The rs2736100 within the telomerase reverse transcriptase gene and rs16847897 within the telomerase RNA component gene moderate the association between internalizing mental disorders and telomere length attrition among HIV+ children and adolescents in Uganda

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Abstract

Background Internalizing mental disorders (IMDs) have been associated with accelerated telomere length (TL) attrition; however, this association has not been investigated in the context of genetic variation. A large genome-wide meta-analysis has implicated several loci that affect mean TL, in particular single nucleotide polymorphisms within the telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) genes. Genetic variations in the solute carrier family 6 member 4 gene (SLC6A4) and the tryptophan hydroxylase 2 (TPH2) genes have also been reported to moderate the association between social environment and TL. The SLC6A4 codes for the serotonin transporter (5-HTT), a protein that recycles serotonin from synapses while the TPH2 codes for tryptophan hydroxylase 2, an enzyme that catalyzes the rate-limiting reaction in serotonin biosynthesis. This case-control study used linear regression models to investigate the moderating effects of selected polymorphisms in TERT, TERC, SLC6A4 and TPH2 genes on the association between IMDs and relative TL (rTL), among Ugandan HIV+ children (7-11 years) and adolescents (12-17 years). Investigated polymorphisms were: rs2736100, rs7726159, rs10069690, and rs2853669 (TERT); rs12696304, rs16847897 and rs10936599 (TERC); 5-HTTLPR, rs35531, 5-HTTLPR/rs35531 and STin2.VNTRs (SLC6A4) and rs1843809, rs1386494, and rs34517220 (TPH2).

Results At baseline, HIV+ children and adolescents with any internalizing mental disorder had significantly longer relative TL compared to controls matched for site, age, sex and socio-economic status (p = 0.001). None of the polymorphisms investigated had any moderating effect on the observed association between IMDs and baseline rTL. At 12 months, we observed no statistically significant difference in 12-month rTL between baseline cases and controls (p = 0.117), however, on modeling the effects of each of the selected polymorphisms, we observed that the interaction of IMDs and each of rs2736100 and rs16847897 significantly influenced rTL (p = 0.007 and p = 0.012 respectively).

Conclusions The rs2736100 and rs16847897 polymorphisms moderate the association between IMDs and rTL among Ugandan HIV+ children and adolescents over time. The T-allele for rs2736100 and a C-allele for rs16847897 are associated with accelerated rTL attrition among cases of IMDs. The mechanisms under which these alleles interact with IMDs to moderate rTL require further investigations.

Background

Children and adolescents living with HIV suffer a considerable burden of internalizing mental disorders (IMDs) (namely, post-traumatic stress disorder and mood and anxiety disorders, such as depressive disorders, generalized anxiety and separation anxiety disorders) [1-3]. Studies undertaken both in the developed (Europe and the United States) and developing world (sub-Saharan Africa) among HIV infected (HIV+) children and adolescents (HCA) have documented rates of major depressive disorder of between 12.7% and 40% [2-8] and rates of anxiety disorders of between 9% and 32.2% 1-3, 6]. For IMDs combined, rates of between 12% and 27% have been documented in Uganda and South Africa, respectively [1, 9]. Among people living with HIV/AIDS, IMDs have been associated with a number of other negative outcomes, including accelerated cellular aging [10], faster HIV disease progression [11,12], poor adherence to medication [12, 13], risky sexual behavior [13-15], poor linkage to care for newly diagnosed HIV+ persons [16], increased HIV transmission (through the promotion of HIV drug resistance) [17] and impaired academic and social functioning [3, 15]. Twin studies have estimated a genetic heritability of 35% for depression [18] and 30-50% for post-traumatic stress disorder (PTSD) [18, 19], while a meta-analysis of family and twin studies estimated a genetic heritability of 31.6% for generalized anxiety disorder [20]. While considerable research has investigated the psychosocial risk factors for IMDs among HCA, there is a paucity of research on biological risk factors, including genetic factors. A recent genome-wide association study (GWAS) identified 44 loci for depression [21]. Depression has also been reported to genetically overlap with schizophrenia and bipolar disorder in European samples [22]. The underlying etiology, and biochemical and molecular events in particular, of IMDs are, however, mostly unknown, although polymorphisms in the solute carrier family 6 member 4 gene (SLC6A4), catecholamine-o-methyl transferase and the brain-derived neurotropic factor genes have been linked to depression [23-25] while polymorphisms in the tryptophan hydroxylase 2 (TPH2) gene have been associated with depression and anxiety traits [26, 27]. Several studies have investigated telomeres, the protein-bound deoxyribonucleic acid (DNA) repeat structures at the ends of chromosomes, in relation to IMDs [28]. Telomeres are important in protecting chromosomes from fusing together during mitosis, thus preventing loss of genetic data [29, 30]. Telomeres shorten progressively with each cell division eventually leading to

