaches distinguishing average TL to accurately diagnose patients with TBDs, perhaps due to a mismatch between approaches to TL measurement (e.g. measurement of average TL, rather than shortest) and clinical mechanisms underlying TBDs.

To address this gap, the second part of this presentation provided data from individuals with TBD using TeSLA measurement. TeSLA is able to more accurately assess the distribution of TL than Southern blot-based measurement. This study involved 47 individuals, consisting of 22 healthy controls, 18 TBD patients, and 7 healthy relatives of individuals with TBD who were carriers of monoallelic mutations in *RTEL1*. All TeSLA TL measurement parameters were moderately correlated with mean lymphocyte TL measured by Flow-FISH, including mean TeSLA TL ($r=0.53$, $p=0.03$), average TL of the 20% shortest telomere ($r=0.48$, $p=0.05$), the percent of telomeres under 3.0 kb ($r=-0.55$, $p=0.002$), and the percent of telomeres under 1.6 kb

(r=-0.46, p=0.06). These parameters were also significantly different in patients with TBD relative to healthy controls, with TBD patients exhibiting significantly lower mean TL (p<0.0001), shorter average TL in the shortest 20% of telomeres (p<0.0001), and greater percentage of telomeres less than 3 kb (p<0.001) and 1.6 kb (p<0.001). Notably, TeSLA parameters also distinguished between patients according to disease severity, with patients with severe bone marrow failure (n=11) exhibiting shorter mean TL (p=0.046) and greater percent of telomeres under 1.6 kb (0.027) than patients without severe bone marrow failure (n=5). Similarly, patients with TBDs involving deficits in multiple organ systems (n=10) exhibited shorter mean TL (p=0.025) and increased percent of telomeres under 1.6 kb (p=0.029) relative to patients with detriments to a single organ system (n=8). Whether TL measurements generated using Flow-FISH were similarly able to distinguish between TBD patients according to disease severity was not discussed. These findings support the hypothesis that bone marrow failure observed in TBDs arises from a disproportionate accumulation of ultrashort telomeres. Moreover, the ability of TeSLA to distinguish between patients with differential severity and clinical manifestations demonstrates the potential of methodologies providing diverse measurements beyond average TL to enhance clinical diagnoses.

Citation:

Raj, HA, Lai, TP, Niewisch, MR, Giri, N, Want, Y, Spellman, SR, Aviv, A, Gadalla, SM, & Savage, SA (2023) The distribution and accumulation of the shortest telomeres in telomere biology disorders. *British Journal of Haematology.* doi: 10.1111/bjh.18945.

Single Telomere Length Analysis by DNA-array-FISH

Yun-Ling Zheng, MD, PhD, Georgetown University Medical Center (remote)

Dr. Zheng, Associate Professor of Oncology, is PI of the U01 study “Standardizing qPCR and developing and testing a new DNA-FISH method for high-throughput assessment of TL using genomic DNA". First generation TL measurement methods (e.g., qPCR, Q-FISH, TRF) assess average TL in a population of cells. Next generation methods seek to measure the lengths of a single telomere to provide a holistic distribution of TL within a sample. Measuring this distribution is critical, as TL is widely variable across chromosome arms, and increases in variation throughout the cell life cycle. For example, younger cells from the IMR90 cell line at population doubling 10 (PD10) exhibited 90% variation in TL across different chromosome arms and 123% in older IMR90 cells at PD50. Current next-generation methods able to measure the distribution of TL include Single Telomere Length Analysis (STELA), TeSLA, digital PCR amplification of single telomeres, Nano and PacBio HiFi long read sequencing. Despite the availability of such approaches, none are ideal for implementation in large population studies, either due to a lack of validation (digital PCR), high cost and labor intensive (Nano and PacBio long read sequencing), or limitations in analyzing long telomeres greater than 8 kb in length (STELA).

Dr. Zheng’s lab developed a new method to measure TL at single telomere resolution, using a combination of DNA microarray followed by telomere specific fluorescent in situ hybridization (FISH), termed DNA-array-FISH. In this design, TL is quantified by the intensity of fluorescence, which is converted to bp units using a standard curve with known quantities of telomere repeats.

Pilot measurements of average TL derived with DNA-array-FISH were highly correlated with TL measurements obtained by sequencing using cloned telomere fragments spanning a range of 400 to 2000 bp (n=6; R^2=0.9994), exhibited strong reproducibility across duplicate assessments in DNA samples from cell culture (n=8; r=0.96) and human genomic DNA (n=87; r=0.92), and were highly correlated with TRF measurements (n=83; r=0.82). In addition to providing estimates of average TL, DNA-array-FISH has the potential to provide information on the distribution of TL in a sample across a range of 200 bp to 300 kb.

Summary and Implications

DNA-array-FISH has several advantages to current approaches to measure TL. The approach is high throughput and low-cost (the cost is similar to qPCR-based TL assay). One DNA microarray chip can assay 112 DNA samples using approximately 500 ng DNA per sample, making it suitable for application in population-based studies. The approach measures multiple telomere parameters in addition to average TL, such as TL variation and the frequency of short telomeres, metrics unavailable to current high-throughput approaches. Challenges to the method include equipment required to create microarray chips and current optimization of the method is on-going.

Telomere Methodological Factors: Lessons from the Contemplative Coping during COVID Study

Jue Lin, PhD, University of California San Francisco
Quinn Conklin, PhD, University of California Davis

The Contemplative Coping During COVID Study was a longitudinal intervention investigating the impact of meditation and contemplative training as coping strategies to mitigate the impact of pandemic-related stressors on change in TL. Relevant to the TRN was the study's secondary aim of investigating the impacts of remote blood collection strategies on DNA sample quality metrics and TL assay precision. At baseline and 1-year follow-up assessments, participants (n= 444) were mailed a remote blood collection kit, consisting of a finger prick lancet and EDTA-treated BD microtainer. Following collection, blood samples were returned via overnight shipping in insulated boxes with cold packs provided by study investigators. Importantly, blood samples from baseline assessments were stored at -80°C for one year until follow-up assessments were completed such that all samples could be extracted and assayed in one batch. DNA extraction was conducted using the GenFindV3 magnetic bead kits and TL assessment was performed by singleplex qPCR, with outcome measures representing the average T/S ratio across three runs, with each run consisting of triplicate replicates.

The volume of blood collected varied widely between samples, ranging between 50 to 1000 µL. However, sample volume was not prohibitive; samples with as low as 50µL blood yielded sufficient DNA for PCR based TL analysis. In addition to volume, returned blood samples also varied in whether they exhibited clotting or not, with approximately 1/3 of samples having some degree of blood clotting. Blood clotting was associated with higher DNA concentration following DNA extraction but was not associated with DNA quality as indicated by 260/280 ratios. Samples

with and without blood clotting also exhibited similar ICCs of TL measurements across repeated DNA extractions ($ICC_{clotted}$ = 0.955; $ICC_{no\text{-}clotting}$ = 0.972), suggesting blood clotting did not impact TL assay precision. Baseline samples stored at -80°C also exhibited similar TL assay precision across duplicate DNA extractions as samples from the 1-year follow-up ($ICC_{baseline}$ = 0.955; $ICC_{follow\text{-}up}$ = 0.961).

Conclusions and Discussion

The study findings suggested blood collected remotely in BD microtainers is a reliable source material for precise TL measurements by qPCR. Moreover, this sample collection method is suitable for long term storage. Baseline blood samples stored at -80°C and extracted at the same time as more recently collected samples exhibited similar DNA concentration, 260/280, 260/230, and ICCs across duplicate extractions as samples from 1-year follow-ups. Presenters also discussed the benefit of BD microtainers relative to dried blood spots (DBS). Although the data was not shown, the presenters indicated ICCs of samples collected via blood spots were lower than ICCs of samples collected with BD microtainers, suggesting BD microtainers would be the preferred sample collection approach over DBS. Attendees questioned the integrity of BD microtainers during shipment relative to DBS, which are more stable at room temperature. Dr. Conklin acknowledged this point and emphasized that use of BD microtainers was made possible by taking care in how the study design approached the return of samples (e.g., overnighted in insulated containers with ice packs). Additional benefits of BD microtainers was the opportunity to have greater sample volume, which could be used to assess additional biomarkers, such as environmental toxicants, that would not be possible in the limited sample mass provided by DBS. Dr. Verhulst noted the higher ICC for singleplex qPCR relative to what was observed in the multi-lab study. Dr. Lin noted that this was likely due to the multiple processing steps and extended storage time involved in the handling, shipping, and processing of the samples from the multi-lab study relative to the work flow in this study, which was all managed by a single lab.

Cross-tissue Comparison of Telomere Length and DNA QC Metrics Across Two Cohorts

Idan Shalev, PhD, Pennsylvania State University
Sarah Wolf, PhD, Pennsylvania State University

Dr. Idan Shalev, Associate Professor of Biobehavioral Health, is PI of the U01 study “The comparability and reproducibility of TL measurements for population-based studies.” This study investigated how TL and metrics of DNA integrity varied between tissues and explored how these factors related to one another to impact external validity of TL measurements. Data for this U01 study was derived from two independent cohorts of adults and children. The adult cohort included samples collected from peripheral blood mononuclear cells (PBMCs), dried blood spots (DBS), saliva, and buccal swabs of 77 individuals (55% female) aged 18-70 years. The child cohort included samples collected from buffy coat, DBS, saliva and buccal swabs of 120 individuals (52% female) aged 8-15. DNA was extracted from all samples using the Qiagen Puregene kit and TL was measured using a qPCR protocol

to measure absolute telomere length (aTL), which provides estimates in kilobase (kb) units. The aTL assay employed in the Shalev lab was optimized using 20 adult cohort samples with paired TRF measurements. The resulting protocol generated aTL data that was highly correlated with TRF (r=0.886) and had moderately high reproducibility (ICC=0.764).

DNA concentration metrics included measures of total DNA concentration from the Nanodrop spectrometer, as well as double-stranded DNA concentration determined from the PicoGreen assay and Agilent TapeStation. DNA quality metrics included 260/280 and 260/230 ratios from the nanodrop spectrometer, which measure protein and organic contamination respectively, as well as less often utilized metrics of DNA integrity derived from the TapeStation. TapeStation DNA metrics included the percentage of DNA in each sample that was unfragmented, quantified as the percent greater than 3 kb in length, the percent of DNA that was highly fragmented, quantified as the percent of DNA less than 3kb and greater than 250 bp in length, and the percent of DNA that was severely fragmented, quantified as the percent of DNA less than 250 bp in length. The final DNA metric was the DNA Integrity Number (DIN) generated by the TapeStation, which serves as a summary measure of DNA quality integrating both the double-stranded DNA concentration and the degree of fragmentation. The DIN ranges in value from 1 to 10, which higher values indicating DNA of greater quality.

TL Variation Across Tissues

Reproducibility of aTL measurements in this study were similar to values observed during assay optimization. The ICC across duplicate measurements was 0.826 and the ICC of samples reextracted and rerun was 0.772. TL measurements were significantly associated with age, and significantly different across tissues. aTL significantly shortened with chronological age, the magnitude of which varied by tissue type. Analyses showed significant age-related decreases from 8 to 70 years in aTL for buccal, DBS, and PBMC, but not for saliva (age 8 to 70 years) or buffy coat (age 8 to 15 years). The lack of observable age effect in the child cohort for buffy coat was likely attributed to the narrow age range in the child cohort aged 8-15 years.

Tissue-specific effects were particularly pronounced for saliva measurements. In the adult cohort, saliva was the only tissue to not exhibit an age-related decrease in aTL. Although adult had longer average saliva aTL, values were not significantly different between the child and adult cohorts. Saliva TL measurements tended to be shorter than all other tissues, whereas buccal measurements were both longer than measurements in saliva and shorter than aTL measurements in DBS and buffy coat/PBMCs. aTL measurements generated in DBS and buffy coat/PBMCs tended to be similar in length. Despite differences in raw length, aTL measurements were significantly correlated in all pairwise comparisons (ρ = 0.23 – 0.51) except saliva-PBMC comparisons in the adult cohort (ρ = 0.18).

DNA Quality and Integrity Across Tissues

DNA metrics were also related to one another in hypothesized ways. DIN and metrics of DNA fragmentation derived from the TapeStation instrument were all highly correlated with one another (|r| = 0.44 – 0.93). DNA concentrations were also all significantly associated with each

other (r = 0.63 – 0.75). Notably, 260/230 ratios, but not 260/280 ratios, were significantly correlated with DNA concentration and DIN.

DNA integrity was significantly variable across tissues. Blood-based tissues (i.e., DBS, Buffy coat, PBMCs) have higher DIN than buccal and saliva, both of which were characterized by left-sided tails of lower DIN values. 260/280 ratios, indicative of protein contamination, were more variable in all non-invasive tissues (i.e., buccal, DBS, and saliva). 260/230 ratios were also significantly variable between tissues, but were generally low overall, having values less than 1.50 on average. Patterns of DNA concentration were similar across nanodrop, PicoGreen, and TapeStation metrics, with blood-based tissues of buffy coat and PBMS having the highest concentrations, followed by buccal cells.

Relationships Between DNA Metrics and TL Across Tissues

Although DNA metrics were related to aTL measurements, these relationships were not always consistent across tissues. DIN values were positively associated with aTL measurements for buccal, DBS, and saliva. The degree of unfragmented DNA in sample, quantified as the percentage of DNA greater than 3 kb in length, was also positively associated with TL in saliva and DBS. By contrast, the degree of severely fragmented DNA, quantified as the percent of DNA less than 250 bp in length, was negatively associated with aTL in saliva and DBS samples. 260/230 ratios were also negatively associated with aTL measurements in saliva, DBS, and also buccal samples. Higher 260/280 ratios were associated with shorter aTL in buccal and DBS but were associated with longer aTL in saliva. DNA metrics were generally not associated with aTL measurements in buffy coat or PBMC samples.

Researchers next tested whether incorporating DNA metrics could improve the quality of TL measurements, quantified as whether the measures improved the fit of models predicting TL above and beyond the value explained by age and sex alone. The selected metrics varied by tissue. For example, DIN values, percent of highly fragmented DNA, and A260/230 improved the model fit for buccal, while only A260/280 improved fit for saliva. Importantly, the association between aTL and age in saliva, previously unobserved in original models, was recaptured following control for DNA QC metrics.

