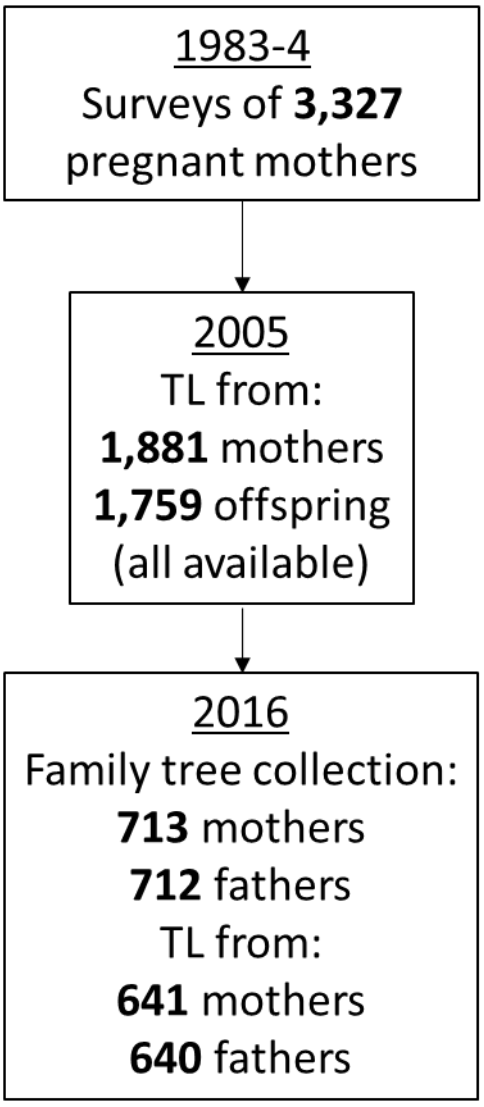
Supplementary Material



Supplementary Figure 1. Flowchart outlining sampling strategy. TL=telomere length.

Additional methods details

Blood collection and telomere length analysis. Venous blood samples were drawn into EDTA tubes, and then were processed up to the cell lysis step of the Gentra Puregene DNA extraction protocol in the Philippines. These stabilized samples were stored at room temperature until shipped back to the US for completion of extraction.

Telomere lengths (TL) for 2005 gathered samples were measured using the monochrome multiplex quantitative polymerase chain reaction assay (MMQPCR; 1) as described in detail previously (2, 3). A subsample of 190 of these samples show a correlation between MMQPCR

measures and southern blot of terminal restriction fragments (r=0.663) that is on par with recent qPCR TL validation efforts (2, 4).

TL for 2016 samples were assayed on a BioRad CFX 384 real-time PCR detection system (Hercules, CA, USA) using a similar protocol as the 2005 samples. Prior to plating, all samples were diluted to 8 ng/µl. DNA was quantified using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA). High quality DNA extracted from whole blood was used to create an eight-point, two-fold serially diluted standard reference curve (from 100 ng/reaction to 0.78 ng/reaction). All samples, standards, and negative controls were run in triplicate. Some DNA from the same high quality stock as the standard curve was also diluted to 8 ng/µl and used as a positive control. Twelve positive controls were included on each plate. The final reaction volume was 15 µl. Standard curves had average R^2 values of 0.97 and 0.99, and average efficiency values of 90.63% and 92.06%, for T and S respectively.

Since the coefficient of variation (CV) has recently been recognized to be an invalid statistic to assess TL measurement reliability, we instead used the intraclass correlation coefficient (ICC) (5, 6) which estimates the percent of variation attributable to individuals versus to measurement error. ICC(1) gives an estimate of the reliability of measures of samples analyzed on one run (in triplicate), while ICC(k) gives an estimate of the reliability of the average TL estimate of a sample measured across multiple runs. While considerable numbers of samples in the 2005 analyses were included on multiple runs, these samples were re-run because of initially high intra-assay CVs. Of these samples, 873 were run separately in triplicate on two separate runs and had an individual ICC(1) of 0.81 (95% CI: 0.79-0.84) and average ICC(k) of 0.89 (95% CI 0.88-0.91). For the 2016 samples, an additional plate of samples (n = 95) was assayed an additional time to assess inter plate reproducibility: ICC(1) = 0.79 (95% CI: 0.70, 0.86), ICC(k) = 0.88 (95% CI: 0.82, 0.92). Of these 95 samples 61 where fathers with an ICC(1) of 0.79 (95% CI: 0.68-0.87) and 34 were mothers with an ICC(1) of 0.79 (95% CI: 0.63-0.89). Intra-assay coefficient of variation measures for 2016 father and mother samples were 0.09 and 0.10 respectively.