DNA damage responses, replicative senescence, or programmed cell death, and thereby provide a marker of biological aging [31]. TL is also influenced by environmental factors, with research suggesting that the pathophysiological processes underlying infectious disease and stress exposure are capable of increasing TL attrition (i.e., increasing cellular age independent of chronological age) [28, 32]. TL has been positively associated with more years of healthy life [33] and TL measurements provide both a proxy of cumulative environmental exposures as well as an indication of increased risk for poor health [28, 32] IMDs are highly comorbid with both psychiatric and somatic disorders, including those associated with advanced aging [34]. Depression has, for example, been reported to be associated with chronic diseases (e.g., type 2 diabetes mellitus and cardiovascular disease) [34, 35], as well chronic inflammation [36]. In addition, higher mortality rates have been reported among patients with mental disorders (e.g., depression and other affective disorders) compared to the general population, with this mortality mainly due to the same age-related diseases, such as cancer, cardiac and cerebrovascular disease [37-39]. The observed comorbidity between IMDs and age-associated diseases suggests that IMDs may be mediating accelerated TL attrition or accelerated TL attrition may be causally involved in IMDs. Compared to controls, shorter TL has been reported in studies of depression [40-44], anxiety disorders [45, 46] and PTSD [47, 48]. A longitudinal study by Shalev et al. (2014) [42] reported that persistence of IMDs from 11 to 38 years predicted reduced leucocyte TL at 38 years of age among male participants. There is, however, a dearth of data on the mechanisms by which IMDs lead to TL attrition. IMDs act as chronic stressors [49, 50], producing long-lasting biological adaptations that could potentially explain TL attrition due to IMDs [51]. Stress-induced increases in inflammatory responses have also been suggested to mediate telomere shortening [52]. TL is maintained by a telomerase enzyme component known as Telomerase RNA Component (TERC) and a reverse transcriptase enzyme known as the telomerase reverse transcriptase (TERT) [30, 53]. Evidence that IMDs could be driving TL attrition emanates from in vitro and in vivo animal model experiments in which manipulation of telomerase activity was found to influence depressive-like behaviors and even reversed neurodegeneration [54-56]. In a mouse model, chronic mild stress led to a decrease in TERT levels and telomerase activity in the hippocampal region of the brain that was reversed by treatment with antidepressants [55], highlighting the effects of depression on the TL maintaining enzyme. Evidence for the role of TL among patients with internalizing mental disorders (IMDs) with age-related diseases such as cardiovascular disease may offer a novel potential mechanism for the excess morbidity and mortality associated with IMDs [57]. As mean TL is highly heritable with heritability estimates ranging between 44–80 % [58, 59], it is likely that genetic variation substantially contributes to telomere maintenance. We therefore assessed IMDs and TL in a sample of HCA and investigated whether genetic variation moderated the association between IMDs and TL. Specifically, we investigated single nucleotide polymorphisms (SNPs) rs2736100, rs7726159, rs10069690, and rs2853669 in the telomerase reverse transcriptase gene (TERT); rs12696304, rs16847897 and rs10936599 in the telomerase RNA component gene (TERC); rs1843809, rs1386494, and rs34517220 in the tryptophan hydroxylase 2 gene (TPH2); 5-HTTLPR, rs35531, 5-HTTLPR/rs35531 and STin2.VNTRs in SLC6A4. A large genome-wide meta-analysis of 37,684 individuals found several loci to influence mean leucocyte TL (LTL), with loci at TERT and TERC genes contributing the leading SNPs [60]. These loci have been shown to be involved in telomere biology [60]. The TERT and TERC loci are also involved in the formation and activity of telomerase enzyme [60]. Telomeres are maintained by the telomerase enzyme, an enzyme whose catalytic protein component and RNA template is encoded by the TERT and TERC, respectively. This warrants investigation of the effect of variations in these genes on TL. Minor alleles for rs12696304 and rs16847897 of the TERC have been associated with shorter LTL [61, 62]. For the TERT, minor allele of rs2736100 has been associated with shorter TL [60], while minor alleles in rs2853669 and rs7726159 have been associated with longer TL [63, 64]. Since IMDs were found to be associated with relative telomere length (rTL) attrition in our sample [10], other risk factors interacting with IMDs may well influence rTL attrition. Serotonergic (5-HT) neurotransmission has been implicated in IMDs [65-67]. The SLC6A4 codes for the 5-HTT, a protein that recycles the serotonin from the synapses while TPH2 codes for tryptophan hydroxylase 2, an enzyme that catalyzes the rate-limiting reaction in serotonin biosynthesis [68]. Genetic variations in SLC6A4 and TPH2 have also been reported to moderate the association between social environments and TL in children [69]. We investigated 5-HTTLPR, rs35531, 5-HTTLPR/rs35531 and STin2 VNTRs in SLC6A4. 5-HTTLPR is a functional polymorphism that comprises either 14 (short, S-allele) or 16 (long, L-allele) copies of a 22-23 base pair (bp) repeat [70]. The L-allele has two to three times higher basal transcriptional activity compared to the S-allele [71]. Within the 5-HTTLPR is a functional SNP, an A to G substitution at position rs25531. The presence of G is associated with reduced expression of the gene [72]. We analyzed this SNP together with 5-HTTLPR as a haplotype. Upstream, the SLC6A4 is a second intron (STin2) polymorphism which consists of a variable number of tandem repeats (VNTR) with multiple repeated copies of a 16-17 bp element [73, 74] which repeats either 10 or 12 times [71]. We also investigated the rs1843809, rs1386494, and rs34517220 in the TPH2. The rs1843809 and rs1386494