Conclusions, Implications, and Discussion

This work supported previous research showing significant correlation and variation in TL across tissues. It also highlighted how processes of sample collection and DNA extraction procedures can impact metrics of DNA quality and purity. aTL significantly shortened with chronological age for all tissues except saliva and buffy coat, the latter of which had a restricted age range (i.e., 8 to 15 years). aTL varied by tissue, particularly between blood and non-blood tissues. Despite this variation, aTL was correlated across most tissue pairs. Cross-tissue variation in DNA qualities may help drive variation in aTL, and the investigators provided evidence that longer aTL is linked to higher DIN, DNA concentrations, and to some extent, A260/230 values. Model comparisons suggest that incorporation of DNA metrics significantly improves predictions of aTL, although important metrics vary by tissue. These results highlighted

potential considerations for tissue selection in future population-based studies of TL and the value of incorporating quality DNA metrics as control variables to improve TL prediction.

Discussion then centered on the relevance of these data for future population-based studies of TL. As shown in limited previous work, blood-based samples exhibited the highest quality DNA and therefore, may be preferred for reliable measurement of TL. Buffy coat and PBMCs exhibited high DNA integrity and more acceptable A260/280 and A260/230 values compared to less invasive tissues like buccal and saliva, which exhibit more variable and lower quality DNA metrics. DBS, as a minimally invasive tissue, had similar aTL values to PBMC and buffy coat, and results suggest this as an appropriate alternative to blood-based samples, especially in pediatric and remote populations. Saliva had lower DNA integrity, aTL values that were strongly influenced by metrics of DNA quality, and aTL did not significantly decrease with age despite being measured in both the child and adult cohorts. Taken together, this suggests saliva collection processes specific to children can result in low quality DNA, perhaps stemming from a higher proportion of non-viable cells. This conflicts with previous work supporting saliva as an acceptable alternative to blood and suggests the need for additional tissue comparisons of DNA quality metrics and evaluation of saliva TL precision. However, not all new or ongoing studies can rely on blood-based tissues. In such instances, these results show that quantifying sample-specific metrics of DNA quality for use in model predictions of TL can improve model fits of the data, thereby strengthening the signal of exogeneous predictors of TL and the utility of TL as a proxy for health-related outcomes. Alternative to controlling for variation in DNA metrics, standardizing DNA extractions to yield consistent concentrations could also minimize methodological impacts on TL measures. The investigators encouraged further study of variation in quality metrics of DNA across tissues and how it may mediate variation in TL, which can help inform how to select tissues and/or control for differences in DNA quality in future population-based telomere studies.

Citation:
Wolf SE, Hastings WJ, Ye Q, Etzel L, Apsley AT, Chiaro C, Heim CM, Heller T, Noll JG, Schreier HMC, Shenk CE, Shalev I (2024). Cross-tissue comparison of telomere length and metrics of DNA integrity, quality, and quantity among individuals aged 8 to 70 years. *PLoS ONE*. doi: 10.1371/journal.pone.0290918.

Open Forum: Next Methodological Questions for the Telomere Research Network and Recommendations for Additional Cross Laboratory Studies

Chair: Stacy Drury, MD PhD, Tulane University School of Medicine

In this open forum TRN meeting attendees reflected on results presented from U24 collaboratory and U01 laboratory studies to identify important methodological considerations for TL measurement to be address by future TRN collaboration. This session focused on brainstorming next steps for the TRN and how to integrate all this information from this year's conference and previous years into the next 2 years of funding for the U24. To start, Dr. Drury highlighted the key takeaways from the TRN as well as the conference. Cross laboratory data was discussed first, where several methods demonstrating relatively strong reproducibility. In fact, there is likely

no upper limit for reproducibility as this metric can be bolstered with ever increasing size of reproducibility samples. Moreover, the data also demonstrated there is no lower limit to reproducibility. In other words, when poorly implemented or initiated with low quality samples, even the most robust measurement approaches can exhibit low reproducibility. For example, even Southern blot, often described as the silver standard measurement of TL, due to the absence of a true TL accurate measurement, is susceptible to wide variation in measurement precision and is also subject to measurement challenges due to individual differences in subtelomere regions. Thus, regular checking and reporting on the reproducibility, using the ICC, of these assays and ensuring utilization of rigorous reporting guidelines for scientific dissemination is a critical need for the field. Ensuring the reporting of between DNA extraction ICCs for each and every cohort, inclusion of DNA quality metrics, and careful consideration of methods used for cross sectional compared to longitudinal studies is needed. The utility of population nomograms was then brought up by an audience member, in which TL can be measured and compared to the relative TL of an individual of that same age in a population.

Discussion next focused on how researchers can be more thoughtful of telomere biology when choosing which measurement approach to utilize. One example explained by Dr. Verhulst is further consideration on an epidemiological level whether shorter or longer TL is a better predictor of significant endpoints compared to average TL. In contrast to human work, work in avian models suggest it is the longest telomeres that seemed to be the most predictive of outcomes such as mortality. Dr. Ling Zheng related this to her work with cancer research, where longer telomeres are associated with increased risk for bladder and lung cancers, but are less predictive for other cancer etiologies, highlighting the complexity of telomere biology in cancer. By contrast, work presented by Dr. Savage using the STELA methodology demonstrated the importance of the shortest telomeres as the predictors of health outcomes. Drawing on evidence from Drs. Gadalla and Aviv's work, where Southern blot outperformed qPCR in evaluating clinical response to HCT transplant, she posited that methods with high sensitivity to detect very short telomeres such as STELA, Flow-FISH, and Southern Blot would be best suited for research into telomere biology disorders, but such sensitivity not be necessary for studies investigating other health outcomes. To address these needs, it was suggested that the TRN could support and encourage studies that investigate what metric of TL and stability is most predictive of different types of outcomes and exposures, drawing on data from key cohorts with robust data for environmental exposures, health outcomes, and TL quality control. This suggestion received wide support from attendees.

Dr. Hastings proposed that advancing the goal of determining which telomere metric is predictive of an outcome requires more laboratories to be engaged and utilizing advanced measurement techniques beyond qPCR. He recommended that the TRN support training aimed at increasing the number of laboratories that can implement complex measurement techniques providing more advanced telomere metrics (such as TeSLA and STELA). Dr. Drury responded to this recommendation by saying if laboratories were to be trained in these techniques then standardization and continued assessments to make sure the methodological rigor of these techniques is maintained would be essential. Challenges would include the onboarding of labs,

the need for guidelines to ensure precision of new methods added to laboratories, issue of the DNA extraction method, and then issue of the accuracy of TL measurements. Dr. Eisenberg added that this effort could begin from the ground up where a laboratory that is proficient in a specific technique can pair up with a laboratory that is interested in improving its technique and have buy-in from both to improve methodological rigor and consequent sharing of data. Dr. Eisenberg also noted the availability of existing data to meet this challenge. For example, stored images of Southern blot gels that could be reanalyzed with more diverse telomere metrics (e.g., distribution of TL) as a means to increase the diversity of metrics associated with health outcomes while simultaneously being used as an avenue to introduce more researchers to the method.

Attendees then discussed the development of new assays, highlighting the need for a training model to ensure quality control, precision, accuracy, and reproducibility of new methods developed or when existing methods are operationalized for the first time in a new laboratory. This led some to suggest the need for a TL standard that can be replicated, shared, and used for calibration and development of new assays. A call to action was made because no such standard currently exists, but is sorely needed, especially for future studies working with samples expected to have relatively short telomeres (e.g. <3kb). Important factors to consider when optimizing new or existing approaches, as highlighted by Dr. Shalev's investigation, include quality of the DNA, quantity of DNA, assay throughout, and imaging analysis (where applicable). Dr. Eisenberg noted the need to consider additional confounds in addition to these factors, noting by way of example that T/S ratio measurements generated using qPCR are influenced through different forms of DNA extractions. Dr. Lin added that there is not currently a way to know how accurate TL can be measured and that in some respects all current measurements are relative. Thus, even with a universal standard open questions about accuracy of the resulting measurements would remain. To address this, Dr. Alder suggested that if the same type of extraction method was used for all samples and the universal standard, then a deviation from this standard could still be calculated and used as a relative TL metric comparable to other approaches, so long as they also maintained equivalent processing between the universal standard and analytical samples. Claims about the lack true accuracy in TL measurements were challenged by Dr. Ahmad, who added that long-read sequencing can be used to measure TL explicitly, as is done in his research with *C. elegans* and others' work with yeast telomeres.

Keynote – Exposome Exposures and Health Effects: Identifying Exposure Priorities to Improve Health and Health Equity

Tracy Woodruff, PhD, MPH, University of California San Francisco

Spanning from around 1945 until the present, there has been an increase in both the variety and quantity of industrial chemicals manufactured and used in the US. Globally, 350,000 chemicals and mixtures are registered for use and/or production. The U.S. has 40,000 chemicals registered for use, with 9.5 trillion lbs. of chemicals being produced or imported per year, amounting to roughly 30,000 lbs. per person per year. Some of these chemicals produce unknown byproducts, and only a fraction have been measured or evaluated for health effects in

pregnant women or children. Efforts to further characterize chemical exposure are impaired by US laws that enable chemical companies to disregard disclosure regarding where they're using the chemicals that are in their product. Due to this lack of reporting, emerging compounds are increasingly being found in pregnant women, including sumilizer GA 80, a plasticizer used in production of plastic polymers, and other polyfluoroalkyls (PFAs) not included within the 6 currently regulated including 2-perfluorooctyl ethanoic acid, a derivative of long-chain fluorotelomer products used as surface protectors like in textiles and carpet.

Data collected through the NIEHS EPA Children's center indicated that the increased detection of these chemicals during pregnancy and in the environment around children likely contribute to the disproportionate rates of chronic diseases and developmental disorders in children, particularly those of Black and Latinx origin. Compounds responsible for such outcomes vary from perfluorochemicals (PFCs/PFAs), plasticizer-related chemicals like phthalates or Bisphenol A (BPA), and flame retardants, to only name a few. By participating in analytic chemistry efforts to incorporate biomonitoring as a measure for evaluating chemicals in biological samples, Dr. Woodruff, Endowed Professor of Reproductive Health and Environmental Research at University of California San Francisco, seeks to enhance opportunities for decision-making entities within industry and government to create new policies that mitigate damaging effects of environmental exposures on public health.

Scientific Approach to Evaluating the Intersection Between Chemical Exposures and Social/Structural Stressors

Alongside NIEHS, Dr. Woodruff is seeking to advance a new direction for exposure research, viewing humans as living in an "Exposome" reflecting the totality of human environmental exposure, recognizing that there are many exposures occurring that we don't currently measure, from conception throughout the lifespan. The specific environmental inputs of interest will vary between fields, but the goal is to view the exposome as interactive effects between environmental toxicants/chemicals, individual factors, and life experiences at home and work. For the last five years Dr. Woodruff worked to disentangle interactions between environmental exposures and structural inequities such as redlining, financial strain, food insecurity, and psychosocial stress as part of a larger consortium of cohorts across the US that deals with Environmental influences on Child Health Outcomes (ECHO). Work from ECHO has found that there are disproportionate levels of chemical exposure among communities in the US, and that these two factors of social/structural inequities and chemical exposures do occur in combination, ultimately compounding negative effects on health. This work includes research showing high prevalence (85%) of environmental toxicants in both low- and high-income pregnant women within the San Francisco Bay Area, and that these toxicants acted in concert with psychosocial stress to productive additive risks for low birthweight and adverse pregnancy outcomes. In coordination with Dr. Rachel Morello-Frosch and Dr. Stephanie Eick, Dr. Woodruff looked deeper into whether these interactive and additive effects are influenced by biological susceptibility, and they found that markers indicating increased risk for chronic disease, can make individuals more susceptible to environmental exposures.

Ensuring that chemicals of concern to pregnancy and health can be accurately identified requires methodology that is precise and reliable. Advancing work on this front, Dr. Woodruff along with her collaborators, Dr. Abrahamsson and Dr. Morello-Frosch, are funded to advance non-targeted high-resolution mass spectrometry. This methodology was utilized in previous research defining the mechanistic role of PFAs in pathways related to cholesterol development, providing evidence that silicone-based compounds, found in biotic samples, interfere with fatty acid metabolism. It is investments into improved methodology that empower future discovery into the impacts of environmental exposure on disease. This approach is well reflected by work between UC Berkeley, the State of California, and the International Agency on Research on Carcinogens, who collaborated to develop a system to categorize the cancer and other health risks of novel compounds based upon previous mechanistic data, including whether similar chemicals have been found to be electrophilic, genotoxic, or associated with increased oxidative stress. These categories serve as mechanistic pathways linking environmental exposures to cancer, making it simpler to sort information while also identifying the potential health risks of novel compounds based on data from related compounds. Collaborating with basic scientists is also essential to promote early identification of chemicals that are potentially harmful. This work is highlighted by collaborations with labs at UCSF and UCLA using the high-throughput screening reproductive and developmental toxicants (HART) assay, which combines toxicity exposure and organismal culture in a 96-well format. This assay allows labs to rapidly screen a diverse set of chemicals in multiple organisms, including one study where HART was used to identify biotic effects from chemicals extracted from 180 different environmental samples on reproductive health outcomes in yeast and *C. elegans*, indicating that in both species toxins like BPA were prevalent in the environment and potent in the organism.

Research Collaboration

Collaboration opportunities are essential to understand and address environmental contributors to disease. In addition to collaborative research, the ECHO program also focuses on avenues to consolidate data and methods for population studies, aggregating data from psychometric questionnaires, child health outcomes, and biological samples on over 50,000 participants, half of which are non-white, which creates an important opportunity to evaluate different health outcomes and exposures specifically regarding Black and Latinx participants.

Dr. Woodruff also assisted in the development of a new consortium being funded by the NCI and NIEHS called the Seeker Consortium. The Seeker Consortium consists of five cohorts across the US who are working to understand environmental influences on cancer. Their work involves recruiting pregnant participant from Fresno CA, a highly polluted area, and looking at the impact of these exposures on intermediate biomarkers of cancer, including telomeres. In addition to the ECHO and Seeker data repositories, entities like the EPA, USDA, FDA, and NHANES possess similar databases, further contributing literature on the products containing various chemicals to allow for more thorough computational toxicology approaches.

Engaging the Clinical Community

Dr. Woodruff aims to translate population and mechanistic research into meaningful changes in policy and practice that improve human health. Toward this end, she works with several entities that have moved to integrate this science of environmental exposures on health into their professional practice and deliberations: The American College of Obstetricians and Gynecologists (ACOG), the American Society of Reproductive Medicine (ASRM), and the Federation International of Obstetricians and Gynecologists (FIGO). The clinical community has become increasingly concerned about the lack of clarity regarding what different environmental exposures mean for their patients' health. To address these concerns, Dr. Woodruff has facilitated increased engagement with professional communities, working to integrate learning on environmental reproductive health as components of physician training and patient interaction, emphasizing that physicians should knowledgeable about how chemicals can adversely influence developmental reproductive health, especially among underserved communities of color with higher rates of exposure.