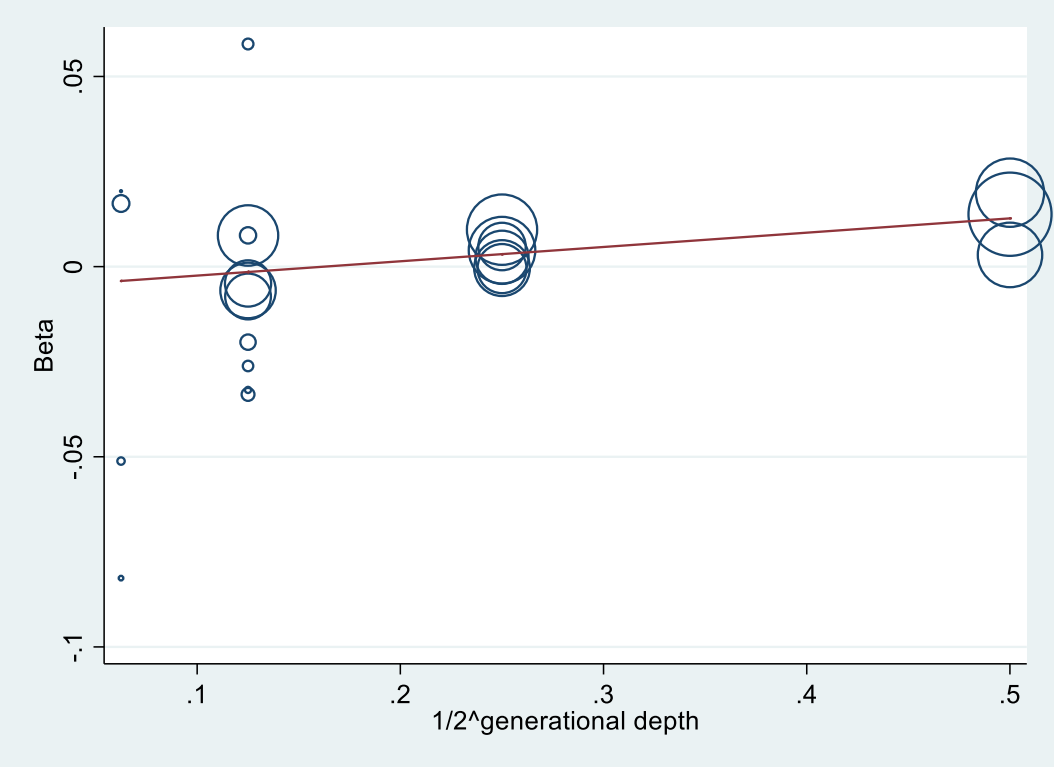
For the 2016 samples, T/S ratio was calculated using the estimated starting quantity of each sample based on the standard reference curve. In order to improve statistical power, T/S ratios were adjusted by average well position effect (Eisenberg et al. 2015). Specifically, average T/S was calculated for each well across all plates assayed. Next, we calculated the total mean T/S for the entire 2016 sample, which we used to determine well deviance (T/S_{total_mean}-T/S_{well_avg}). A uniformity assay was then run. The deviance values for each well in the uniformity assay (i.e. T/S_{avg}-T/S_{obs}) were then averaged with the respective well deviance values for the 2016 sample plates. We then subtracted this average deviance from the well average T/S, which yielded the mean well-adjusted T/S. For each observed sample T/S ratio, we subtracted the well-adjusted T/S and added the total mean T/S. Finally, T/S ratio was averaged for each sample triplicate. This specific adjustment method was settled on because they yielded the tightest correlation with both measures of external validity (age and 2005 TL). We note that the lowest correlation between 2005 and 2016 TL measures from adjustment methods considered was 0.43 while the highest was 0.47.

Confounding by SES, Urbanicity or Ancestry. The PAC effect could be due to social and other factors that influence both TL and PAC. To explore this, we examined the changes in PAC β values before and after the inclusion of several potential confounding variables. Like PAYC/PAOC analyses, for comparability, we first ran a regression model without any additional control variables, but restricting the set of individuals for which complete case control variable data were available (Supplementary Table 5, Model 1). Then the control variable(s) are added in. We tested the effects of social variables log-household income and urbanicity in 1983 on PAC effects of offspring, mothers and fathers (grandpaternal age effects are not examined) (Supplementary Table 5, Model 2).

Principal components (PCs) of genome-wide genetic variation index population structure/ancestry. These may index social and/or biological differences among individuals which may affect both PAC and TL. As in previous analyses (7), the bivariate association between the first ten principal components and TL were tested. The top principal components up to and including the last one showing a significant bivariate association with TL were included as control variables. Substantial attenuation of the PAC β would suggest potential confounding necessitating further exploration. Results are shown in Supplementary Table 5, Model 3.