have been associated with depression [75], while rs34517220 has been associated with reduction in depressive symptoms among patients treated with flouxetine [76]. Our a priori selection of these polymorphisms was thus based on their role in telomere biology or association with IMDs. We hypothesized that selected polymorphisms in TERC, TERT, SLC6A4 and TPH2 would moderate the association between IMDs and rTL attrition among our study population.

Results

Socio-demographic factors were evenly distributed between cases and controls (Table 1) and none of these factors was significantly associated with baseline rTL.

Table 1 Distribution of socio-demographic factors in cases and controls

Variable (n)	Case n (%)	Control n (%)	P-value
Sex			P = 0.111
Male (342)	160(43.6)	182(49.5)	
Female (393)	207(56.4)	186(50.5)	
Site			P = 0.941
Urban (415)	208(56.5)	207(56.3)	
Rural (321)	160(43.5)	161(43.7)	
Age			P = 0.374
7-11 years (389)	202 (57.6)	187(54.2)	
12-17 years (307)	149(42.4)	158(45.8)	
Education level			P = 0.371
No formal education (13)	9(2.5)	4(1.1)	
Primary (648)	323(88.0)	325(88.8)	
Secondary (72)	35(9.5)	37(10.1)	
Socioeconomic status			P = 0.459
Low (332)	171(46.5)	161(43.8)	
High (404)	197(53.5)	207(56.2)	
Mean CD 4 count at baseline			
	947.04	944.02	P = 0.939

CD4 = cluster of differentiation 4; primary = 0 – 7 years of formal education; Secondary = 8 – 14 years of formal education; Low socioeconomic status = 0 – 13; High socioeconomic status = > 13. All numbers that do not add up were due to missing data.

Difference in rTL between cases and controls at baseline

We found a positive but non-statistically significant correlation between rTL and age among cases (p = 0.379) and controls (p = 0.854). At baseline, mean rTL was longer in cases compared to controls (p = 0.001) (Table 2). None of the investigated SNPs or polymorphisms significantly moderated the association between IMDs and baseline rTL.

Table 2: Association between IMDs and baseline rTL

Group	Obs	Mean rTL	Std. Dev	95% CI	P-value
Cases	307	1.198	0.364	(1.157, 1.239)	
Controls	306	1.097	0.354	(1.057, 1.137)	<0.001

Difference in 12-month rTL between baseline cases and controls

At bivariate analysis level, we observed no significant difference in 12-month rTL between cases and controls (p = 0.117) (Table 3). However, in cases, we observed rs2736100 and rs16847897 to significantly moderate the association between IMDs and rTL (p = 0.007 and p = 0.012 respectively) (Table 4). For rs2736100, mean rTL were different between cases and controls for the *GG* genotype while mean rTL was similar between cases and controls for both the *TG* and *TT* genotypes (Figure 1). For rs16847897, mean rTL differed between cases and controls for the *CC* genotype while mean rTL was similar between cases and controls for both the *GC* and *CC* genotypes (Figure 1). Both the *GG* genotype for rs2736100 and the *CC* genotype for rs16847897 were more prevalent among cases (Table 5).