Conclusion, Implications, and Areas for Expansion

Equitability and the reduction of toxic chemical exposures requires systematic changes in order promote lasting effects, a sentiment largely shared by the voting public. In a survey of 1,000 people spanning diverse political, income, and sociodemographic strata, all groups overwhelmingly agreed that companies should be reducing harmful chemicals from products, that the government has a role to play, and that this is an important consideration in terms of people's health. Intervention on chemical exposure generates significant economic and public health returns. Previous updates to EPA standards, such as reducing acceptable amounts of PFAs in drinking water, generated an estimated savings near $1 billion annually, largely through reduced incidence of cardiovascular events, pregnancy complications, and infant mortality.

Scientists can do much to enhance these processes. First, they can enhance the quality of research with improved methodology and collaboration. Methodological improvements allow for more efficient chemical identification, knowledge which can be then shared with basic scientists working to determine the impact of these chemicals on physiology using *in vitro* models, especially those suited to predict reproductive outcomes, where toxicants can be especially potent. Collaborative research also enhances sample size and the power to distinguish interactive effects, with larger consortium studies being able to look associations across thousands of outcomes and exposures. In addition to enhanced research quality, can assist policy makers to make use of the best available science to make decisions to protect public health. Despite working with professionals at the EPA, government entities, and other policymakers, Dr. Woodruff highlighted how structural issues still exist in translating science into use. Nevertheless, she concluded her talk with five principles for decision-makers to use while approaching chemical exposures and health hazards: 1) industries pay for data collection, 2) companies should make clear that having a lack of data does not mean lack of risk, 3) prioritize identification of populations at risk, 4) exposures are not homogenously distributed, so do not safety thresholds at the population level, and 5) risk assessments should include and account for possible conflicts of interest.

Questions and Discussion

Following the presentation Dr. Woodruff addressed questions from the audience, beginning with a question from Dr. Tom Welsh about whether TL was measured in the ECHO study, and if so, was it affected by the combined impacts of stress and pollutant exposure in a manner resembling the additive effects on adverse pregnancy outcomes. Dr. Woodruff indicated that TL was measured in this study, but that it was conducted on a smaller sample than was used for the larger analyses. Overall, the relationship was contrary to expectations, increased pollutant exposure was associated with longer TL in cord blood and pregnant women when measured at the 2nd trimester. Dr. Woodruff acknowledged that they are unsure how to interpret this finding, suggesting that the longer TL might be a proxy for increased cancer risk following pollutant exposure, adding that they aim to address this question within the Discovering cancer Risks from Environmental Exposures And Maternal/child health (DREAM) cohort. Dr. Stacy Drury followed this question, drawing on data from their study of mothers and infants in Suriname in which some chemicals were associated with longer telomeres, while others were associated with shorter telomeres. Both Dr. Drury and Dr. Woodruff agreed about the importance of distinguishing between different pollutant exposures, highlighting the need to contextualize pollutants within their effects on different biological systems.

Dr. Verhulst followed this discussion by asking Dr. Woodruff to provide her thoughts about whether outcome measures could be aggregated to form a more complete picture of health impacts in a manner similar to how the many environmental (e.g., pollutants) and psychosocial (e.g., stress) factors are aggregated to conceptualize a complete exposome. Dr. Woodruff responded that they selected their approach toward aggregating the exposome because in addition to looking at the combined effects on a given health outcome, it also allowed them to parse out the relative weights of different individual factors to the overall effect observed on a health outcome. With respect to adopting a similar aggregation for health outcomes, Dr. Woodruff used the example of metabolomics, where they can look at groups of metabolites that are all affected by a particular exposure or group of exposures, and then conduct functional enrichment on these metabolites to identify particular biological pathways or systems impacted. Dr. Woodruff cited the combination of metabolomics data to measure endogenous molecules within individuals with targeted approaches to measure pollutant exposure as an exemplar way for researchers to identify potential mechanisms mediating the impact of pollutants on health, citing by example an analysis they had conducted showing a distinct relationship between siloxanes and fatty acid metabolism. Researchers can then use data generated from human samples, for example relative combinations of different pollutants, to design in-vitro studies in model organisms to discern the actual impacts of these compositions on biological function. Dr. Woodruff concluded by reiterating that the diversity of pollutants and biological functions implicated in human health makes the problem of identifying mechanistic impacts incredibly complex, emphasizing that making headway requires partnerships across the basic and population sciences, as well as between governmental entities to promote translation of findings into meaningful societal change.

Session 2: Telomere Network Analysis Awardee Presentations

Chair: Elissa Epel, PhD, University of California San Francisco (remote)

To date the TRN has awarded three cycles of pilot analysis awards, supporting 8 projects in 2020, 6 projects in 2021, and 3 projects in 2022. These projects have emphasized research on the environmental impacts of pollutants and early life stress on TL, dynamics of TL in newborns, and methodological validation of new and emerging TL measurement methodologies. These TRN analysis awards have led to 8 accepted or submitted publications, supported pilot data collection for grants, and promoted professional collaboration leading to postdoctoral appointments with TRN PIs. TRN pilot awards are continuing for the 2023 cycle, emphasizing projects with new or optimized measurement methodologies, large scale studies to examine impacts of environmental exposures on multiple telomere parameters, and studies examining associations with disease outcomes and telomere stability.

Predictors of Newborn Telomere Length and Efforts to Define Early Life Trajectory of Telomere

Dries Martens, PhD, University of Hasselt

Dr. Dries Martens, Centre for Environmental Sciences, presented work highlighting the importance of measuring newborn TL. It is well known that TL shortens over time and that the risk of developing age-related disease increases with chronological age, with robust evidence provided by the UK Biobank linking average TL and the development of age-related diseases independently of chronological age. Importantly, there is wide variation in TL at birth, both when TL is measured with qPCR or the TRF method, and this variation at birth is strongly related to later life TL. Two studies by Benetos et al. published in 2013 (*Aging Cell*) and 2019 (*FASEB*) demonstrated the stability and predictability of later life TL based on TL measured earlier in live, suggestive that TL at birth might be a strong predictor of later-life TL. This finding was extended by the Martens et al. 2021 (*EBioMedicine*) study where rank-order TL at age 4 did not change dramatically compared to newborn TL ($n=273$, $r=0.73$, $p<0.0001$). A difference was noted between qPCR and TRF, where qPCR demonstrated a slightly lower rank-order stability in TL compared to the TRF method potentially due to measurement error. As later life TL is highly determined at birth, the importance of measuring newborn TL was discussed in relation to the potential impacts on later-life health, markers of disease susceptibility, and life expectancy. More specifically, initial TL was discussed as a molecular explanation linking adverse prenatal conditions to later life health and disease under a Developmental Origins of Health and Disease (DOHaD) model. Most studies to date investigated predictors of newborn TL, but few studies focus on trajectories of TL following birth. Relevant to the TRN is the current work to fill these knowledge gaps by focusing on larger prospective birth cohorts, confirming predictors of newborn TL and trajectories, and studying health changes in relation to initial TL such as the FINNBRAIN cohort and ENVIRONAGE studies.

Conclusions and Discussion

Overall, Dr. Martens found that attrition during childhood and adulthood does not strongly change the rank order of TL within a population in adulthood. Further, it is likely that TL at birth and the change in early life TL are stronger predictors of adult health outcomes than TL attrition observed later in life. The heritability of TL was briefly discussed in addition to its stability in relation to genetic and epigenetic factors. To answer these questions the shared parental environment as well as the early life environment should be investigated while measuring TL at birth and across development.

Citation:

Martens DS, Van Der Strukken C, Derom C, Thiery E, Bijnens EM, Nawrot TS (2021) Newborn telomere length predicts later life telomere length: Tracking telomere length from birth to child- and adulthood. *EBioMedicine.* doi: 10.1016/j.ebiom.2020.103164.

Joint Effects of Telomere Length and Social Environment in Predicting Youth Delinquency

Darlene Kertes, PhD, University of Florida

Dr. Darlene Kertes, Associate Professor of Psychology, operationalized TL within a developmental framework that considers cellular age as a cumulative biomarker of the impact of life adversities. Her work investigated the interplay of cellular age, sex, and TL to inform modifiable interventions of the social environment that impact overall health. The presented data was drawn from an investigation on associations between TL and behavioral outcomes in a cohort of 411 adolescents aged 11-17. Given the knowledge gaps surrounding the relationship between TL and behavioral problems in youth, a Bayesian approach was used to determine predictors. The phenotype assessment in this study included the Externalizing Behavior Problems self-reported inventory to assess violent versus non-violent delinquency, the Social Environment Array to assess social contexts, and demographic covariates such as age and season of collection. TL assessment was conducted using singplex qPCR in genomic DNA extracted from passive drool saliva. To analyze this data a Bayesian Poisson regression model was used to quantify the data and identify the strongest contributors to the direct effects of social environment and the interactions with TL and sex. Positive direct effects of the social environment on delinquency unrelated to TL were found such as witnessing violent victimization in their everyday life, attitudes towards violence, and whether they've experienced physical aggression at the hands of their parents. By contrast, religiosity was observed to have a negative direct effect on delinquency. Three interactions were observed regarding effects of social environment and TL on non-violent delinquency. First, T/S ratio is associated with nonviolent delinquency for girls regardless of friend support, whereas for boys it is only related under conditions of low support. Second, the association of T/S ratio with non-violent delinquency is strong under conditions of low family support for boys only. Third, a smaller T/S ratio is associated with nonviolent delinquency for girls regardless of caregivers' level of social support.

Conclusions and Discussion

This study suggested that social environment factors and TL interact to predict externalizing-type behavioral health problems, as demonstrated by the magnitude to which T/S ratio is associated with delinquency being somewhat dependent on social support that individuals feel. Sex differences in these associations are also apparent, as interaction effects of TL and social environment were demonstrably stronger for boys. Dr. Kertes' study highlighted the importance of incorporating the social environment and cellular aging in studies investigating risk factors among vulnerable populations, such as delinquency in adolescents. A discussion was held regarding the limitations of self-report measures and the honesty of male versus female respondents on the Phenotype Assessment. While noting that the sample is almost equally split (54% were male), Dr. Kertes highlighted that levels of non-violent delinquency are equal among boys and girls in the general population, but in this study the self-report measure showed higher rates among girls.

Citation:

Kertes DA, Clendinen C, Duan K, Rabinowitz JA, Browning C, Kvam P (2024) The social environment matters for telomere length and internalizing problems during adolescence. *Journal of Youth and Adolescence.* doi: 10.1007/s10964-023-01848-w.

Associations Between Early Life Adversities, Ambient Air Pollution, and Buccal Telomere Length in Children

Rosemarie de la Rosa, PhD, MPH, University of California Berkeley (remote)

Dr. Rosemarie de la Rosa, Assistant Professor of Environmental Health Sciences, presented data from the Pediatric ACEs Screening and Resiliency (PEARLS) Study aimed to investigate the association between TL and air pollution and childhood adversity in Black and Latinx communities, exposures which are highly concentrated in these communities due to structural policies and inequities. Epidemiological evidence, such as that presented by Dr. Woodruff, has implicated psychosocial stress as a modifier and potential amplifier of the association between air pollution and negative childhood health outcomes such as asthma. One of the guiding questions in this study was: What are the mechanistic biological pathways that are resulting from those cumulative effects of chemical and psychological stressors? Hypothesized pathways included the effect of air pollution on increasing reactive oxygen species (ROS) levels, which can contribute to an observable aging phenotype via damage to telomeres. Similarly, higher instances of Adverse Childhood Experiences may also be associated with telomere shortening. Together, these hypotheses led to the primary research question of the study: Does exposure to childhood adversity modify the association between fine particulate matter ($PM_{2.5}$) and TL? In a cohort of 197 PEARLS participants, experiences of abuse, neglect, household challenges, and related life events were identified using the 17-item ACEs and Related Life Event Screener tools. Buccal TL was measured by singleplex qPCR and normalized to control DNA from the 1301 cell line. Pollution was measured as exposure estimates generated from monthly ground-level $PM_{2.5}$ data. Exposure to childhood adversity was not directly related to differences in TL. There was a however a direct association between pollution exposure and TL, such that each one unit

increase in 12-month $PM_{2.5}$ was associated with a 0.02 decrease in TL as indicated by the T/S ratio. Additionally, the association between the 12-month PM2.5 and shorter TL was stronger among children that reported greater Related Life Events.

Conclusions and Discussion

Overall, Dr. de la Rosa's study highlighted how children with higher levels of reported adversity may be more susceptible to the effects of chronic PM2.5 exposure on TL shortening. During discussion, the age at which these exposures to adversity was questioned by the audience. Dr. de la Rosa noted that due to the cross-sectional study design employed they were not able to pull the age of exposure but now that this study is employing a longitudinal design those data points will be collected and directionality will be more thoroughly examined.

Citation:

de la Rosa R, Le A. Holm S, Ye M, Bush N, Hessler D, Koita K, Bucci M, Long D, Thakur N (2024) Associations Between Early-Life Adversity, Ambient Air Pollution, and Telomere Length in Children. Psychosomatic Medicine doi: 0.1097/PSY.0000000000001276.

Anti-aging Effects of Elite Football and Team Handball Trainings

Muhammad Asghar, PhD, Lund University

Dr. Asghar, Ragnar Soderberg Fellow and Associate Professor of Biology, presented aimed to investigate the impact of elite sport training on measures of cellular aging. Changes in cellular aging involve a dynamic interplay between genetic and environmental factors. Elite athletes offer one avenue to explore the effects of behavior on aging as they naturally adopt a pro-healthy lifestyle without external intervention. This study sample consisted of 129 elite women football players and lifetime handball players with no known disease. Participants were divided into four groups: young football players, young controls, elderly handball players, and elderly controls to include age-matched controls. Physiological aging was measured with body composition, bone mineral density, cardiovascular disease parameters, and physical performance. Biological aging was measured as TL in whole blood as well as leukocyte subsets of lymphocytes and monocytes. Additional biological aging indicators included mitochondrial copy number and mitochondrial biogenesis. Results showed significant differences in physiological aging between athletes. Young football athletes and elderly handball players showed significantly greater lean muscle mass, lower body fat, and improved VO_2 max relative to age-matched counterparts. Results for biological aging metrics were mixed. Younger football players showed longer TL, but there were not differences observed for older athletes. Mitochondrial content was higher in both young elite football players and elderly controls. However, despite having higher mitochondrial content, elderly controls exhibited lower mitochondrial function.