<u>Deviations from pre-registration</u>

- Meta-regressions using metareg in Stata were random effects not fixed effects models.
- In meta-regression models intermediate male ancestors were transformed in the same manner as generational depth (i.e. as $1/(2^{\wedge\text{intermediate male ancestors}})$.
- Checking if PAC effect varied with own age was not in pre-registration.
- PAC association with TL attrition was not proposed in pre-registration.
- For comparison purposes in MAC analyses we re-ran PAC models without including MAC but limiting to the same sample for which MAC was also available to make values comparable
- In our preregistration, we hypothesized that "..partial PAC effects might attenuate due to PAYC or PAOC having no causal relationship with TL but being correlated with PAC (correlations range from 0.64 to 0.75)." However, subsequent simulation analysis suggested showed that we should not expect such attenuation (i.e. PAC β increased when controlling for PAYC when assuming no PAYC effect in 50.09% of simulations).
- Beta values from different regression models were compared used Stata's suest followed by test commands. Correlation coefficients were compared using a Fisher transformation technique (8, 9).



Supplementary Figure 2. Meta-regression results of generational depth of PAC association predicting variability of PAC estimates. Larger circles indicates estimates with greater precision (inverse of within-study variance). Each circle represents an estimated ancestral PAC effect on a descendant's TL (from Table 2). For example, there are six estimated GPAC effects (FF and MM in each of cohorts 1, 2 and 3). The β value for the ½^generational depth term=0.0376175, and the y-intercept=-0.0061226. This implies a predicted PAC effect of 0.0127 (0.0376175*(1/2^1)-0.0061226) and GPAC effect of 0.00328 (0.0376175*(1/2^2)-0.0061226)

Simulation analyses

To better understand the stability and expectations of several of our regression models, we ran a series of simulations. In all cases, simulations were conducted in Stata by drawing 10,000 multivariate normal distributions from set correlation structures and then running relevant regression models. Weighted mean correlations were calculated using Fisher's r to Z-transformation (10). The code for the simulations are provided as an additional supplementary file.

Change in effect size between PAC and GPAC. To simulate this change, the weighted PAC-TL correlation across each of the three cohorts was calculated (r=0.101) and then divided in half to generate a GPAC effect. We then drew a sample of 3,282 with this covariance structure and ran a regression of PAC predicting TL. This test was followed by a second regression on this same dataset, with the first 2,914 samples of PAC and GPAC predicting TL in the same model. These sample sizes were picked to match the actual sample sizes of our study (see Table 2). The PAC effect was then divided by the GPAC effect. The median effect was 2.16 (implying a PAC effect 2.16-fold greater than GPAC) with a 95% CI from 0.96 to 9.61. This slight increase over the expected 2x median effect appears to be due to controlling for PAC when calculated GPAC

causing an attenuation of the GPAC effect. When not including PAC in the model when calculating GPAC, the median effect was 1.98 with a 95% CI of 0.97 to 7.16.

Maternal versus paternal age. The correlation structures for these analyses were derived from combining weighted mean correlations for statistics calculated from all three cohorts. Specifically, these values were:

- PAC with offspring TL: r=0.101
- PAC with MAC: r=0.753
- MAC with offspring TL: r=0.086

Then three different sets of correlation structures were derived from these observed statistics.

1. *Observed PAC effect on offspring TL, but assuming no MAC effect on offspring TL*. MAC correlation with offspring TL is due entirely to the correlation of PAC with MAC (r=0.101*0.753=0.076). This calculated value is similar to the observed MAC-TL correlation of 0.086.
2. *Observed MAC effect on offspring TL, but assuming no PAC effect on offspring TL*. PAC correlation with offspring TL is due entirely to the correlation of PAC with MAC (r=0.086*0.753=0.064).
3. *Observed PAC effect on offspring TL, but assuming a negative MAC effect on offspring TL*. We choose a negative MAC effect of half the magnitude of the PAC effect which leads to an expected MAC correlation with offspring TL of r=√ ((0.101*0.753)^2-(0.101*.5)^2)=0.057.

In each of these simulations we calculated the percentage of simulations in which the following occurred:

1. PAC β increased when controlling for MAC over model without MAC in it
2. PAC β > MAC β
3. PAC β > 0
4. MAC β > 0

Supplementary Table 1. Maternal versus Paternal age effect on offspring TL simulations. Values are % of times out of 10,000 simulations for which this was true.