Table 3: Distribution of 12month rTL by baseline IMDs

Group	Obs	Mean rTL	Std. Dev	95% CI	P-value
Cases	278	0.925	0.336	(0.886, 0.965)	
Controls	274	0.884	0.275	(0.852, 0.917)	0.117
Combined sample	552	0.905	0.307	(0.879, .931)	

Group = baseline cases and controls, Obs = number of observations, Std. Dev = standard deviation, CI = confidence interval

Table 4: Two-way analysis of variance for the interaction of IMDs with rs2736100 and with s16847897 on rTL at 12 months

SNP	Obs	Variable	F	P > F
rs2736100	511	Mental disorder	6.03	0.014
		rs2736100	0.44	0.645
		IMDs * rs2736100	4.95	0.007
rs16847897	515	Mental disorder	7.25	0.007
		rs16847897	3.00	0.050
		IMDs * rs16847897	4.44	0.012

SNP: single nucleotide polymorphism; Obs: number of observations;

IMDs*rs2736100: interaction of internalizing mental disorders with rs2736100 on relative telomere length;

IMDs *rs16847897, interaction of internalizing mental disorders with rs16847897 on relative telomere length.

Figure 1: Mean rTL between cases and controls by genotype for rs2736100 and rs16847897

Mean rTL were different between cases and controls for both the *GG* and *CC* genotypes of rs2736100 and rs16847897 respectively.

Table 5: Distribution of rs2736100 and rs16847897 genotypes between cases and controls

SNP	Genotype	Cases (n)	Controls (n)	P-value
rs2736100	*GG*	82	57	0.017
	TG	149	181	
	TT	106	95	
rs16847897	*CC*	27	17	0.349
	GC	137	128	
	GG	186	187	

SNP = single nucleotide polymorphism

Discussion

In this sample of Ugandan HCA, we previously documented both longer rTL at baseline and accelerated rTL attrition over 12 months among cases with IMDs compared to controls [10]. The current study investigated whether selected polymorphisms within the *TERT*, *TERC*, *SLC6A4* and *TPH2* genes moderated observed associations between IMDs and rTL at baseline and 12 months. As hypothesized, *TERT* rs2736100 and *TERC* rs16847897 significantly moderated the association between IMDs and rTL. To our knowledge, this is the first sub-Saharan African study to investigate these interactions among HCA.

Since TL is an independent predictor of chronological age [77], we expected a negative correlation between TL and age. The positive correlation between rTL and age in the combined sample, although not statistically significant, and the lack of a statistically significant association between rTL and age among participants is potentially due to the age group sampled (7-17 years) as TL have been shown to shorten rapidly from birth to age 5. Stabilization then occurs between age 4 to 5 and young adulthood and then telomere loss resumes at a slower rate later during adulthood [78, 79].

In line with previous studies that have reported associations between IMDs and TL in European populations [41-48], we also found an association between IMDs and rTL at baseline (p = 0.001). However, contrary to these previous studies, rTL was longer among cases than controls (p = 0.001) and we propose that elevated levels of telomerase in our cases, an enzyme that is responsible for maintaining length of telomeres to be the potential reason for the discrepancy, as elevated telomerase levels have been reported among people with depression than among healthy matched controls at baseline [80]. We recommend future studies to investigate for that possibility.

None of the investigated polymorphisms significantly influenced the association between IMDs and rTL at baseline. We however observed significant interactions between *TERT* rs2736100 and *TERC* rs16847897 with IMDs on rTL at 12 months. These results are in line with results from a large GWAS study where *TERT* and *TERC* were among the loci that were found to be associated with mean TL [60].

In our study population, presence of a *T*-allele for the *TERT* rs2736100 and a *G*-allele for the *TERC* rs16847897 were respectively responsible for no statistical significant difference in rTL between cases and controls at 12 months indicating that these alleles were responsible for the accelerated rTL attrition observed among the cases. Our findings are in line with previous studies where both the *TERT* rs2736100 and the *TERC* rs16847897 were each associated with shorter rTL [60, 62]. Each copy of the major allele rs16847897 was also associated with shorter mean TL in a Han Chinese population [81].

Although rs2736100 and rs16847897 have been associated with shorter rTL [60, 62], the functionality of these SNPs is not known and the mechanisms through which IMDs act with them (rs2736100 and rs16847897) to influence TL are also not known. Studies have shown that telomerase activity and expression of *TERT* may be related to the mechanisms of action for psychopharmacological interventions such as antidepressants and antipsychotics [82, 80] and thus experimental studies on telomerase, *TERT* and *TERC* among IMDs may provide insights on how IMDs interact with the SNPs to influence TL. Future experimental studies are needed to elucidate these mechanisms.