Conclusions and Discussion

This study provided mixed evidence for the impact of elite physical training on physiological and biological aging. Although physiological parameters like muscle mass, lean fat, and respiratory activity (i.e., VO_2 Max) were shifted toward anti-aging phenotypes, biological aging indicators were less indicative of decreased cellular aging. Questions from the audience regarding the

social nature of a team sport like football or handball and the self-selection of participants that actively join these teams were brought up to address potential confounds to the conclusion of causality. The study's cross-sectional study design was noted by Dr. Asghar, who expressed a desire for continuing this study longitudinally and increasing the sample size to rule out potential confounds and increase power to demonstrate clear directionality.

Citation:

Hagman M, Fristrub B, Michelin R, Krustrup P, Asghar M (2021) Football and team handball training postpone cellular aging in women. *Scientific Reports.* doi: 10.1038/s41598-021-91255-7.

Session 3: Novel Method Development for Telomere Length Measurement

Chair: Stacy Drury, MD, PhD, Tulane University School of Medicine

The purpose of this session was to highlight work conducted by TRN affiliates toward establishing novel approaches and analytical strategies aimed at improving the precision and diversity of TL measurements.

Introductory Remarks

Richard Hodes, MD, National Institute on Aging (remote)
Richard Woychik, PhD, National Institute of Environmental Health Sciences (remote)

Dr. Richard Woychik, Director of the National Institute of Environmental Health Sciences (NIEHS), provided opening remarks for Day 2 of the conference, highlighting the overlapping goals of the TRN and NIEHS in fueling research on how the environment positively and negatively affects human health. The need to study the intersection of multiple environmental exposures through interdisciplinary collaboration was also discussed, utilizing the "exposome framework" and noting the importance in individual differences in response to exposures. This concept was framed as 'precision environmental health' which aims to investigate the ways in which people respond to compounding environmental exposures in unique ways. Relevant to the TRN was Dr. Woychik's remarks on TL as a crucial biomarker for stress, environmental exposures, and more importantly resilience against exposures to best understand disease risk at the cellular and community level. Dr. Richard J. Hodes, Director of the National Institute of Aging (NIA), seconded these remarks, adding comments on the need to look at the integrated impact of environmental exposures on TL. Dr. Hodes also emphasized the unique role the TRN plays as a model for collaborative interdisciplinary research across the domains of biology, sociology, neuroscience, and public health.

Assessment of Amplification Efficiency and Efficiency of Mismatched Primer Pairs to Amplify Vertebrate Telomere Repeats via qPCR

Eugenia Xu, PhD, Princeton University

Traditional qPCR operates under the principle that changes in fluorescence are related to changes in the quantity of target DNA, which increases with each cycle of PCR amplification.

The quantity of target DNA after a given number of PCR cycles (N_c) is related to the number of amplification cycles (c), the quantity of target DNA at baseline (N_0), and the amplification efficiency (E) by the formula $N_c = N_0 * E^c$. Under conditions of perfect amplification, the efficiency takes a value of 2, reflecting a perfect doubling of target DNA with each PCR cycle.

The amplification efficiency is affected by many factors, including the match of PCR primers to target DNA template. The qPCR approach to TL measurement originally developed by Richard Cawthon utilizes primers that are slightly mismatched to telomere repeats. This is because perfect match telomere primers to repetitive telomere repeats can form primer dimers, wherein primers preferentially bind to each other instead of target sequence, preventing extension by Taq polymerase and impairing TL measurements. There are three sets of mismatched telomere primers used in the predominate amount of studies using qPCR-based measurements of TL: tel1/tel2 [5'-**ggtttt**(t**g**aggg)$_5$t-3'] / [3'-**tcccga**(cta**t**cc)$_5$cta-5'], tel1b/tel2b [5'-**cggttt**(gtt**t**gg)$_5$gtt-3'] / [3'-**ggcttg**(cct**t**ac)$_5$cct-5'], and telg/telc [5'-**acactaa**(ggtt**t**g)$_4$ggttag**t**gt-3'] / [3'-**tgttagg**(ta**t**ccc)$_5$taac**a**-5'], bolded text indicates mismatched bases. These primer sets exhibited significantly different estimates of baseline telomere content when using the same amount of oligonucleotide telomere duplex as sample input. Moreover, the degree of baseline content estimated was significantly lower for all three primer sets relative to estimates of baseline content generated using perfect match primer sets aligned to oligonucleotide duplexes corresponding to the IFNB1 and 36B4 reference genes commonly utilized in TL qPCR protocols. This implies that 1) mismatched primers underestimated baseline content of target nucleotide sequence and 2) the degree of underestimation was variable between different primers sets.

To address this observation, Dr. Xu, in collaboration with Dr. Daniel Notterman and Dr. Lisa Schneper, sought to determine an approach to 1) better select mismatched primers for qPCR measurement of TL, and 2) improve quantification accuracy of estimates derived from mismatched primers. Since estimates of baseline content were different despite sample input and cycle number being maintained between different primer sets, the team hypothesized that mismatched primers would impact baseline estimates through varied ineffective amplification or usage of target DNA. Importantly, newly synthesized amplicons generated during PCR would be perfectly matched to primers, since Taq polymerase would generate DNA corresponding to the primer template. Thus, impacts of primer mismatch on ineffective amplification or use of target DNA would be most acute during early PCR cycles when the proportion of mismatched to matched amplicons is highest. Importantly, these differences are not captured by qPCR instrumentation, which quantify amplification efficiency during the exponential measurement phase, at which time target quantity reaches a sufficient level to be detectable via fluorescence.

Amplification Efficacy

To address the impact of mismatch primers on baseline estimates of telomere content, the team proposed the concept of amplification efficacy (*f*), defined as how effective the initial target DNA is amplified in a qPCR assay, and quantified as $f = N_0/N_i$, where N_0, is the observed quantity of the target amplicon estimated from qPCR and N_i is the actual quantity of the target amplicon within the input sample. This parameter would then be integrated into existing kinetics formula

to correct for mismatched primers with inadequate use of input sample in early PCR cycles, resulting in an updated formula as $Nc = f*N_i*E^c$. The question then becomes, how can we determine the value of amplification efficacy *(f)* for different sets of mismatched primers?

To determine this, the team used oligo duplexes containing the telomere sequence as well as sequence corresponding to reference genes *IFNB1* or *36B4*. In this manner, the same oligonucleotide can be used for reference gene and telomere primer sets, ensuring baseline copy number is maintained across conditions. Thus, any difference in predicted baseline estimates could be confidently attributed to differences in amplification efficacy resulting from primer mismatch. In addition, the team employed a control duplex containing the sequence of both reference genes. Any differences in baseline estimates derived using each reference gene set, both of which match perfectly and should have similar efficiency and efficacy, would be attributed to experimental noise.

Results and Conclusions

Results indicated that all mismatched primer sets had similar amplification efficiency to perfectly matched reference gene primers, as expected. However, all three primer sets had reduced amplification *efficacy* relative to reference primer sets. Moreover, the order of primers was maintained irrespective of whether the efficacy was calculated with respect to IFNB1 or 36B4 amplicons. Primer set tel1b/tel2b exhibited the best efficacy, followed distantly by tel1/tel2 and telg/telc which had efficacy comparable to one another. This implies that the tel1b/tel2b primer set would generate the least biased estimates of telomere DNA content. The team also discussed the impact of primer concentration on amplification efficacy, noting that 500nM and 900 nm concentration showed better amplification efficacy than 100 nM, with 900 nM slightly better than 500 nM concentration. The team recommended incorporating oligonucleotide duplexes containing reference gene and telomere sequence to generate estimates of amplification efficacy for the primer sets utilized in a given study prior to running analytical samples to improve the validity of measurements derived for analytical samples.

Citation:

Xu E, Schneper LM, Notterman DA (2023) A novel metric to improve mismatched primer selection and quantification accuracy in amplifying DNA repeats for polymerase chain reactions. *PLOS One.* doi: 10.1371/journal.pone.0292559.

Nanopore telomere sequencing -NanoTelSeq- Enables Accurate Length Measurement of Telomeres

Riham Smoom, The Hebrew University of Jerusalem (remote)

Riham Smoom is a doctoral student in the lab of Dr. Yehuda Tzfati, developing the use of long read nanopore sequencing for accurately measuring TL, termed NanoTelSeq. The lab turned to this approach to address curious findings in Riham's work with mouse samples. Previous work by Peter Lansdorp's lab, using crossbreeds between long TL (40-50 kb) M. musculus mice and short TL (5-15 kb) M. spretus mice, identified the Regulator of Telomere Elongation helicase 1

(*RTEL1*) gene as genetically associated with the different TL in the two mouse species. However, the functional difference between the two RTEL1 proteins that contributes to mechanisms leading to differences in TL had not been found. Follow-up work in Dr. Tzfati's lab suggested that the dramatic difference in TL between the two species of mice is due to a single amino acid variation in RTEL1. To test this, they established an experimental M. musculus strain of mouse in collaboration with Dr. Kaestner's lab at the University of Pennsylvania Perelman School of Medicine. This mouse model, carrying a single amino acid change of methionine 492 in RTEL1 to a Lysine, was termed 'Telomouse' or K/K. Pulse Field Gel Electrophoresis (PFGE) showed gradual TL shortening in mouse embryonic fibroblasts (MEFs) derived from Telomouse, decreasing in average TL from 28.3 kb to 15.3 kb between population doubling (PD) 10 and PD 250. By contrast, the wild-type MEFs had longer TL to start (46.9 kb), which only mildly shortened to 37.8 at PD 250. These findings are consistent with the function of RTEL1 as a regulator of TL.

However, this study's results generated more sources of inquiry, as instead of simply decreasing in migration (indicative of shorter average TL), the overall banding pattern of Telomouse MEFs also decreased in intensity as TL decreased. This occurred despite the same amount of DNA being input to each well, indicating a loss of telomere DNA in addition to decreases in average TL. To investigate further, they mixed Telomouse MEFs at PD250 with human fibroblasts and compared TL using quantitative metaphase FISH (Q-FISH), easily differentiating between the cell types by their vastly different centromere position. Subsequent Q-FISH analysis showed that Telomouse MEFs and human fibroblasts had comparable telomere DNA signals. However, Telomouse cells exhibited a greater heterogeneity in TL and a larger fraction of very short telomeres, below the limit of Q-FISH detection. To further characterize these very short telomeres, they conducted regular (not pulse-field) in-gel hybridization, revealing a smear of telomere DNA signal below 5 kb – below the range of detection in PFGE and Q-FISH.

As the shortest telomeres are often hypothesized to be particularly relevant to cellular function and senescence, detection of the very short telomeres is relevant. Thus, Dr. Tzfati's lab, realizing the need for a more sensitive approach to read very short TLs, developed a long-read nanopore sequencing method for measuring TL (NanoTelSeq). The steps of NanoTelSeq are as follows: 1) ligate the 5' end of the telomere to a telorette oligonucleotide complementary to the G-rich overhang, 2) anneal teltail oligonucleotide complementary to the telorette to make a sticky end (this can be done before the ligation as well), 3) ligate the sticky end to a nanopore sequencing adaptor, which is attached to a helicase motor protein that unwinds the double stranded DNA and drives the 5' end through the nanopore during sequencing. To optimize the protocol, Dr. Tzfati's lab tested 6 different telorette primers, with the six possible permutations of the six nucleotides composing the telomere DNA repeat at their 3' end. (3'-CAATCC-5', 3'-TCCCAA-5', 3'-CCAATC-5', 3'-CCCAAT-5', 3'-ATCCCA-5', 3'-AATCCC-5'). Telorette #3 (3'-CCAATC-5') consistently had the highest percentage of telomere DNA reads across human fibroblasts (75.6%), wild-type MEFs (59.2%), and Telomouse MEFs (75.7%), followed by telorette #1 (3'-CAATCC-5'). That such a high percentage of total reads are aligned to telomeres confirmed that the telorette ligates to the native 5' end of the telomere and not to any random broken or nicked DNA. After these findings, the lab used NanoTelSeq to measure TL on the same Q-FISH

samples of human fibroblast and wild-type and telomouse MEFs. Results of NanoTelSeq analysis confirmed that the MEFs bear many very short telomeres less than 3 kb in length, supporting their initial hypothesis that decreased total amount of telomere DNA on PFGE was the result of a loss of these smaller bands with pulse field gels and that these shorter sequences were below the detection limits of Q-FISH.

Chromosome-Specific TL

One unique potential strength of the NanoTelSeq approach is the possibility to explore variability in chromosome-specific TL by aligning long read sequences to chromosome arm specific subtelomere sequences using well annotated reference genomes. In their analysis of chromosome-specific TL in the human fibroblast line, Dr. Tzfati's lab was able to successfully align 473 telomere DNA reads to characterize TL on 38 of 46 chromosomes. Notably there was wide heterogeneity in the length across chromosomes, with some specific chromosomes exhibiting the shortest TL (i.e., 8q ~ 7.2 kb) while others had TL nearly twice as long (i.e., Xq ~ 13.5 kb). To explore translation of the findings to ecological human samples, the group collaborated with the TRN to conduct NanoTelSeq on 5 genomic DNAs selected from the set of 107 PBMCs utilized in the second network collaboration experiment. Although there was variation in average TL between the five samples, they were all characterized by having many telomeres detected below 3kb and 2kb, corroborating earlier work in mouse and human cell lines. Very few telomeres were measured as having length less than 1 kb, which is consistent with experimental evidence positioning this length as the point at which telomeres become critically unstable and lose function. Average TL measured by NanoTelSeq was much shorter than average TL generated using gel electrophoresis approaches, an observation explained by the NanoTelSeq's unbiased incorporation of much shorter telomeres into the overall average, in addition to the fact that NanoTelSeq measures only the length of the telomere repeat tracts without the subtelomere regions included in the TRF approach. Surprisingly, NanoTelSeq was also able to detect some very long telomeres (> 30 kb), that were not detected by gel electrophoresis approaches.

Comparing chromosome mapping for these 5 samples allowed the group to generate preliminary data on patterns of chromosome-specific TL that may be conserved across individuals. For example, the length of the 2p telomere tends to correspond with the average TL generated by averaging NanoTelSeq reads across all chromosomes. Some telomeres were consistently shorter than average (e.g., 2q), whereas others were consistently longer than average (e.g., 3p, 12q, 13q). Moreover, the different arms of the same chromosome do not have the same TL, suggesting that chromosome specific methods of TL should endeavor to quantify both the p and q arms.