	+ PAC, 0 MAC	**0 PAC, + MAC**	**+ PAC, - MAC**
PAC β increase when controlling for MAC	50.52	0.16	95.35
PAC β>MAC β	97.99	4.51	99.98
PAC β>0	100.00	50.53	100.00
MAC β>0	50.54	99.92	4.22

Paternal age versus paternal age at youngest sibling. The correlation structures for these analyses were derived from combining weighted mean correlations for statistics calculated from all three cohorts for the sample which PAYC was available. Specifically, these values were:

- PAC with offspring TL: r=0.080
- PAC with PAYC: r=0.649
- PAYC with offspring TL: 0.029

Then two different sets of correlation structures were derived from these observed statistics.

1. Assuming no PAYC effect on TL, we would expect a PAYC-offspring TL correlation due to the correlation of PAC with PAYC (r=0.080*0.649=0.052). This is larger than the observed PAYC-TL correlation of 0.029.
2. Assuming no PAC effect and a PAC-TL association only driven by the association of PAYC with PAC we would expect a PAC-TL association of r=0.029*0.649=0.019

Supplementary Table 2. Paternal age at youngest child effect on offspring TL simulations. Values are % of times out of 10,000 simulations for which this was true.

	+ PAC, 0 PAYC	**0 PAC, + PAYC**
PAC β increase when controlling for PAYC	50.09	12.52
PAC β>PAYC β	95.96	26.48
PAC β>0	99.90	50.00
PAYC β>0	50.29	86.87

Paternal age versus paternal age at oldest sibling. The correlation structures for these analyses were derived from combining weighted mean correlations for statistics calculated from all three cohorts for the sample which PAOC was available. Specifically, these values were:

- PAC with offspring TL: r=0.066
- PAC with PAOC: r=0.645
- PAOC with offspring TL: r=0.046

Then two different sets of correlation structures were derived from these observed statistics.

1. Assuming no PAOC effect on TL, we would expect a PAYC-offspring TL correlation due to the correlation of PAC with PAOC (r=0.066*0.645=0.045). This is similar to the observed PAOC-TL correlation of 0.046.
2. Assuming no PAC effect and a PAC-TL association only driven by the association of PAOC with PAC we would expect a PAC-TL association of r=0.046*0.645=0.032

Supplementary Table 3. Paternal age at oldest child effect on offspring TL simulations. Values are % of times out of 10,000 simulations for which this was true.

	+ PAC, 0 PAOC	**0 PAC, + PAOC**
PAC β increase when controlling for PAOC	49.00	4.69
PAC β>PAOC β	89.84	18.98
PAC β>0	99.09	50.37
PAOC β>0	50.38	94.71

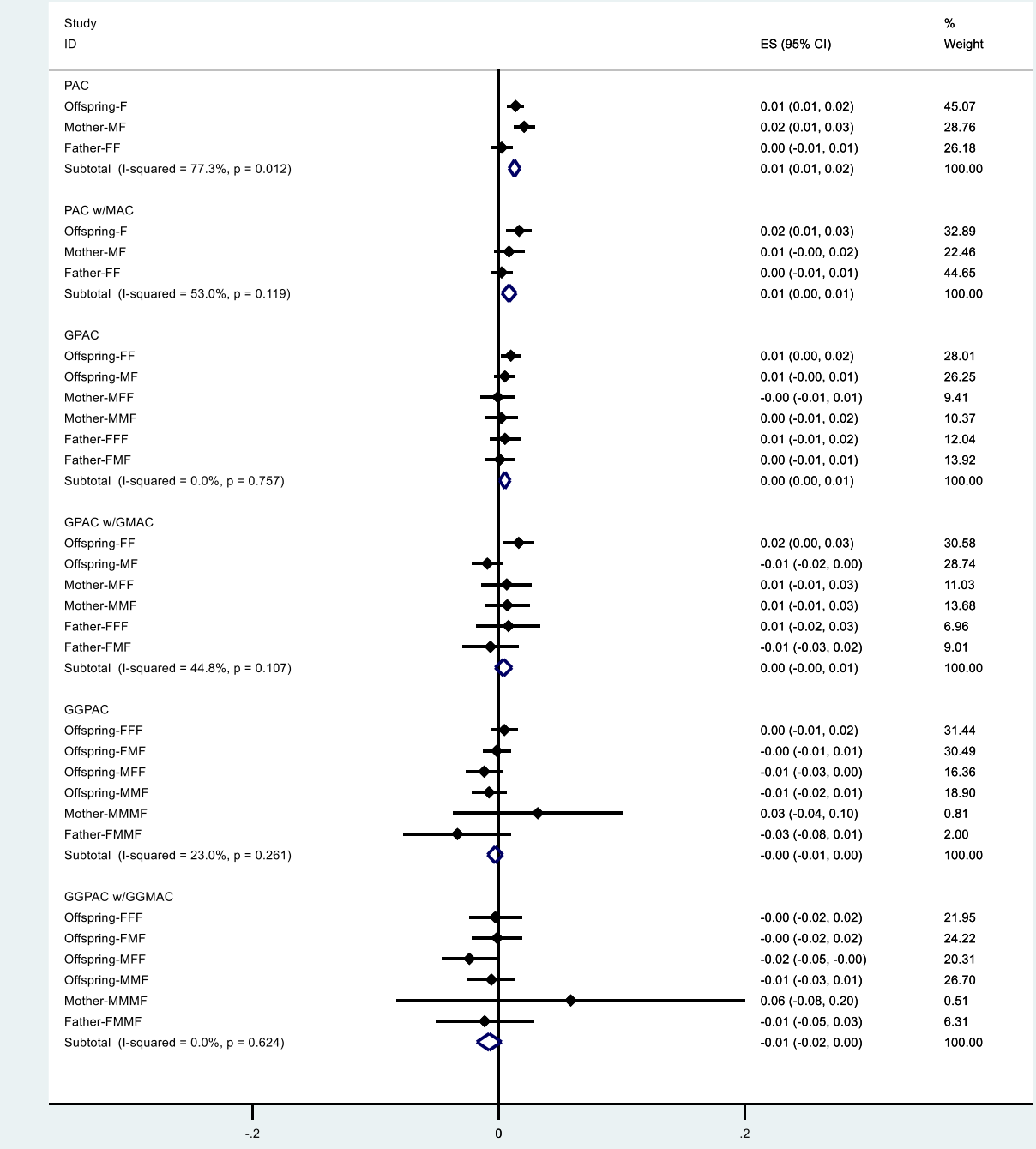
<u>Does sperm TL actually increase with age- PAYC/PAOC analyses?</u>