We did not find any moderating role for selected variants within the *SLC6A4* and *TPH2* on the association between IMDs and rTL. A study by Mitchell *et al.* (2014) [69] reported genetic variants in serotonergic and dopaminergic pathways as moderators of the association between exposure to disadvantaged environments and TL among 9-year-old boys. These results suggest that variants in *SLC6A4* and *TPH2* moderate the association between exposure to socially disadvantaged environments and TL but not the association between IMDs and TL. However, methodological differences between the two studies should be noted where Mitchell *et al.* (2014) [69] investigated moderation among boys only.

Conclusion

The *TERT* rs2736100 and *TERC* rs16847897 polymorphisms temporally moderate the association between IMDs and rTL among Ugandan HIV+ children and adolescents. The *T*allele for rs2736100 and a *G*-allele for rs16847897 are associated with accelerated rTL attrition among cases of IMDs. The mechanisms under which these alleles interact with IMDs to moderate rTL require further investigations.

Methods

Study design

This case-control study was carried out in children (7-11 years) and adolescents (12-17 years). A total of 368 cases with any IMD and 368 age- and sex-matched controls were included. Both cases and controls were Ugandans. This study was nested within the previously described CHAKA study [1, 15], which enrolled 1339 HCA (855 children and 484 adolescents) in Uganda.

Study population

Study subjects were recruited from two HIV clinics in urban Kampala (Joint Clinic Research Centre (JCRC) and Nsambya Home Care) and three HIV clinics in rural Masaka (The AIDS Support Organisation (TASO), Kitovu Mobile Clinic and Uganda Cares). All study subjects were on anti-retroviral therapy.

Procedures

As part of CHAKA study, children and assenting adolescents, as well as their caregivers, were interviewed using a structured questionnaire and provided a blood specimen (4 ml) for genetic analyses. The questionnaire included, amongst others, socio-demographic characteristics and depression, PTSD and anxiety modules of the DSM-5 referenced Child and Adolescent Symptoms Inventory-5 (CASI-5) [83]. The CASI-5 was administered by trained psychiatric nurses and psychiatric clinical officers at two time points (baseline and 12 months). The CASI-5 lists the symptoms of a wide range of psychiatric disorders including MDD, generalized anxiety disorder, PTSD and attention-deficit/hyperactivity disorder among others. Individual CASI-5 items are rated on a four-point frequency of occurrence scale ranging from never (0) to very often (3). Though there are several CASI-5 scoring algorithms, in the present study we used symptom count cut-off scores, which reflect the prerequisite number of symptoms for a clinical diagnosis. At each study visit, 4 ml of blood from each study participant was collected via venipuncture into an EDTA vacutainer and subsequently stored at -80°C pending DNA extraction.

Inclusion and exclusion criteria

Inclusion criteria: i) HIV-infected outpatients, registered with the HIV Clinics at JCRC or Nsambya Home Care at the Kampala study site and TASO, Kitovu mobile or Uganda Cares clinic at the Masaka site; ii) aged between 7 and 17 years at the time of enrolment; iii) conversant in English or Luganda, the language into which the assessment tools were translated; and iv) able to provide written informed consent/assent. Cases were HCA who had any depressive disorder (depression or dysthymia [persistent depressive disorder]), anxiety disorder or PTSD. Controls were age- and sex- matched HCA without any psychiatric disorder. Persistent IMDs were baseline cases that remained cases at 12 months while remitted ones were baseline cases that no longer qualified for a diagnosis at 12 months. *Exclusion criteria*: i) seriously ill and unable to understand study procedures; and ii) any other psychiatric disorder other than the IMDs listed above.

Ethical considerations

The study complied with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The CHAKA study obtained ethical and scientific clearance from the Uganda Virus Research Institute (UVRI) Science and Ethical Committee (#GC/127/15/06/459) and the Uganda National Council of Science and Technology (# HS 1601). The present study obtained approval from the Higher Degrees Research & Ethics Committee, School of Biomedical Sciences, College of Health Sciences, Makerere University (# SBS 421) and the Health Research Ethics Committee of Stellenbosch University (#S17/09/179). Study subjects who were diagnosed with significant psychiatric problems were referred to mental health units at Entebbe and Masaka government hospitals.

Selection of cases and controls

All baseline cases of IMDs (368) in the parent study, CHAKA were considered and thus cases were HCA who had any internalizing mental disorder (IMD). All cases at baseline were ascertained, and the cases were then stratified by site (one of two sites), sex, age category (one of three categories) and Socio-economic status (SES) (one of three SES categories). This resulted in a total of 36 strata (2X2X3X3). In each stratum the number of cases was ascertained (e.g. for males in site 1 in the youngest age category and the lowest SES group there were 9 cases). An equal number of controls (HCA without any psychiatric disorder) were then randomly sampled from the stratum concerned (so for males in site 1 in the youngest age category and the lowest SES group we sampled 9 controls), thus the controls were frequency matched to the cases on site, sex, age and SES.