Conclusions and Discussion

In conclusion, Dr. Tzfati's lab established an approach, NanoTelSeq, which generates measurements of very long and very short telomeres in mice and human cells using long read nanopore sequencing. NanoTelSeq does not rely on PCR amplification or any sequence enrichment and, if shown to be precise, has the potential to be uniquely informative for

longitudinal TL studies, as well as studies examining dynamic change of specific chromosome arms or studies determining whether the shortest telomeres stay shortest or if they are preferentially targeted by telomerase and elongated. That said, the approach is not yet suitable for application in population-based studies and further exploration of the association with SB and qPCR-based TL estimates is needed. Currently, the method requires 1-2 µg DNA, is run on chips that can accommodate only 5-10 samples, and requires 3-4 days for full data collection. Concerns expressed by attendees included whether DNA fragment size influences observed TL. Although the team has yet to test this experimentally, Dr. Tzfati points to initial quality control and statistical tests they have conducted to rule out this possibility. Telomere reads are only included in calculations if they can be reliably confirmed to be long enough to not create a bias for short telomeres and influence the final estimate of the median TL.

Citation:

Smoom, R., May, C.K., Ortiz, V., Tigue, M., Kolev, H.M., Rowe, M., Reizel, Y., Morgan, A., Egyes, N., Lichtenal, D., Skordalakes, E., Kaestner, K.H., & Tzfati, Y. (2023) Telomouse – a mouse model with human-length telomeres generated by a single amino acid change in RTEL1. *Nature Communications*. doi: 10.1038/s41767-023-42534-6.

Absolute Telomere Length Quantification with CRISPR-Cas12a

Waylon J. Hastings, PhD, Tulane University School of Medicine

In this methodological presentation Dr. Hastings outlined a protocol for a new method to determine average TL using Clustered Regularly Interspaced Short Palindromicic Repeats (CRISPR) gene editing technologies. Specifically, the approach leveraged the CRISPR associated enzyme 12a (Cas12a), which, upon binding to target DNA, changes conformation to gain non-specific single-stranded DNAase activity. The assay workflow involved plating DNA in 96-well plates and adding a reaction mix to each well using a multi-channel pipette. The reaction mix contains the Cas12a enzyme, guide RNA (gRNA), and a single stranded DNA reporter. During an incubation period Cas12a forms a complex with the gRNA and is directed to the target DNA. Upon binding to target DNA, Cas12a will induce cleavage of the single-stranded reporter containing a 5' fluorescent tag and a 3' black hole quencher separated by a series of Adenine nucleotides. The single-stranded reporter exhibits no fluorescence in its native form. Cleavage by Cas12a releases the 5' tag from the 3' quencher, resulting in a fluorescent signal detectable by standard kinetic plate readers. Fluorescent signal is related to target DNA quantity through use of standard curves comprised of double stranded oligomers in an approach resembling that employed in the qPCR method for absolute TL. Telomere content was estimated using 84 bp oligomers containing 16 copies of the canonical telomere repeat sequence. The concentration of telomere standards was constructed to align with expected telomere DNA content in human genomic DNA samples at a concentration of 1-5 ng/µL. Genome copy number estimates were derived using 83 bp oligomers containing sequence within the single-copy gene *IFNB1*. The concentration of IFNB1 standards was constructed to correspond to expected genome copy numbers following 15-20 PCR cycles of amplification. Telomere gRNA contained a 23 bp sequence aligned to telomere repeats and the *IFNB1* gRNA contained a 23 bp sequence aligned to a region within the *IFNB1* oligomer standard. Double-stranded oligomer standards and gRNA

were designed to have similar length, GC content, and melt temperature between reactions targeting *IFNB1* and telomere repeats.

Piloting using a series of five standards following a 3-fold were distinguishable from a no-template control (NTC) and exhibited an R^2 of 0.971 following 11 minutes of incubation. However, there was only a small window in which standards were distinguishable from the NTC and had yet to plateau. To address this concern, Dr. Hastings attempted to increase the detection window by adjusting the concentration of the single-stranded probe, demonstrating that lower probe concentration delays signal takeoff and allows for better discernment between lower contracted standards and the NTC. To test specificity and whether the assay could be multiplexed, Dr. Hastings incorporated an off-target control into the design, wherein telomere standards were added to a reaction mix containing the gRNA aligned to *IFNB1* and *IFNB1* standards were added to a reaction mix containing the gRNA aligned to telomere repeats. In this design, the off-target control was indistinguishable from the no-template control. Moreover, each standard set could be quantified on the same plate in a highly precise manner, exhibiting a CV less than 5% across triplicate measurements of each standard and an R^2 greater than 0.99 for each standard curve.

After initial assay optimization, Dr. Hastings conducted initial pilot tests for quantification of analytical samples using DNA from the Jurkat E6.01 cell line, which is commonly utilized as a short telomere control. Results demonstrated the ability of the approach to quantify telomere DNA content using 2µL DNA run at concentrations of 5 ng/µL and 2 ng/µL. However, estimates of genome copy number were too low to be discernable from the no-template control. This was attributed to the much lower frequency of *IFNB1* target sequence relative to telomere sequence in each DNA sample. Specifically, each cell would be expected to contain several thousand copies of telomere repeats while the IFNB1 target sequence would only be present in a single copy per cell.

Conclusions and Discussion

Highlighted strengths of this approach included minimal DNA requirements (6-15 ng), low cost ($10-$30), and throughput comparable to existing qPCR-based approaches. Moreover, the CRISPR-based measurement offers the possibility of enhanced precision, able to discern telomere DNA content in native DNA without the need for qPCR amplification. This circumvents analytical concerns about diminished precision involved in relating changes in fluorescence happening on an exponential scale to between-samples differences occurring on a linear scale. Moreover, the approach provided an estimate in objective base-pair units. This can be determined via qPCR amplification of single-copy gene target sequence, which if conducted would take less cycles of amplification than that required to quantify genome copy number within MMqPCR, STELA, and TeSLA approaches. Alternatively, researchers can presume genome copy number without direct measurement using reported averages of 152 genome copies per ng DNA, although resulting measurements would need to be cross validated against methods providing similar objective estimates like Southern Blot.

Limitations to be addressed included further optimization of probe concentration and detector sensitivity to maximize the window of detection, validating the specificity of telomere gRNA-binding as it relates to telomere-like repeat elements characterizing subtelomere and interstitial regions, and further testing using analytical samples extracted using various procedures. In open discussion Dr. Alder additionally suggested using multi-copy sequences such as Alu elements which have several thousand copies per cell, as alternatives to discern genome copy number without the need for PCR amplification. This suggestion was met with intrigue and slight pushback, as the degree to which the number of these elements are maintained between individuals is currently unknown.

Precise Measurement of Telomere Length in T-cell Derived Extracellular Vesicles

Bo Ning, PhD, Tulane University School of Medicine

Dr. Ning's work in the Center for Cellular and Molecular Diagnostics specializes in using CRISPR-based technologies for small molecular detection across several domains including detection of pathogenic genomes in infectious disease (e.g., RSV SARS-CoV-2), drug resistant mutations in cancer (e.g., pancreatic and lung cancers), and protein and peptide detection in neurological disorders. The application of CRISPR-based technologies to infectious disease is particularly notable, exhibiting increased sensitivity to detect and diagnose COVID-19 relative to traditional PCR-based tests, in some instances resulting in confirmatory diagnosis necessary to assign treatment and save patient lives. Further, CRISPR-based detection of viral DNA can be utilized in plasma samples to track changes in viral load within patients up to 100 days after initial infection, which has utility in identifying individuals at risk for symptoms of long-COVID. Dr. Ning's work with CRIPSR technologies extends beyond assay development, venturing into portable device creation for a quantitative blood test for tuberculosis.

Liposome Packaging of CRISPR-detection Systems

More recently, his lab worked on liposome packaging of the CRISPR detection system to improve sensitivity of nucleic acid detection in extracellular vesicles (EVs). The rationale for this approach is to increase sensitivity by allowing increased interaction between reaction mix components are target nucleic acid by decreasing the volume scale in which the reaction takes place. In this design, EVs are captured using well plates coated with antibodies with homology to EVs excreted from CD81 cells (or other cells of interest). Once captured, liposomes containing reaction mix components, including CRISPR enzymes, primers, signal probes, and components necessary for room-temperature rolling polymerase amplification are added to each well. Instead of circulating in the micro-scale well volume, these liposomes fuse with the captures EVs due to their functionally similar bi-lipid membranes. Should the target nucleic acid be present in the EVs, the reaction will then be mediated within the liposome-EV fused particle, which exhibits a nano-scale total volume. In this manner, the CRISPR-gRNA complex has significantly greater chances of binding and producing signal indicating the presence of target DNA than if the reaction were to take place in an open microwell system. Dr. Ning has applied this technology to detect micro RNAs (miRNAs) indicative of pancreatic cancer, and is now interested in utilizing

this same technology to detect telomere DNA exported in EVs, which has been implicated as a mechanism to protect T-cells from senescence and promote long-term immunological memory (Lanna et al., 2022, *Nature Cell Biology*).

Conclusions and Discussion

The lab has is currently working to implement liposome packaging for detection of telomere DNA in EVs. The challenge in quantifying telomere DNA relative to miRNAs is telomeres are much longer, which makes it difficult to package into vesicles at a high quantity. To address this challenge, the team wants to increase the size of the packaging liposome to allow for binding of multiple EVs to a single liposome to enrich telomere signal. Further, instead of allowing for undirected fusion of liposomes with EVs, the team plans to capture the reaction mix containing liposome first, and then use antibody directed fusion to capture specific EVs of interest known to contain telomere DNA, essentially reversing the approach used to detect cancer miRNAs in which EVs were captured and liposomes were allowed to fuse indiscriminately knowing only the target EVs were present in the sample. Beyond detection of telomere DNA in EVs, this technology would have application in tracking senescence of CD28 cells, which are characterized by specific antibodies that could be recognized and fused with liposomes, allowing for fine-tuned determination of how telomere shortening contributes to senescence associated cellular phenotypes within specific cell populations.

Audience deliberation included questions of the exact length of telomere DNA stretches present within EVs that are many times smaller than whole cells. Dr. Ning posited that these stretches must be quite small in length, which makes detection a challenge since they would be present in much lower quantity per EV than miRNAs. Even so, the repetitive nature of the target DNA would allow greater numbers of enzyme complexes to bind to each target molecule, in contrast to work with miRNAs where each complex bind each target at a 1:1 ratio. Thus, the larger liposomes could contain greater concentration of the Cas12a-gRNA complex to increase signal detection. Further discussion questioned what parameters of T-cell functioning would be indicated by the presence of telomere DNA in exported EVs since they have many functions including secretion of cytokines and mediating cytotoxicity. To address this, Dr. Ning suggested distinguishing between activated and inactivated T-cells to determine at what stage telomere DNA is exported from T-cells and relating this back to their activity at that stage to unravel possible function.

Open Forum: Guidelines of Validation of Novel Telomere Measurement Assays

Chair: Stacy Drury, MD, PhD, Tulane University School of Medicine

In this open forum TRN meeting attendees reflected on proposed methodological and analytical techniques presented in the previous session with the goal of identifying important considerations and validation metrics necessary in the establishment of novel telomere measurement approaches.

There continues to be a need, given the wide use of qPCR-based TL measurement, to improve repeatability and precision with this and other methods. There is also a clear need to develop novel methods able to be (1) high throughput (2) repeatable (3) capture the full dynamic range of TL (short to long) and (4) potentially to be able to measure chromosome specific TL. To date there is still an absence of a true gold standard and future efforts to ensure repeatability of nanopore sequencing is needed to determine the feasibility of nanopore sequencing to be a true gold standard. CRISPR-based technologies also hold promise as an approach able to provide estimates of the true length of telomeres and has the potential to distinguish the length of telomeres of specific chromosomes. However, optimization of both methods is expected to take additional time and careful attention to reproducibility and rigor is required. In the interim, work like that presented by Dr. Xu can be immediately implemented to improve existing approaches through enhanced analytical and methodological rigor.

Also discussed was the lingering question of how to utilize patients' TL measurements in the context of point of care testing. If it were possible to produce a portable TL measurement device resembling the tuberculosis test designed by Dr. Ning, how would clinicians and patients utilize that information to improve healthcare? Attendees agreed that such a TL measurement would not have much utility in the context of healthcare at the current time, because so little is known about the exact value of a single TL measurement for most health conditions, however clinical utility of individual TL measurement may be relevant for bone marrow transplant and individuals with telomere biology disorders. A portable, point of care, direct TL measurement, would be of greatest utility in remote settings and for decreasing participant burden. Sharing the NIEHS perspective, Dr. Michelle Heacock spoke about the desire to utilize telomere parameters as a readout indicative of chemical exposures that are themselves difficult to measure, using as an example 8-Oxo-Guianine, which has a short half-life but leaves lingering measurable oxidative damage to telomeres. Following up on this point, Dr. Lisbeth Neilson spoke on the continued interest in combined exposures and how new methods could be used to determine their cumulative impact and increased susceptibility for future exposures, for example if and how psychosocial lifetime stress makes one increasingly susceptible to damaging effects of pollutants, whether certain telomere parameters be it average TL, chromosome-specific TL, or telomere damage are more predictive, and whether specific points across the lifespan when theses exposures occur are particularly important in determining effect sizes on TL.

Interactive Debate: Fundamental Questions on the Role of Telomeres as Sentinels in Human Population Studies

Chair: Simon Verhulst, PhD, University of Gronigan
Panelists: Duncan Baird, PhD, Cardiff University (remote), Stacy Drury, Veryan Codd, PhD, University of Leicester (remote), Jon Alder, PhD, University of Pittsburgh

The interactive debate led by Dr. Simon Verhulst had assigned roles for the panelists to present support for or against three statements related to TL and measurement. Each statement was shared with the zoom and in-person audience at the TRN prior to discussion to ascertain preliminary perspectives as to whether the audience agreed or disagreed with each statement.

After both sides expressed their views, a second vote would be taken to see if audience members were persuaded one way or the other.

Statement 1: Single measurements of TL are meaningless because it is the rate of telomere shortening that is relevant to human health and disease.