For PAYC analysis only non-last born children were included. While for PAOC only non-first born children were included. For comparability, regression models without PAYC or PAOC but

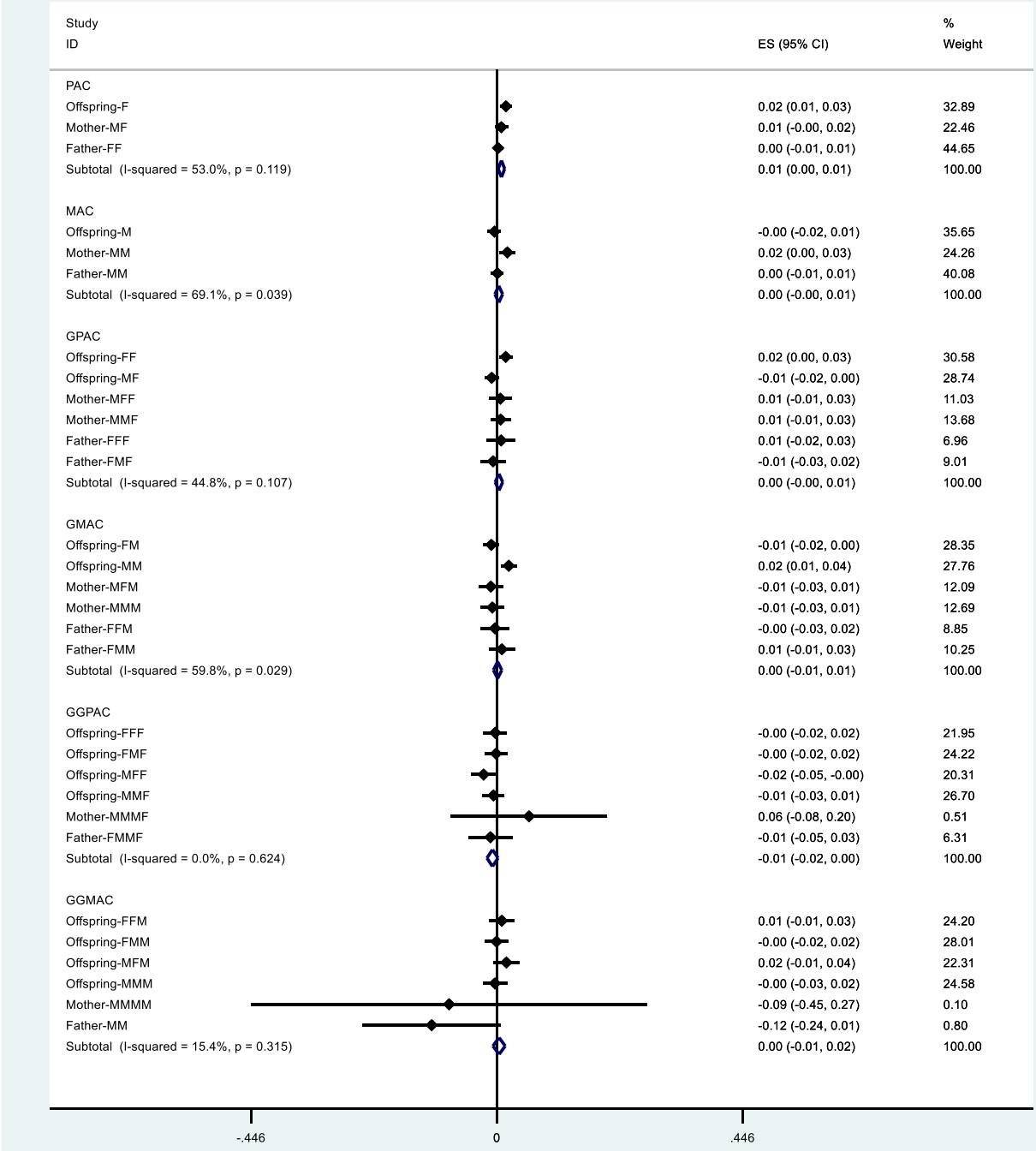
restricting the sample to be the same as for which PAYC or PAOC data are available were run first (Supplementary Table 6, odd numbered models). Then PAYC or PAOC was added in (Supplementary Table 6, even numbered models). We predicted that PAC would be more strongly associated with offspring TL than PAYC or PAOC when included together in the same models.