Analysis of relative telomere length

DNA was extracted from blood collected from each participant, using the QiAmp Mini DNA Extraction Kit (Qiagen GmbH, Germany). Extracted DNA was quantified by 260/280 and 260/230 ultraviolet spectrophotometry on the NanoDrop 1000 spectrophotometer V3.7 (Thermo Fisher Scientific, Wilmington, MA). The DNA was subsequently diluted to 5 ng/µL and amplified using the KAPA SYBR FAST qPCR Master Mix (Merck, Darmstadt, Germany) per Cawthon, (2002) [84], with slight modifications. Primers specific for telomeric repeats were adapted from Cawthon, (2002) [84] while those for the stably expressed single copy

reference gene (S) – the human β-globin gene (*HBG*) were adapted from a study by Malan *et al*. (2011) [85]. The adapted primers were used to amplify telomeric repeats and *HBG*, respectively. For the telomere assay, each reaction included 5 µl KAPA SYBR FAST qPCR Master Mix (Merck, Darmstadt, Germany), 1.35 µM and 4.50 µM of forward and reverse primers respectively, 5 ng genomic DNA and water in a 10 uL total reaction volume. The *HBG* assay was identical to the telomere assay except that 2.0 µM of each of the forward and reverse primers were used. The reactions for the telomeric repeats and the *HBG* were amplified on the same 384-well plates. Each participant's DNA sample was amplified in triplicate. If the threshold cycle (Ct) values of the triplicates of particular samples differed by more than 0.5, those samples were excluded. From the triplicate Ct values, the means were calculated for each sample and used in subsequent calculations. Amplification was performed on the ABI 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) using the following thermal cycling profile: 95 °C for 3 minutes; followed by 40 cycles of 95 °C for 3 seconds (s), and 60 °C for 30 s; and a dissociation stage of 95 °C for 15 s, 64 °C for 15 s, and 95 °C for 15 s. A calibrator sample was prepared by pooling equal amounts of DNA from each participant for the construction of a standard curve. The calibrator DNA sample was serially diluted by a factor of 1.68 to produce a nine-point standard curve, with DNA amounts ranging from 50 to 0.79 ng/µL. After amplification of the serial dilutions, a linear plot of the Ct versus the log value of the input amount of DNA (standard curve) was constructed using ABI's SDS v.2.3 software. The efficiency of the reaction was also determined from the standard curve of that reaction. Threshold and baseline values were used as determined by the SDS v.2.3 software. All Ct values were corrected for the PCR efficiency and interplate calibration was also performed using GenEx software (http://www.gene-quantification.de/datan.html).

The mean telomere Ct value (T) was normalized to the reference gene Ct value (S) to control for differences in DNA quantity. The T/S ratio is proportional to the average TL. Thereafter, the factor by which the T/S ratio differed between the experimental sample and the calibrator sample was determined to provide an indication of relative average TL:

$T/S = 2^{-\Delta Ct}$

Relative average TL $= 2^{-\Delta\Delta Ct}$
(Where $\Delta Ct = Ct(T) – Ct(S)$)
A T/S>1 indicates that the average rTL in the sample is greater than that of the average cohort rTL, and a T/S<1 indicates that the average rTL in the experimental sample is below the average rTL.

To address skewness, all outliers revealed by box and whisker plots were removed from the rTL data.

Analysis of *TERT*, *TERC* and *TPH2* genotypes
DNA samples were genotyped for each selected SNP in each of the *TERT*, *TERC* and *TPH2* genes, using a kompetitive allele-specific PCR (KASP) assay (LGC, Middlesex, United Kingdom). This genotyping chemistry allowed for bi-allelic discrimination of SNPs.

Analysis of *SLC6A4* genotypes
All polymerase chain reactions (PCR) were performed in a GeneAmp PCR System 9700 (Perkin Elmer Biosystems, Foster City, CA, USA) and were carried out in 25 µl reaction volumes containing: the DNA template, 200 µM dNTP (Kapa Biosystems, Cape Town, South Africa), 5 µl of 10X Taq DNA polymerase buffer (Kapa Biosystems, Cape Town, South Africa), 1.0 mM magnesium chloride (Kapa Biosystems, Cape Town, SA), 0.625 units (U) Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa), and 0.5 µM of each primer, with bi-distilled water.