Prior to the initiation of the debate, the audience was polled and initial results indicated that 81% of attendees disagreed with the statement. Dr. Veryan Codd opened the debate in support of this statement, citing straightforward evidence that TL changes dynamically with age, as demonstrated by large meta-analyses and reproducibility studies. Moreover, the rate of TL change is subject to between-person differences, with notable influences of lifetime stress and environmental exposures on the rate of change. Toward this end, data from twin studies suggests only 1/3 of TL attrition rate can be attributed to genetic factors, with the other 2/3 subject to environmental factors. Although shorter average TL is associated with increased risk for disease, it is not powerful a predictor as when used in tandem with the rate of shortening. In summary, it is limiting to assume that a single timepoint in which TL is measured is indicative of an individual's exposure to the environment and how their TL has maintained throughout the lifespan. It is important to know where an individual's TL starts at birth or prior to intervention/exposure, but this become increasingly valuable when combined with information about their current TL later in life of after intervention/exposure.

To counter this position, Dr. Stacy Drury began by citing studies demonstrating that the variation of TL at birth between individuals is far greater than the differences in the rate of attrition over the course of the lifespan, referencing in particular the Landsorp 2022 study utilizing precise measurements of TL generated using Flow-FISH. This one measurement at birth, compared to many across the timespan, is arguably more indicative and insightful to TL differences and consequent associations observed across the lifespan. As reinforced by earlier data presented by Dr. Dries Martens, TL at birth may vary as much as 3.5-4.0 kb between individuals, and this rank-order in TL at birth is maintained throughout adulthood. In the context of annual TL attrition rate, estimated to be between 30-100 bp, it would take nearly a 100-fold difference in TL decline to would need to occur to close gaps observed at birth in a meaningful time span. What we start with, Dr. Drury concluded, is essential, and as such, this reinforces the importance and value of using data from cross-sectional studies to answer important research questions about the relationship between TL and human health and disease.

Panelists then presented rebuttals. Dr. Codd began by clarifying that not all environmental exposures are going to have the same impact on TL attrition. Thus, it cannot be assumed that attrition rates will be maintained across the life course, arguing against Dr. Drury's point that the attrition rate in not profound enough to exert meaningful impacts on health and disease. Dr. Drury used this opportunity to suggest that estimates of TL attrition are limited by measurement capacity, which in many cases has insufficient precision to detect meaningful differences in TL change over short time periods, and by extension has limited precision to reliably quantify meaningful differences in TL attrition rate, which would require even greater resolution. To do so, researchers would need to observe individuals over a sufficiently long time frames, especially

when considering individuals at older ages when stem cell replication is notably slower relative to earlier in life. As a result, cross-sectional studies are going to be much more representative of intergenerational impacts on TL prenatally and will also highlight the relationship between health and disease in relation the risk for individuals with short telomeres.

Dr. Verhulst then opened the debate to in-person and online attendees for comment. Dr. Dan Eisenberg elaborated on how measurement error can underestimate the heritability of TL attrition. Specifically, measurement error increases the amount of between-person differences in observed TL. Although this error is equally applied to all samples, there will always be more individuals unrelated to one another than are closely related in a heritability study. Due to this disproportion, the contribution of measurement error to population variation in TL is unequally attributed to being a result of non-shared environment than to genetic relatedness. Thus, the heritability of TL attrition rates is likely higher than the 1/3 estimated and cited by Dr. Codd. Because of this limitation, TL attrition rate is less meaningful in relation to health and disease because it is a static quantity of the individual, as a result Dr. Eisenberg opposed the preliminary statement, and stated that single measurements of TL are meaningful, and in fact more meaningful than TL attrition. Dr. Simon Toupance added to the discussion in support of a single measurement and highlighted that this discussion and many discussions during the whole TRN meeting continually over emphasize mean TL, when biologically the shortest telomeres are the most relevant. Although mean TL is influenced by shortest TL, is remains a poor proxy given the diversity of TL between chromosomes. Dr. Zheng redirected the conversation, suggesting that a single measurement of TL in an individual, such as 7 kb, is meaningless without any comparison references to interpret this measurement. In other words, a measurement is only valuable in so far as how it compares to the population distribution. This is true because we still lack a ‘normative’ TL estimate for the healthy population, which renders single measurements of TL generally useless. After these statements were concludes, a second vote was cast to assess audience views with the statement. The results demonstrated that participants were somewhat swayed, but still generally disagreed with the statement (74% post vs. 81% pre), suggesting strong support for the relevance of single TL measurements to health and disease.

Statement 2: No environmental or psychosocial stress exposure has sufficient effect on TL to result in cellular functional or health consequence.

Initial poll results were heavily one sided, with 87% of participants disagreeing with the statement. Dr. Jonathan Alder began the second debate in defense of this statement, citing evidence from genetic and familial studies that inheritance of TL shortening occurs over generations, not within lifetimes. It is also important to clarify between ‘shorter’ telomeres and ‘short’ telomeres. For example, many studies have demonstrated significant telomere shortening versus an exposure group or control group in the case of intervention studies. However, when these lengths are plotted on a population nomogram, the TL for all individuals was well within the normal range, irrespective of control status, exposure status, or intervention status. Dr. Alder also added that short telomere mediated disease is caused only when TL reaches a functional threshold around 4 kb, before which telomere-mediated functional consequences will not be

observed. Thus, accurately stating that environmental and psychosocial stress has sufficient effect on TL requires demonstration that the functional threshold has been met.

The opposing arguments were provided by Dr. Duncan Baird, who began by referencing the various cross-sectional studies in TL in the human population ranging in cardiovascular disease and cancer to exposure and environmental and psychosocial stress. Many of these studies utilized small cohorts (<100 participants) with error-prone study designs which could make it difficult to draw conclusions on the effect of environmental and psychosocial stress on TL. Moreover, a meta-analysis by Dan Nettle has demonstrated a publication bias where small underpowered studies show more significant associations than larger studies. This would lead one to believe environmental or stress exposure in fact do not have sufficient impacts on TL to produce increase risk for disease. However, studies with larger cohorts (such as the UK BioBank with 470,000 participants analyzed for TL) now demonstrate significant effects of psychosocial stressors on TL as well as associations between TL and disease. Large meta-analyses also support effects of psychosocial stress on TL, such as one with 21,000 participants providing evidence for significant associations between shorter TL and depression. In addition to such large-scale cross-sectional analyses, longitudinal studies with over 2000 participants provide convincing evidence of the effect of violence and abuse in childhood on TL and shortening. As more studies with large cohorts and more robust study designs are developed, the evidence in favor of effects of psychosocial and environmental stress on TL will continue to accumulate. Even so, the evidence does suggest that effects of psychosocial stress are more pronounced in childhood than in adults. Although the data for environmental exposures is slightly less convincing, there are still significant associations observed between shorter TL and exposure to small environmental particulates. Lastly, while observational studies cannot provide evidence of causation, Mendelian randomization studies examining impacts of genetic variation can provide significant evidence for associations between TL and health outcomes. These studies highlighted how some polymorphisms associated with differences in TL are similarly associated with increased risk of disease, such as coronary heart disease. This suggests that TL is important for human disease, and if we see TL differences that are robustly associated with environmental or psychosocial stress exposure, then there is the potential for these same exposures to have functional health consequences.

Dr. Alder started his rebuttal by stating that only specific genotypes are associated with telomere dysfunction phenotypes that manifest at birth, and experiences through life have yet to produce similar telomere dysfunction phenotypes. Dr. Baird did not entirely disagree with Dr. Alder's point but drew on instances of patients that have short telomeres and have these mutations in those disease genes, but for whom dysfunctional phenotype only manifests to disease later in life. So, while the TLs are in the normal range it is not until late in life that these pathologies develop. Dr. Ezra Susser then suggested that the statement at hand is too broad, and that the definition of life does not always include the prenatal period which could involve large changes in TL. Dr. Baird reinforced this point, citing various studies that demonstrated the association between prenatal adversity and TL, suggesting prenatal impacts of exposures on TL are even more

profound than that observed in childhood which shows environmental exposures very early in life influencing TL.

Dr. Verhulst then redirected the debate by positing whether implicit in this statement is the assumption that there is a causal relationship between environmental and stress exposures, telomere dynamics, and cellular function/health. Dr. Shawn Ahmed opposed this suggestion and goes on to say that human beings are complex and do not experience single exposures at any point during the lifespan. In studies where associations are demonstrated, there may be a significant effect, but it is hard to disentangle other confounding environmental and genetic influences. Dr. Sarah Wolf also opposed the statement. Addressing the causality link, Dr. Wolf encouraged the group to not automatically assume that functional consequences only occur at certain thresholds of TL that are associated with senescence, and noted some mechanisms where short TL early in life can lead to immediate changes in physiology. Early life exposure and consequential changes in telomere dynamics can translate into these functional changes throughout the lifetime. Dr. Quinn Conklin also joined those opposing the statement on the basis that some of the studies, even those in large cohorts, do not have sufficiently robust measurements of psychosocial stress to make a conclusion. Dr. Bo Ning took a methodological stance, stating that relationships between predictors and outcomes may be both observed and not observed because the technology is not developed enough for precise TL measurement, thus it is hard to make a case that measurement technology is influencing the evidence one way or another. At the conclusion of these sentiments, a second poll was taken, with results showing that even more participants disagreed with the original statement, suggesting participants strongly believed not only that stress and environmental exposures influence TL, but also that these impacts are sufficiently strong to produce functional and health consequences.

Statement 3: The association between TL and mortality is driven by infectious diseases only.

Results of the initial poll were heavily one sided, with 96% of attendees disagreeing that associations between TL and mortality were driven by instances of infectious disease. Dr. Drury started the debate in favor of the statement citing the high prevalence of infectious disease worldwide, including the 296 million people infected with Hepatitis B, as well as other highly infectious diseases such as herpes and CMV that are highly prevalent globally. A vast majority of telomere studies use human leukocytes, which are heavily involved in the immune response, as the primary measurement of TL. Such immune responses cause further cell division every time an infection occurs, especially in recurring infectious diseases such as herpes, and this constant cell division leads to shorter telomeres. Infectious diseases are also the leading cause of death around the world following the COVID-19 pandemic. These infectious diseases cause recurrent infection and continued inflammation in humans, ultimately shortening telomeres. Moreover, early evidence suggested strong associations between infections and cardiovascular disease, the age-related disease most consistently associated with TL. Thus, it might be more informative to study infection as the driver of associations between disease and TL.

Dr. Codd took the opposing stance, citing Mendelian randomization studies that have suggested that the genetic basis of TL can also predispose individuals to specific diseases and pathologies. Although observational data can be confounded by viral infections, such confounds would not impact the results of Mendelian randomization data that only draws upon individuals' genetically mediated TL. In fact, the genetics of longevity suggest that genetic predisposition to a long life is mediated by decreased risk for common diseases such as coronary disease, not infectious disease, and these common diseases are in fact the diseases that TL is associated with.

Dr. Drury then began her rebuttal by incorporating studies conducted in wild birds by Dr. Muhammad Asghar that showed TL shortening in response to chronic malaria was observed across multiple tissues, suggesting infectious disease can mediate TL shortening beyond just leukocytes. She also noted that meta-analyses have indicated that viral infections also increase the risk of pulmonary fibrosis, diabetes, and hepatitis C. This suggests that these chronic viral infections or early viral infections are leading to predispositions to diseases independently from genetic predispositions. Although disease predisposition may still be mediated by shorter TL, it is the infection driving the shortening, and in the absence of infection such shortening may not occur or may not be impactful. Simply having these viral infections present in the body, Dr. Drury added, and having T cells that have been exposed to them before, will lead to antigen production which leads to T cell stimulation and ultimately telomere loss.

Dr. Codd defended her position by stating that there is no debate that viral infections shorten telomeres. Rather, the important aspect is that they are not the sole route of shortening telomeres. Short TL as predicted by genetics and genetic variance are linked to telomere biology and not the immune response, which can and has been similarly investigated in Mendelian randomization. If these TL associated variants are not linked to the immune response but are associated with risk for disease, then the association between TL and disease cannot be solely through infection.

Dr. Verhulst then opened the discussion to the audience and asked the crowd to challenge the extent to which Mendelian randomization studies that highlight associations between alleles and disease are demonstrating effects on telomeres, or instead reflect a polygenic effect of those alleles. Dr. Eisenberg, defending Dr. Drury's argument, stated that Mendelian randomization demonstrated that individuals with shorter telomeres are more predisposed to infectious diseases that then further shorten telomeres, which in turn which makes them more susceptible to non-communicable diseases. Dr. Sharon Savage brought up a question referencing the strengths and weaknesses of Mendelian randomization studies. Specifically, Dr. Savage questioned to what extent the limitations of qPCR measurements used to generate TL measurements from which polygenic GWAS scores of TL are inferred influences the reliability of the estimated genetic effects. In other words, are the estimated effects of alleles on TL unreliable due to being derived from imprecise TL measurements. Dr. Savage stated that she is neutral regarding the statement of TL, infectious disease, and mortality. In her opinion it is circular, as infectious diseases induce inflammation and telomeres and tissues are both sensitive to inflammation, so there is more to this statement than simply looking at telomere biology.

Assuaging Dr. Savage's concern about the implications of qPCR on GWAS studies of TL, Dr. Codd stated that the lack of qPCR precision or accuracy is overshadowed by the size of GWAS cohorts like the UK BioBank and others, while in smaller studies it would be more of an issue. Dr. Zheng stated that she is against the statement and adds that individuals who have inherited telomere dysfunction or shortening have compromised immune systems and that poses a potential cause of susceptibility to infectious diseases and other diseases. Short telomeres may be responsible for disease susceptibility whether infectious or not, which related back to Dr. Savage's stance on the statement's circular nature.

Dr. Drury and Dr. Verhulst ended the discussion stating that it is difficult to parse out the effects of infectious diseases and non-communicable diseases, as it is rarely a single factor leading to the death of an individual. Moreover, Dr. Drury made an important connection between latent infectious disease, which can resurface when immune function is compromised by psychosocial stress and/or environmental exposures. It truly represents an ongoing interaction between internal and external factors. Although some individuals were swayed by Dr. Drury's case, a large proportion (~85%) of the audience still disagreed with the initial statement, suggesting most researchers believed the relationship between TL and mortality was not solely driven by infectious disease.

Session 4: Novel Models and the Importance of Cross Species Collaboration

Chair: Stacy Drury, MD, PhD, Tulane University School of Medicine

The TRN is uniquely situated to foster transdisciplinary collaboration by bringing together scientists working in pre-clinical and animal models with researchers working with human clinical samples and population-based studies. This session highlighted emerging work from TRN partners working with model organisms as an opportunity to enhance the bidirectional translation between model organisms and human studies to better inform research related to telomere dynamics in both directions.