On average, the PAC β maintained a significant positive value and actually increased (although non-significantly so) when controlling for PAYC (Supplementary Table 6: models 1 & 2). PAC β value estimates were positive in all cases and larger than PAYC β values which were mostly negative. The same pattern was observed with GPAC (models 3-6). Simulation analyses suggest that these patterns are more consistent with a PAC effect than a PAYC effect, but that statistical power to distinguish these was limited.

The PAC effect, on average did not change with inclusion of PAOC in the model versus not (models 7 & 8) and PAOC had no discernible overall effect. PAC β value estimates were mostly positive and larger than PAOC β values which were mostly negative. However, there was very high heterogeneity in PAOC estimates across cohorts (I^2=81%), with a significant positive effect in the offspring cohort, a significant negative effect in the mother cohort and a non-significant negative trend in the fathers. Consistent with effects on the fathers and mothers being relayed to offspring, the GPAC effect non-significantly increased with inclusion of GPAOC (models 9-12). GPAOC showed an overall significant negative effect. That is, for every year older the grandfathers were at the conception of the intermediate parents' oldest sibling, grandchildren had an estimated 0.016 SD shorter TL. Overall, while these results are generally more consistent with a PAC than PAOC, simulation analyses suggest that these analyses have limited ability to reliably distinguish with certainty.



Supplementary Figure 3. PAC effects controlling and not controlling for MAC.



Supplementary Figure 4. Comparing PAC and MAC effects (PAC and MAC included in same models together).

Supplementary Table 4. Evaluating non-linearities in PAC effects. Beta is main effect and beta-sq is squared beta effect.

cohort	Beta	se	p	n	predictor	Beta-sq	se-sq	p-sq
z_ic2005tl	0.01323	0.003951	0.000829	1738	C_a_F	9.17E-05	0.000321	0.774908
z_mom2005tl	0.016488	0.004608	0.000365	913	C_a_MF	0.000478	0.000318	0.13333
z_dad2016tl	0.004742	0.0053	0.371303	631	C_a_FF	-0.00017	0.000307	0.577661
z_ic2005tl	0.010261	0.004675	0.028453	833	C_a_FF	-6.5E-05	0.000292	0.824477
z_ic2005tl	0.005906	0.004785	0.217469	906	C_a_MF	-0.00024	0.00033	0.469747
z_mom2005tl	-0.00252	0.006988	0.719166	307	C_a_MFF	0.00015	0.000291	0.606211
z_mom2005tl	0.001681	0.006686	0.801674	276	C_a_MMF	0.000446	0.000364	0.221538
z_dad2016tl	0.000959	0.005812	0.869045	297	C_a_FFF	-0.00011	0.000241	0.635195
z_dad2016tl	-0.00063	0.005745	0.912931	294	C_a_FMF	0.000554	0.000379	0.1445
z_ic2005tl	0.004669	0.005514	0.39784	325	C_a_FFF	0.000262	0.000214	0.2208
z_ic2005tl	-0.00885	0.00547	0.10677	326	C_a_FMF	0.000493	0.000367	0.179557
z_ic2005tl	-0.01202	0.007533	0.111547	306	C_a_MFF	0.000309	0.000313	0.324495
z_ic2005tl	-0.00878	0.006808	0.19809	278	C_a_MMF	0.000551	0.00037	0.137599
z_mom2005tl	0.045704	0.018885	0.094153	9	C_a_MFFF	0.007051	0.002969	0.098087
z_mom2005tl	-0.01716	0.025836	0.626785	6	C_a_MFMF	-0.01639	0.006599	0.24366
z_mom2005tl	-0.00509	0.014294	0.745277	7	C_a_MMMF	0.001346	0.000635	0.124204
z_dad2016tl	-0.01722	0.024759	0.536666	9	C_a_FFFF	-0.00046	0.002216	0.848589
z_dad2016tl	-0.36638	0.158553	0.260011	6	C_a_FMFF	-0.07475	0.034646	0.276303
z_dad2016tl	-0.02626	0.032739	0.506634	6	C_a_FMMF	0.001485	0.003934	0.742061
z_ic2005tl	0.022541	0.024636	0.528257	10	C_a_FFFF	-0.00119	0.002382	0.705276
z_ic2005tl	-0.0964	0.071587	0.406627	9	C_a_MFFF	0.006963	0.008562	0.565337
z_ic2005tl	-0.07607	0		6	C_a_MFMF	-0.03823	0	
z_ic2005tl	0.032878	0.031366	0.48502	7	C_a_MMMF	0.003048	0.000963	0.194709