For the *5-HTTLPR* and *5-HTTLPR*/rs25531 polymorphisms, an initial denaturation step was performed at 95 °C for 3 minutes. Thereafter, a denaturation step was performed at 95 °C for 15 seconds (s), followed by the

primer annealing step, at 60 °C for 15 s, and an elongation step, performed at 72 °C for 15 s. A final elongation step, at 72 °C for 10 minutes, was then performed. The denaturation and extension steps were repeated for 35 cycles using primers that were adapted from Voyiaziakis *et al*. (2011) [86]. The forward and reverse primers were labeled with FAM and HEX dyes respectively at the 5' terminus end. After amplification, about 10mL of the products were electrophoresed on 2.0% agarose gels, in sodium borate buffer at 120 volts for about 40 minutes, using ethidium bromide stain. The *L*- and *S*-alleles were fragments of 419 bp and 375 bp respectively. In order to discriminate between the rs25531 *A* and *G* alleles, 5 µl of the remaining PCR products were digested using 5 units of mspI restriction endonuclease (New England Biolabs, United Kingdom) in a 10 µl reaction volume overnight at 37 °C. 5ul of the digested product were run on a 2% agarose gel to ensure success of the digestion reaction, while the remaining 5ul were saved for capillary electrophoresis.

For the *STin2* VNTR polymorphism, an initial denaturation step was performed at 95 °C for 2 minutes. Thereafter, a denaturation step was performed at 95 °C for 30 s, followed by the primer annealing step, at 60 °C for 30s, and an elongation step, performed at 72 °C for 30s. A final elongation step, at 72 °C for 5 minutes, was then performed. The denaturation and extension steps were repeated for 35 cycles using primers adapted from Battersby *et al*. (1996) [74]. The forward and reverse primers were also labeled with FAM and HEX dyes respectively at the 5' terminus end. 15ul of the PCR products were run on a 1.5% agarose gel in order to determine the success of the PCR.

5 ul of the digested products were multiplexed with 5 ul of the PCR products for the *STin2* VNTR. The multiplexed samples were sent to the central analytical facility of Stellenbosch University from where they were subjected to capillary electrophoresis on the ABI 3130 genetic analyzer (Applied Biosystems). Electrophoresis results were analyzed using the peak scanner software which is freely available online (https://www.thermofisher.com). Fragment sizes of the alleles at the *5-HTTLPR*/rs25531 locus were as follows: S_A = 281 bp, L_A = 325 bp and S_G/L_G = 151 bp, resulting into the following genotypes: S_A/S_A = 281 bp; L_A/L_A = 325 bp; S_G/S_G, L_G/L_G, L_G/S_G = 151 bp; L_G/S_A = 151 bp + 281 bp and L_A/S_G, L_A/L_G = 325 bp + 151 bp. Fragment sizes of the alleles at *STin2* VNTRs locus were as follows: *10-repeat* = 265 bp and *12-repeat* = 300 bp, resulting into the following genotypes: *10/10* = 250 bp, *10/12* = 265 bp + 300bp, and *12/12* = 300 bp.

Power for the study

We calculated the *post hoc* power for our study based on results from a study by Epel *et al.* (2004) [87]. We used the formula of sample size and power for difference in means in case–control studies. We worked on the assumption that cases (individuals with IMDs) would have higher levels of stress than controls (individuals without IMDs). Epel *et al.* (2004) [87] found a 15% reduction in mean rTL among cases compared with controls. Given a 1:1 ratio of cases to controls and using a 5% level of significance, with 368 cases and controls, our study was well powered (power greater than 80%) to detect any reduction above 4.75% in mean rTL between cases and controls. For instance, a reduction of 5% in mean rTL between cases and controls provided a power of 83.8%.

Statistical methods

Statistical analyses were conducted using Stata 15 (StataCorp, TX, USA). Socio-demographic characteristics (including socio-economic status) were described between cases and controls. Socio-economic status (SES) was generated from a scale of 9 household items owned (car, motorcycle, refrigerator, electricity, bicycle, radio, telephone, cupboard and flask). Each item was weighted in the respective order, the car carrying a maximum weight of 9 and a flask a minimum weight of 1. A total score of items was generated; whose median cut off was used to classify low and high SES. A t-test was used to compare CD4 counts between cases and controls to account for any disparity in HIV disease progression.

The distribution of rTL at baseline and at 12 months was determined using a standardized normal probability plot (P-P plot). Associations between the different socio-demographic factors and rTL were tested using one-way analysis of variance (ANOVA) to determine potential confounders. Independent sample t-tests were used to assess the association between IMDs and rTL both at baseline and 12 months. A two-way ANOVA was used to assess for interaction between each of the polymorphisms and IMDs on rTL both at baseline and 12months. Mean rTL at 12 months were plotted against genotypes for the interaction terms to elucidate the nature of the interaction terms. Where required, 95% confidence intervals were calculated.