Lymphocyte Telomere Length in a Bovine Model of Parturition and Prenatal Stress

Tom Welsh, PhD, Texas A&M University

Dr. Tom Welsh, Regents Professor in the Departments of Animal Science and Veterinary Integrative Biosciences, conducts research on telomere dynamics using Brahman cattle as a model organism. Brahman calves are a valuable model organism due to their world-wide prevalence, availability of phenotypic data, and their tolerance of environmental stressors, particularly their adaptability to climate change, which will become increasingly important in the years to come. Dr. Welsh's team follows a One Health model to understand how maternal and prenatal stress impact the health and performance of cattle, which is of relevance due to the value of animal-derived protein in the human diet.

The team's long-term objective is to identify the effects of prenatal stress (PNS) on health and productivity of calves. In this study, Brahman cows were artificially inseminated with semen from the same Brahman bull and then separated into control or PNS groups upon pregnancy diagnosis. PNS was operationalized as the stress associated with transportation in confined trailers for 2 hours each at 60, 80, 100, 120, and 140 days into gestation. The cows were matched by temperament, age, and parity between groups (16 PNS cows/16 control cows with 8 heifer and 8 bull offspring in each group). For the entirety of the study the groups were kept in the same pasture and were only separated during the trailer rides. Data collection included pen score to measure temperament, body weight, and blood collection and tissue biopsies at 25 days after birth. DNA was extracted from white blood cells using a spin column kit with DNA quality assessment at Texas A&M University's Institute for Genomic Sciences and Society Genomics Core. TL was measured by multiplex qPCR using the bovine telomere sequence and the β-2 globulin gene. Serum cortisol, ACTH, and IgG were also assessed.

Preliminary results showed that animals that were exposed to prenatal stress had greater concentrations of circulating cortisol and shorter TL. Moreover, known sexual dimorphism in TL, with females exhibiting greater TL than males, was also reproduced in the Brahman model. There were also inverse relationships between cortisol and TL, with animals exhibiting greater basal cortisol being characterized with shorter telomeres. Animals with increased temperament, e.g., more behavioral conflicts in the pen, were noted as having shorter TL and decreased IgG.

Conclusions and Discussion

Maternal stress was associated with TL in Brahman offspring, with measures of stress hormones exhibiting an inverse relationship with TL, replicating well established results observed in human models of early life and prenatal stress. Dr. Welsh discussed the potential application of these findings among others to use TL as a population health marker and as an indicator of well-being in wild populations. Future directions for this study will involve looking at different tissues collected from the offspring to examine tissue-specific differences between the PNS and control groups that may relate to differences in behavior and reproductive fitness. Preliminary results from an in vitro fertilization experiment indicated that sperm from sires that were prenatally stressed had about 10-15% fewer embryos to develop to the blastocyst stage. Additional discussion about parturition and time point of TL measurement (weeks before parturition, close to parturition, and after parturition) revealed that multi-parity and raising a calf coincided with shorter TL in the dam. *Supported by the USDA-NIFA grant 2019-67015-2957 and the Multi-State Hatch Project W4173.

Epigenetic Inheritance of a Telomere Capping Defect Triggers Longevity in *C. Elegans*

Shawn Ahmed, PhD, University of North Carolina

Dr. Shawn Ahmed, professor of Biology and Genetics, aimed to investigate the transgenerational impact of stress on *C. elegans* telomeres, which may operate through a combination of classical Mendelian inheritance and epigenetic inheritance. Transgenerational epigenetic inheritance conceptually involves exposure to an environmental stressor in a parent that reprograms the

germ cells and is subsequently transmitted to offspring across multiple generations. This has been seen in humans in famine situations where transgenerational phenotypes expressed have included diabetes, obesity, cardiovascular disease, and body size. Dr. Ahmed and his team utilized *C. elegans* to study single-stranded telomere binding proteins, specifically Pot1, which stabilizes single-stranded telomere overhangs. Pot1 is a single protein in humans with three homologues in C. elegans, Pot-1, Pot-2, and Mrt-1. The binding proteins in *C. elegans* have action in mediating telomerase activity (Mrt-1), repressing telomerase (Pot-1 and Pot-2), and repressing alternative lengthening of telomeres (Pot-2).

Using a Pot-1 protein tagged with mCherry, the team was able to identify tagged countable telomere foci in fertilized nuclei. However, these foci disappeared at the 1 cell stage of development, precluding telomere tracking into further stages of zygote development. To address this, *POT-2* was mutated and in the resultant fertilized embryo the telomere foci did not disappear, allowing quantification of telomere foci. To examine multigenerational inheritance, the POT-2 mutant male was crossed with a wild-type animal that contained POT-1 mCherry. The resulting progeny demonstrated a phenotype consistent with that of the parent POT-2 male for up to six generations, demonstrating multigenerational epigenetic inheritance through sperm. The team next reversed the design, tagging Pot-2 and mutating Pot-1 with epigenetic modifications. In contrast to results with Pot-1, the F1 progeny showed complete loss of Pot-2 mutations. There might be several mechanisms mediating this inheritance, such as histone silencing, smalls RNAs, or cytosine methylation, the latter of which occurs in other systems but not in *C. Elegans.* After testing different mechanisms, histone silencing marks emerged as a potential mechanism of transgenerational inheritance. This was further supported when genome silencing defective mutants all had low levels of foci, indicating impaired transgenerational inheritance. Using the reverse approach, clones with high levels of silencing were characterized by high levels of foci, indicating enhanced inheritance. When looking at longevity specifically, Pot-1 mutants were living longer compared to wild-type Pot-1, providing evidence of transgenerational inheritance of longevity related traits.

Conclusion and Discussion

Dr. Ahmed and his team concluded that the Pot-2 mutations create susceptibility to higher levels of silencing at telomeres and in doing so create an epigenetic state that is heritable for a number of generations, even in the presence of wild type protein. The same is true for Pot-1, except it produces a de-silenced state that is inherited. More specifically, Pot-1 mutants and their cross-progeny live longer, and this is possibly a result of a stress response transcription factor, which has been implicated as a longevity transcription factor by other researchers. Overall, it was concluded that Pot-1 and this stress response transcription factor co-localize at telomeres and work together to impact activity of longevity related genes, suggesting their effects may have other consequences other than simply affecting the length of telomeres. The main difference between human Pot-1 mutations and C. elegans Pot-1 mutations was discussed, with Dr. Ahmed noting that in worms Pot-1 is split into three different proteins with opposite phenotypes for Pot-1 and Pot-2 whereas in humans presumably all mutations are wrapped up in Pot1 which means it might not be as simple to compare the two. The mechanism could potentially still be analogous,

perhaps with some mutations in human Pot1 promoting decreased silencing and others promoting increased silencing.

Keynote – The Energetic Cost of Telomere Maintenance and Mitochondria

Martin Picard, PhD, Columbia University (remote)

It has been well established that exposure to psychosocial stressors accelerates aging phenotypes and increases risk for age-related disease. Dr. Picard's research situates mitochondria as the central mediator linking these two processes. Mitochondria play a complex role within a cell, serving as both the cell's energy transformation network as well as its central information processor. In addition to being proximally located within a few hundred nanometers of the nucleus, mitochondria exhibit the capacity to receive, sense, and integrate information, possessing numerous sensors on their surface, including glucocorticoid receptors. Mitochondria adapt to these various signals, physically interacting with one another and changing size through fusion and fission. Numerous mitochondrial proteins are encoded inside the nucleus, while the nucleus in turn is rendered inert without the energy and information provided by mitochondria.

Cellular Lifespan Model

To understand this mito-nuclear partnership in the context of stress and aging, Dr. Picard'slab developed a cellular lifespan model to profile *in vitro* longitudinal changes in mitochondrial and nuclear integrity within the larger gene-environment paradigm. In this model, human (dermal) fibroblasts are collected from regions like the forearm and cultured in media. Every five to seven days a sample of this culture is transferred into a new dish, resulting in up to 42 time points from a single biopsy over the course of the 9-month experiment. The remaining cells from the previous culture dish are then assayed for some feature of interest such as cellular anthropometrics, mitochondrial bioenergetics, DNA, RNA, or secreted factors. Cellular anthropometrics include cell counts, which allows for estimations of cellular division rate, as well as cell size, which has important consequences for the efficiency of cellular metabolism. DNA samples are used to measures parameters of DNA methylation, TL, and mitochondrial DNA copy number (i.e., number of copies of mitochondrial genome per cell). The team can also integrate sequencing to identify mitochondrial damage and stability by quantifying mitochondria DNA deletions and mutations. RNA samples are particularly insightful for assessing the genetic program of cells across time and in concert with changes to cellular metabolism. Secreted factors are assessed by sampling culture media to extrapolate how cells would influence surrounding tissue, with emphasis on molecules associated with senescence and telomere instability like interleukin 6, and GDF15.

Contrary to traditional *in vitro* approaches working with one or two cell lines, Dr. Picard's lab generated deep longitudinal profiles for lines derived from six individuals, three from healthy controls, and three from donors who had known genetic defects in the mitochondrial gene *SURF1*, which encodes an assembly factor for complex IV of the electron transport chain. This mutation results in early mortality due to inability of cells to effectively produce ATP, and these cell lines were derived from donors who all died before age 10. In addition to comparing

longitudinal profiles between healthy control and individuals with mitochondrial disease arising from genetic background, the team also pharmacologically induced mitochondrial dysfunction in cell lines derived from the same healthy controls using oligomycin, a compound known to disrupt function of Complex V of the electron transport chain. This resulted in a total of 9 different cell lines, three derived from healthy donors, three derived from patients with mitochondrial disease, and three derived from the same healthy donors where mitochondrial dysfunction was pharmacologically induced. Longitudinal cellular lifespan data are available for more cell lines and additional experimental treatments (described in Sturm et al., *Sci Data* 2022).

Mitochondrial Defects Decrease Cellular Lifespan and Induce Cellular Hypermetabolism
Initial results showed stark differences between cell lines derived from healthy individuals and patients with mitochondrial disease. Cells from patients had a 53% lower Hayflick limit, the total number of times a cell can divide before activating cell death or senescence pathways. Moreover, healthy cell lines in which mitochondrial dysfunction was induced pharmacologically had cellular lifespan 40% lower than their non-treated counterparts, resembling those of patients with mitochondrial disease, suggesting mitochondrial function was a driving force behind reduced cellular lifespan. In looking at TL measured by the T/S ratio, cells with mitochondrial dysfunction had an increased telomere attrition rate, such that average TL decreased at greater increments for each cell division. Similar findings were observed for epigenetic clocks, which accelerated 131% and 54% faster in cells with mitochondrial dysfunction from disease or pharmacological intervention, respectively.

Pharmacologically manipulated and diseased cell lines also divided at a slower rate (~40%), which could be an adaptation to conserve energy in the context of decreased ATP synthesis, as cell division represents the largest demand for which cells must provide energy. To assess energetic consumption, the team quantified oxygen consumption, a measure of electron transport chain activity, and extracellular acidification, a proxy for glycolytic activity. Together these represent an estimate of the total energetic supply via the two main pathways (electron transport and glycolysis) by which cells can produce ATP. The energetic production in cell lines from diseased patients was widely variable across the cellular lifespan, while energetic production from healthy donor cells was tightly coupled both within a cell line and across the three donors. Contrary to expectation, cells with mitochondrial dysfunction from disease or pharmacological manipulation produced more ATP across their cellular lifespan relative to cells from healthy donors (~91% & 108% more respectively). Together, this implies that mitochondrial disease widely decreases the *efficiency* at which cells can produce and utilize their energy. Dr. Picard termed this gross overproduction of energy a state of cellular hypermetabolism.

An important question arising from this observation is where the cells are utilizing this excess energy, if not toward cell replication. To answer this, the team profiled extracellular factors secreted by cells into the surrounding media, revealing that cells with mitochondrial dysfunction produced much more inflammatory cytokines, especially toward the end of cellular lifespan, consistent with the senescence associated secretory phenotype (SASP). In further looking at gene expression, mitochondrial defects were shown to cause a time-dependent activation of the

integrated stress response (ISR), a pro-survival homeostatic program, which during extreme stress can drive signaling toward cell death. In mapping the time-dependent induction of ISR, Picard showed that this activation was induced through the integration of two specific stress response genes, *CHOP* and *DDiT3*. These genes serve as important regulators for the ISR, as well as for GDF15, a senescence associated protein whose levels increased 8- to 32-fold with mitochondrial perturbation. Overall, dysregulated cell lines exhibit a global pattern of gene expression distinct from that observed in healthy controls. Using functional enrichment, Picard showed that dysregulated cells are downregulating genes related to cell division, consistent with their decreased cell division rate. However, these cells are not transitioning to an energy conservation state. Instead they are upregulating genes related to transcription and translation. Moreover, DNA methylation patterns show decreased repression of loci involved in energetically expensive processes of cell-cell communication, organ differentiation, and developmental processes that are all energetically expensive.

Using In-Vitro Models to Study the Impact of Chronic Stress on Cellular Allostatic Load

In the data presented so far Dr. Picard focused on how baseline perturbation to mitochondria function impact cellular lifespan and bioenergetics. Next, he shared work leveraging this cellular lifespan model to investigate the impacts of chronic stress. This work adopts an Energetic Model of Allostatic Load, which posited that exposure to stressors induces hormonal and signaling cascades that change the physiology of organs, cells, and parts of cells in a manner that increases the energy required of the whole-body system. As the central processor of each cell, these changes will thus impact mitochondrial function and increase *cellular allostatic load*, quantified as the cells' energy expenditure. When stressors are chronic, dysregulation occurs, leading to allostatic overload and break down of the mito-nuclear system.

To investigate this phenomenon, the lab employed the same cellular lifespan model, but instead of directly impairing mitochondria with oligomycin, they modeled exposure to chronic stress by maintaining constant concentration of glucocorticoids (dexamethasone) in the culture media. On average, stress exposed cells increased energetic production by approximately 62%, resembling the state of hypermetablism in cells with dysfunctional mitochondria. These cells also had 20% shorter cellular lifespan (cell divisions), an 8% increase in TL shortening rate, and a 36% increase in epigenetic clock aging. However, because there was no direct manipulation of mitochondria, it remained uncertain whether accelerated cellular aging is due to increases in global energy production (hypermetabolism) or specifically due to changes in the energy production mediated by mitochondria.