Supplementary Table 5. Paternal ages predicting offspring's telomere lengths controlling for potential confounders[a]

	Cohort	1[b]	2[c]	3[d]
Model	Control variables	β	β	β
1	minimum	0.013***	0.020***	0.003
2	1 + income + urbanicity	0.013***	0.020***	0.003
3	1 + Ancestry	0.013***	0.020***	-

[a]All models control for age and intermediate paternal ancestors as applicable. [b]cohort born in 1983-84 and TL measured in 2005. models additionally controls for sex and age X sex, n=1720. [c]mothers of 1983-84 born cohort members with TL measured in 2005, n=868. [d]fathers of 1983-84 born cohort members with TL measured in 2016, n=630. †<0.1; *P < 0.05; **P < 0.01; and ***P < 0.001. '-' indicates cells intentionally left blank because of insufficient data to model.

Supplementary Table 6. Paternal ages predicting descendants' telomere lengths controlling for paternal age at youngest child (PAYC) or paternal age at oldest child (PAOC)[a]

	Cohort	1[b]		2[c]		3[d]		**Combined**	
Model	Ancestor[f]	β	n	β	n	β	n	**β**	**n**
1	PAC	**0.014****	1296	**0.022*****	774	0.002	536	**0.013*****	**2606**
2	PAC	*0.010†*	1296	**.029*****	774	0.008	536	**0.015*****	**2606**
2	PAYC	0.005	1296	*-0.011†*	774	-0.009	536	-0.003	**2606**
3	MF	0.004	779		-		-		
4	MF	0.004	779		-		-		
4	MFYC	-0.001	779		-		-		
5	FF	**.010***	652		-		-		
6	FF	*0.011†*	652		-		-		
6	FFYC	-0.002	652		-		-		
3 & 5	**GPAC**	*0.007†*	1431	meta-analysis of FF and MF from models 3 & 5					
4 & 6	**GPAC**	*0.008†*	1431	meta-analysis of FF and MF from models 4 & 6					
4 & 6	**GPAYC**	-0.001	1431	meta-analysis of FFYC and MFYC from models 4 & 6					
7	PAC	**0.014****	1291	**0.012***	620	0.001	467	**0.010*****	**2378**
8	PAC	0.006	1291	**0.024****	620	0.005	467	**0.010****	**2378**
8	PAOC	**0.018***	1291	**-0.022***	620	-0.008	467	0	**2378**
9	MF	0.003	618		-		-		
10	MF	**.018***	618		-		-		
10	MFOC	**-0.029****	618		-		-		
11	FF	0.008	530		-		-		
12	FF	0.009	530		-		-		
12	FFOC	-0.002	530		-		-		
9 & 11	**GPAC**	0.005	1148	meta-analysis of FF and MF from models 9 & 11					
10 & 12	**GPAC**	**0.014***	1148	meta-analysis of FF and MF from models 10 & 12					
10 & 12	**GPAOC**	**-0.016***	1148	meta-analysis of FFOC and MFOC from models 10 & 12					

[a]All models control for age and intermediate paternal ancestors as applicable. [b]cohort born in 1983-84 and TL measured in 2005. models additionally controls for sex and age X sex. [c]mothers of 1983-84 born cohort members with TL measured in 2005. [d]fathers of 1983-84 born cohort members with TL measured in 2016. [f]Ancestor or ancestor category (e.g PAC indicates paternal age at conception, GPAC grandpaternal age at conception of intermediate ancestor, MF mothers father's age at mothers conception). †<0.1; *P < 0.05; **P < 0.01; and ***P < 0.001. '-' indicates cells intentionally left blank because of insufficient data to model.