Abbreviations

µL: microliter; CASI-5: Child and Adolescent Symptom Inventory – edition 5; CD4: cluster of differentiation 4; Ct: threshold cycle; DNA: deoxyribonucleic acid; DSM-V: Diagnostic and statistical manual of mental disorders – edition V; *HBG*: human β-globin gene; HCA: HIV+ children and adolescents; HIV/AIDS: human immunodeficiency virus/Acquired immunodeficiency disease syndrome; HIV+: human immunodeficiency virus – positive; IMDs: internalizing mental disorders; JCRC: Joint Clinical Research Council; MDD: major depressive disorder; MRC/DfID: Medical Research Council/Department for International Development; ng: nano gram; qPCR: quantitative polymerase chain reaction; rTL: relative telomere length; s: seconds; S: single copy gene; SNP: single nucleotide polymorphism; TASO: The AIDS Support Organization; TL: telomere length; TPH2: tryptophan hydroxylase 2; UVRI: Uganda Virus Research institute

Declarations

Availability of data and materials
All information gathered about study subjects and their samples is confidential, with access limited to the research team. However, upon request, data from the MRC/UVRI and LSHTM Uganda Research Unit is currently accessed under a data sharing policy via: http://www.mrcuganda.org/sites/default/files/publications/MRC_UVRI_Data_sharing_policy_December2015.pdf.

Consent for publication

No details, images or videos relating to any of the study subjects are included in this manuscript.

Ethics approval and consent to participate

The study obtained ethics approval from the Health Research Committee of Stellenbosch University (# S17/09/179) and the higher Degrees Research & Ethics Committee, School of Biomedical Sciences, College of Health Sciences, Makerere University (# SBS 421). The parent study (CHAKA) obtained ethics approval from the Uganda Virus Research Institute (UVRI) Science and Ethical Committee (# GC/127/15/06/459) and the Uganda National Council of Science and Technology (# HS 1601). All caregivers provided written informed consent for their children or adolescents to participate in the study and for a blood specimen to be withdrawn from them for the rTL and other genetics analyses. Adolescents further provided written informed assent to participate in the study.

Author's contribution

Concept: AK, SMJH, EK, SS; Data collection: AK, EK, SMJH, JSW, SS, Data analysis: WS, AK, RNN, SMJH, JSW, SS, MK, JL, EK; First draft: AK, SMJH, JSW, WS, EK, SS, MLJ; Final revision: AK, SMJH, JSW, EK, SS, WS, MLJ, RNN, PK, MK, JL; All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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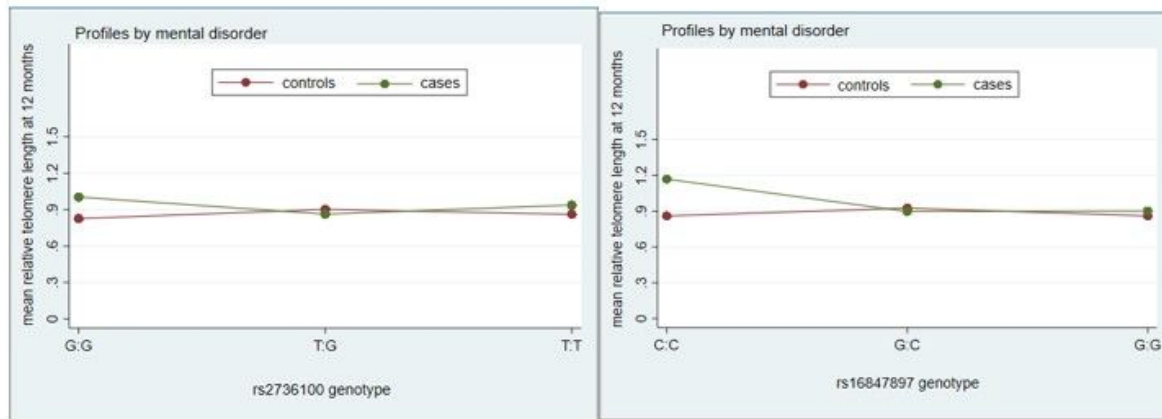
Figures

Figure 1

Mean rTL between cases and controls by genotype for rs2736100 and rs16847897 Mean rTL were different between cases and controls for both the GG and CC genotypes of rs2736100 and rs16847897 respectively.