To address this question, they used they used mitochondrial nutrient uptake inhibitors (mitoNUITs) to block sugars, amino acids, and fatty acids from entering mitochondria, effectively cutting off electron transport chain activity as a source of ATP production. In this design, cells redistributed energy production by increasing ATP production from glycolysis. Moreover, this increases the total energy production by 80% relative to non-treated cells with availability to both energy producing pathways (electron transport chain & glycolysis). Thus, by reducing energy production from electron transport chain, total energy expenditure increased, reflecting a state

of cellular hypermetabolism independent from mitochondria. Using this system, they could now test whether increases in cellular aging are due to global hypermetabolism, in which case cells with impaired mitochondrial uptake would also age faster, or if cellular aging was attributable to changes in energy production specifically mediated by mitochondria, in which case cells with impaired mitochondrial uptake would have a normative aging rate.

Results showed that mitoNUIT treated cells had a further 16% reduction in lifespan and a 50% increase in TL shortening rate, suggesting that cellular hypermetabolism more broadly, and not metabolism attributable directly to mitochondria, was more directly related to decreases to cellular lifespan.

Summary and Conclusions

Studies across different species have found strong relationships between the mass-specific metabolic rate and average lifespan. Whales have a long lifespan and a very low mass-specific metabolic rate, the average amount of energy per cell needed to maintain life for the organism. By contrast, mice have a very high mass-specific metabolic rate and a much shorter lifespan relative to a whale. In this work, Dr. Picard increased the mass-specific metabolic rate of individual cells by either by directly disrupting mitochondrial biology (OxPhos capacity) with disease/pharmacology or exposing them to chronic stress. This perturbation made the human cells behave more like mouse cells, and decreased their cellular life span. His team has also experimented with ways to decrease the mass-specific metabolic rate of cells, either by inhibiting glycolysis, which produces energy with less efficiency than the electron transport chain, or growing them to saturation on the media, which results in cell-to-cell signals that decrease cellular division via contact inhibition. As predicted, these cells had slower rates of telomere shortening, decreased epigenetic aging, and decreased age-related gene expression. Aggregating across all experimental conditions, including controls, manipulations to increase cellular metabolism (dexamethasone, mitoNUITs, mitochondrial disease, & oligomycin-mediated mitochondrial dysfunction), and manipulations to decrease cellular metabolism (glycolysis inhibition & contact inhibition) explained 56% of observed differences in telomere shortening, 95% of observed differences in age-related gene expression, and 24% of differences in epigenetic aging. Collectively, these data suggest cellular metabolism exerts profound, perhaps the most profound, influence on cellular lifespan.

Compared to cells with normally functioning mitochondria, cells that have mitochondrial defects as a result of genetic variation or from environmental perturbations have increased mitochondrial mutations and a hypersecretory phenotype of inflammatory cytokines that also damages nearby cells and tissues. This phenotype seems to be driven by activation of stress responses at the level of gene expression, including the integrated stress response, increases in global transcription and translation, and cell-to-cell communication. Gene expression changes co-occurred with changes in the overall metabolic strategy of cells. Specifically, cells adopted a hypermetabolic state, wherein cells produce more energy but direct it toward processes unrelated to cellular division, which traditionally comprises the largest proportion of cellular

energy expenditure. Despite decreased cell division rate, these cells demonstrated increased telomere shortening, epigenetic aging, and decreased replicative capacity.

This dataset including all measures of cellular aging, cellular lifespan, cellular metabolism, and mitochondrial energetics is publicly available at https://columbia-picard.shinyapps.io/shinyapp-Lifespan_Study/. Related publications are available at http://www.picardlab.org/publications.

Open Forum: Community Guidance to the Telomere Research Network for Research and Dissemination

Chair: Stacy Drury, MD, PhD, Tulane University School of Medicine

The TRN still has banked single DNA extracted samples and PBMCs sets created originally by Nan-Ping Wang at the National Institute of Aging that can be utilized for future studies. Dr. Drury requested input from the audience on how to best utilize these samples to bolster the understanding of the relation between different TL measurement approaches, emphasizing the need for new methods and approaches to integration of indices of mitochondrial function. Specific ideas for next steps included expanding the multi-laboratory methods comparison by re-analyzing data with a 3rd set of TRF measurements, as well as repeating STELA measurements with a second set to assess reproducibility of TL measurements generated with this approach. These STELA measurements could then be compared to nanopore and Flow-FISH results that can similarly generate chromosome-specific measurements. In addition to continued methodological advancement, Dr. Michelle Heacock pushed for the perspective of human population researchers and urged the TRN to assist in providing guidelines for which TL measurement approach can be best suited to answer specific research questions. Weighing in on this, Dr. Drury suggested that data from existing collaborative studies enacted by the TRN shows that PCR-based approaches can be used in population-based studies with a high degree of repeatability, especially MMqPCR. Dr. Karen Sugden echoed Dr. Heacock's sentiment, but questioned whether PCR-based approaches can be implemented in large longitudinal cohorts without knowing the lower limit of TL detection and ability to detect potentially small effect sizes. Responding to these concerns, Dr. Verhulst highlighted published resources from the TRN that can be used that outline the sample sizes needed to detect specific effects depending on the precision of measurements. With such a large sample as Dr. Sugden references, Dr. Verhulst suggested the main challenge is to organize the measurements in a way that you can compare values to each other and within groups, suggesting that for longitudinal studies it would be best to assay TL at the same time, even if the samples were collected at different time points and stored for longer duration. To mitigate potential impacts of storage duration, Dr. Verhulst suggested that larger studies maximize collection, storage, and extraction method uniformity to the extent possible.

In the absence of a perfect high throughput methodology, and a true TL measurement gold sgtandard, qPCR is a reasonable methodology to answer research questions related to human population health and environmental exposures so long as variability in measurement conditions is accounted for, as was seen in the UK BioBank study, and there is sufficient sample size.

Guidelines for and recommendations for storage, as well as analytic approaches to control for different sources of measurement error due to methodologic imperfections have also been published by the TRN and are publicly accessible. Another avenue the TRN is currently reviewing is the ability to generate control DNA standards of known lengths for use by researchers when they are first validating their TL assay, either using an established protocol or developing a new one.

There is consensus that early TL is a strong predictor of later life TL and that there is accelerated TL in the first years of life, however to date there is little longitudinal data in this age range. To address this critical gap, the TRN is collaborating with the FinnBrain cohort and will release pilot funding opportunities to utilize the longitudinal TL data in children birth to age 5 to support future pilot funding initiatives examining the relation between TL trajectory in those years and a wealth of other data related to environmental exposures and intergenerational impact on TL. Dr. Eisenberg stressed the importance of including pre-registration for these pilot analyses to prevent concerns related to prevent p-hacking and data manipulation. This is especially important for studies exploring integrated biomarkers, such as a telomere index suggested by Dr. Hastings. Such an index would include multiple measurements of telomere integrity (e.g., TL, telomerase activity, telomere DNA damage), which could have complex relationships to one another that would be best explored in a hypothesis directed manner to decrease the risk of spurious findings amongst so many interrelated but not well understood metrics.

The 2023 TRN conference covered a wide range of topics from multi-laboratory methods comparison, handling of samples, and future funding proposals for projects involving telomeres. Some challenges for the current state of telomere research were also discussed which included identifying guidelines for longitudinal studies of TL, incorporating and standardizing qPCR reliability, and the need to simultaneously consider sample size and specific assay measurement precision. Approaches to alleviate these challenges included following recommendations for storage, extraction, and sample size published by the TRN, as well as continuing TRN support for proposals validating novel methods and the establishment of new laboratories using banked samples and resources.

Appendix A: Workshop Agenda

	March 30, 2023 (all times in EST)	
08:00-08:30am	*Registration and light breakfast*	
Morning session: Results from the TRN U01 Collaboratory Chair: Stacy Drury, MD/PhD		
08:30-08:45am	Introduction to the TRN and collaboratory	Stacy Drury, U24 PI Janine Simmons, NIA Lisbeth Nielsen, NIA Michelle Heacock, NIEHS Max Guo, NIA
08:45-09:45am	Cross method and cross laboratory results	Simon Verhulst, University of Groningen (U24)
09:45-10:00am	Questions/Discussion	
10:00-10:45am	*Telomere Length Measurements and Opportunities for Clinical Applications (virtual)*	Abraham Aviv, Rutgers (U01 PI) Shahinaz Gadalla, NCI Sharon Savage, NCI
10:45-11:00am	Questions/Discussion	
11:00-11:15am	Coffee Break	
11:15-11:45am	*Single telomere length analysis by DNA-array-FISH*	Yun-Ling Zheng, Georgetown (U01 PI)
11:45-12:00pm	Questions/Discussion	
12:00-12:30pm	*Telomere methodological factors: Lessons from the Contemplative Coping during COVID study*	Jue Lin, UCSF (U01 PI) Quinn Conklin, UC Davis
12:30-12:45pm	Questions/Discussion	
12:45-1:30pm	*Lunch*	Provided by TRN
Keynote: Tracy Woodruff, PhD, MPH Chair: John McLachlan, Tulane University (U24)		
1:30-2:15pm	*Everywhere all at once, exposome exposures and health effects: identifying exposure priorities to improve health and health equity*	Tracy Woodruff, UCSF
2:15-2:30pm	Questions/Discussion	
2:30-3:00pm	*Cross-tissue comparison of telomere length and DNA QC metrics across two cohorts*	Idan Shalev, Penn State (U01 PI)
3:00-3:15pm	Questions/Discussion	
3:15-3:45pm	*Community input regarding next methodological questions for the TRN and recommendations for additional cross laboratory studies*	Stacy Drury, U24 PI **OPEN FORUM**
3:45-4:00pm	Coffee break	
Afternoon Session: Updates on pilot award recipients and flash talks Chair: Elissa Epel		
4:00-4:15pm	Outcomes of TRN pilot awards and next steps	Elissa Epel, UCSF (U24)
4:15-4:25pm	*Predictors of newborn TL and efforts to define the early life trajectory of TL*	Dries Martens*, University of Hasselt
4:25-4:35pm	*Joint effects of telomere length and social environment in predicting youth delinquency*	Darlene Kertes, University of Florida
4:35-4:45pm	*Associations between early life adversities, ambient air pollution, and buccal telomere length in children*	Rosemarie de la Rosa*, University of California, Berkeley
4:45-4:55pm	*Anti-aging effects of elite football and team handball trainings*	Muhammad Asghar*, Lund University
6:00pm	Group Dinner (TBA) and networking	Optional

	March 31, 2023 (all times in EST)	
08:00-08:30am	*Registration and light breakfast*	Provided by TRN
Session 1: Novel Method development for TL measurement **Chair: Stacy Drury, U24 PI**		
08:30-8:45am	NIA/NIEHS Welcome and Introductory Remarks *The importance of collaborative science in establishing standards and scientific rigor*	Richard Hodes, NIA Richard Woychik, NIEHS
08:45-09:00am	Introduction to method development session and goals	Stacy Drury, PI U24
9:00-9:15am	*A novel Metric to Improve Quantification Accuracy and Primer Selection in Quantitative Polymerase Chain Reactions (qPCRs)*	Eugenia Xu, Princeton University
09:15-09:45am	*Nanopore telomere sequencing -NanoTelSeq- enables accurate length measurement of telomeres (virtually)*	Riham Smoom, The Hebrew University of Jerusalem
09:45-10:15am	*Absolute telomere length quantification with CRISPR-Cas12a*	Waylon J. Hastings, Tulane University
10:15-10:45am	*Precise measurement of telomere length in T-cell derived extracellular vesicles*	Bo Ning, Tulane University
10:45-11:00am	Community input on specific guidelines for methodologic validation of novel telomere measurement assays and approaches for effective implementation of these guidelines through funders, peer review and other dissemination outlets	Stacy Drury, U24 PI **OPEN FORUM**
11:00-11:15am	*Coffee break*	
Session 2: Interactive debate about fundamental questions of the role of telomeres as sentinels in human population studies **Chair: Simon Verhulst, University of Groningen (U24)**		
Panelists: Duncan Baird (Cardiff University), Stacy Drury (U24 PI), Veryan Codd (University of Leicester), Jon Alder (University of Pittsburg), Patricia Opresko (Organizing participant, University of Pittsburgh)		
11:15-12:45pm	In this modified debate, panelists **were assigned** by the Chair to present an opinion, either for or against the three statements listed below. These three statements, designed by the TRN organizing committee, were specifically created to instigate dialogue and drive scientific study focused on determining the validity of each statement and/or its counterargument. Panelists agreed to participate and were not permitted to select the position they were supporting. Participants are encouraged, prior to and during the TRN meeting, to consider these statements and the existing data for and against each statement. Specific comments, prior to the meeting, are welcomed on the TRN twitter (@telomerenetwork) or can be sent to the TRN email account "telomerenetwork@gmail.com 1. Single measurements of telomere length are meaningless because it is the rate of telomere shortening that is relevant to human health and disease. 2. No environmental or psychosocial stress exposure has sufficient effect on telomere length to result in cellular functional or health consequence. 3. The association between telomere length and mortality is driven by infectious diseases only.	Debate structure: Panelist: FOR: 3 minutes AGAINST: 3 minutes Panelist Rebuttal FOR rebuttal: 2 minutes (self-response, lifeline (call an expert)) AGAINST: rebuttal, 2 minutes (self-response, lifeline (call an expert)) Open forum: meeting participants may provide responses, individuals selected by chair and meeting team to present opinion limited to 2 minutes each. 10 minutes total. Chair- closing **AUDIENCE VOTE** (via zoom poll)
12:45-1:30pm	*Lunch*	Provided by TRN

Session 3: Novel models and the importance of cross species collaboration Chair: Stacy Drury, U24 PI		
1:30-1:45pm	*Lymphocyte telomere length in a bovine model of parturition and prenatal stress*	Tom Welsh, Texas A&M University
1:45-2:00pm	*Epigenetic inheritance of a telomere capping defect triggers longevity in C. elegans*	Shawn Ahmed, University of North Carolina
Keynote: Martin Picard, PhD Chair: Stacy Drury, MD/PhD		
2:00-2:45pm	*The energetic cost of telomere maintenance and mitochondria (virtually)*	Martin Picard, Columbia University
2:45-3:00pm	Discussion of different models of telomere and mitochondrial interactions, approaches to leverage basic and translational model and their role in aging and disease	Stacy Drury, U24 PI **OPEN FORUM**
3:00-3:15pm	*Coffee break*	
Session 4: Open forum: TRN – Next steps and critical gaps		
3:15-4:00pm	Community guidance for the TRN next research and dissemination steps	Stacy Drury, U24 PI **OPEN FORUM**
4:00pm	Adjourn	