Supplementary Table 7. More specific stats for Table 2						
cohort	Beta	se	p	n	predictor	Group
z_ic2005tl	0.013797	0.003416	5.6E-05	1738	a_F	PAC
z_mom2005tl	0.019466	0.004163	3.37E-06	913	a_MF	PAC
z_dad2016tl	0.003104	0.004407	0.481543	631	a_FF	PAC
z_ic2005tl	0.009739	0.004039	0.016118	833	a_FF	GPAC
z_ic2005tl	0.004338	0.004265	0.309366	906	a_MF	GPAC
z_mom2005tl	-0.0005	0.005791	0.930993	307	a_MFF	GPAC
z_mom2005tl	0.005316	0.005997	0.37616	276	a_MMF	GPAC
z_dad2016tl	-0.00036	0.005094	0.943061	297	a_FFF	GPAC
z_dad2016tl	0.002475	0.005349	0.643928	294	a_FMF	GPAC
z_ic2005tl	0.008211	0.004702	0.081701	325	a_FFF	GGPAC
z_ic2005tl	-0.00625	0.005123	0.22363	326	a_FMF	GGPAC
z_ic2005tl	-0.00783	0.006219	0.209121	306	a_MFF	GGPAC
z_ic2005tl	-0.0044	0.006153	0.474941	278	a_MMF	GGPAC
z_mom2005tl	0.058557	0.026589	0.092416	9	a_MFFF	GGPAC
z_mom2005tl	-0.0324	0.047518	0.565653	6	a_MFMF	GGPAC
z_mom2005tl	0.008245	0.017568	0.66327	7	a_MMMF	GGPAC
z_dad2016tl	-0.01984	0.018608	0.346482	9	a_FFFF	GGPAC
z_dad2016tl	-0.0261	0.027374	0.440989	6	a_FMFF	GGPAC
z_dad2016tl	-0.03358	0.022291	0.228999	6	a_FMMF	GGPAC
z_ic2005tl	0.016617	0.017062	0.432813	10	a_FFFF	GGGPAC
z_ic2005tl	-0.08189	0.063185	0.32438	9	a_MFFF	GGGPAC
z_ic2005tl	0.019859	0.155039	0.918898	6	a_MFMF	GGGPAC
z_ic2005tl	-0.05111	0.039335	0.323445	7	a_MMMF	GGGPAC

References

1. Cawthon RM. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. Nucleic Acids Res. 2009;37(3):e21.
2. Eisenberg DT, Kuzawa CW, Hayes MG. Improving qPCR telomere length assays: Controlling for well position effects increases statistical power. Am J Hum Biol. 2015;27(4):570-5.
3. Eisenberg DTA, Borja JB, Hayes MG, Kuzawa CW. Early life infection, but not breastfeeding, predicts adult blood telomere lengths in the Philippines. Am J Hum Biol. 2017;29(4).
4. Elbers CC, Garcia ME, Kimura M, Cummings SR, Nalls MA, Newman AB, et al. Comparison Between Southern Blots and qPCR Analysis of Leukocyte Telomere Length in the Health ABC Study. J of Gerontol Ser A: Bio Sci and Med Sci. 2013;69(5):527-31.
5. Eisenberg DT. Telomere length measurement validity: the coefficient of variation is invalid and cannot be used to compare quantitative polymerase chain reaction and Southern blot telomere length measurement techniques. Int J Epidemiol. 2016;45(4):1295-8.
6. Verhulst S, Susser E, Factor-Litvak PR, Simons MJ, Benetos A, Steenstrup T, et al. Commentary: The reliability of telomere length measurements. Int J Epidemiol. 2015;44(5):1683-6.
7. Bethancourt HJ, Kratz M, Beresford SAA, Hayes MG, Kuzawa CW, Duazo PL, et al. No association between blood telomere length and longitudinally assessed diet or adiposity in a young adult Filipino population. Eur J Nutr. 2017;56(1):295-308.
8. Cohen J, Cohen P, West SG, Aiken LS. Applied multiple regression/correlation analysis for the behavioral sciences. 3rd ed. Mahwah, N.J.: L. Erlbaum Associates; 2003. xxviii, 703 p. p.
9. DeCoster J, Iselin A-M. Microsoft Excel Spreadsheets: Comparing Correlation Coefficents http://www.stat-help.com/spreadsheets.html2005 [updated 11-05-2005. Available from: http://www.stat-help.com/spreadsheets.html.
10. Strube MJ. Averaging correlation coefficients: Influence of heterogeneity and set size. J Appl Psychol. 1988;73(3):559-68.