**Effect of chronic and heavy drug abuse on biological aging**

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**Abstract.** Telomeres are structures of repeated sequences placed at the ends of chromosomes, which play a major role in various cellular processes. Over the past few years, telomere length has been associated with different environmental and physical factors, as well as with a number of diseases, such as cancer. A number of studies have associated telomere length with many substances that the participants have used, such as anxiolytics and hypnotics. The aim of this study was to investigate the association between telomere length and drug abuse. Following the methodology of quantitative fluorescent in situ hybridization (Q-FISH), we demonstrated that there was a reduction in telomere length in drug abusers. Furthermore, we evaluated this decrease in relation to cell aging and demonstrated that the biological age of drug abusers, particularly the one of opiate users, was by far greater than their chronological age, depending on the difference between the telomere length of abusers and healthy individuals. The findings of this study indicate an association between telomere length and drug abuse, which leads to premature biological aging. The latter warrants further investigation in order to establish this association in different types of drugs with regard to the severity and the chronic abuse of drugs.

**Introduction**

Drug abuse is a well-known, widespread issue and, unfortunately, concerns mainly young individuals. According to the European Monitoring Centre for Drugs and Drug Addiction, in 2017, cannabis users in Europe were estimated to be approximately 23.5 million, cocaine users approximately 3.5 million and opiate users approximately 13.5 million. From the opiate users estimated in 2015, only 38% of these asked for help for detoxification (1). Drug rehabilitation programs aim for the social reintegration of drug-addicted individuals and are based on two methods: The first method is what is termed as ‘dry’ programs, in which the drug abuser receives psychological support and assistance, understands the problem and abstains from any psychoactive substance. The user covers the one third of rehabilitation programs among the member countries of the European Union. The second method is substitution treatment programs in which opioid substances, such as methadone (formerly used) and buprenorphine (currently used) are administered as substitutes for the use and abuse of opiates (2). This type of detoxification covers the remaining 2/3 of rehabilitation programs (3).

One of the cellular aging indexes is the reduction of telomere length. Telomeres are a repetitive 5'-TTAGGG-3' DNA sequence at the end of each chromosome, coated by a complex of six proteins termed ‘shelterin’ proteins [telomeric repeat factor (TRF)1, TRF2, protection of telomeres protein 1 (POT1), TERF1-interacting nuclear factor 2 (TIN2), repressor/activator protein 1 (Rap1) and tripeptidyl-peptidase 1 (TPP1)] (9). These proteins protect the chromosome ends from double-stranded DNA breaks, deletions, inversions and translocations leading to eukaryotic chromosomal stability (10,11). The maintenance of telomere length depends on an enzyme, known as telomerase, which has the ability of a cellular reverse transcriptase and is produced in certain levels in the majority of somatic cells (12). In each cell division, DNA polymerases cannot maintain telomere repeats and telomere length becomes increasingly shorter (13).

In addition to the above-mentioned findings, telomere length has been reported to be a biomarker of cellular aging, associated with age-related diseases, such as diabetes, Alzheimer's disease, hypertension and cancer, as well as with a poor survival (14). It is estimated that telomere length decreases at a rate of 20–40 base pairs each year (15), and factors such as smoking, obesity, a poor diet, drugs, chronic inflammation, oxidative stress, can lead to a further reduction in telomere...
length. The aim of this study was to determine whether drug abuse can cause a reduction in telomere length, and to subsequently estimate the percentage of cellular aging.

Materials and methods

**Sampling.** Blood samples were collected from 16 drug abusers, all of whom were under a ‘dry’ detoxification program at the Therapeutic Center for Persons with Addiction (KETHEA). Blood samples were received within the first 15 days following the integration of the participants to the detoxification program. As a control group, we used 70 healthy individuals who voluntarily attended the Laboratory of Toxicology, Medical School, University of Crete (Crete, Greece). A written informed consent form was completed by all participants. This study was approved by the Ethics Committee of the University of Crete (07/01.11.2018).

**Quantitative fluorescent in situ hybridization (Q-FISH) analysis.** Peripheral blood samples (2.5 ml) were collected from all the participants. Leukocytes, from all samples, were isolated and cultured in RPMI-1640 culture medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin and streptomycin for 72 h in a CO₂ incubator. Chromosome preparation was performed using standard methods. Briefly, the leukocytes were treated with a hypotonic solution and fixed using an acetic acid:methanol ratio of 1:3. A few drops of fixative solution containing cells were used for each slide. The slides were dried and incubated on a hot plate (55°C) overnight prior to hybridization. Slides with chromosome preparations were hybridized with a (C3TA2)₃ peptide nucleic acid (PNA) probe (PANAGENE, Daejeon, Republic of Korea). Following hybridization, digital images were acquired using a Leica LCS Lite Confocal Microscope (Leica Microsystems, Wetzlar, Germany). Image acquisition was performed with a charge-coupled device camera (HCX PL APO CS 63X objective; Leica Microsystems, Wetzlar, Germany) at a 1,024x1,024 pixel resolution and 8-bit depth. Images were processed with Leica Q-FISH software, which allows for the quantification of telomere length (Fig. 1). To ensure that these Q-FISH experiments were reproducible, we used the L5178Y-S cell line (cat. no. 93050408; Culture Collections, Public Health England, Salisbury, UK) with a stable and known mean telomere length, which was estimated to be approximately 7 kb (16).

**Statistical analysis.** The telomere length for whole chromosomes (TL) and the telomere length from the short telomeres (TL<20th percentile; TLS) were estimated through Q-FISH fluorescence data. Descriptive statistics, such as the means, percentiles and quartiles were calculated using a newly developed spreadsheet termed BIOTEL, which specializes in such calculations (Fig. 2). Additional analyses were performed using IBM SPSS Statistics 24.0. The associations of discrete data were made using Pearson's Chi-square test. Differences in TL or TLS between the controls and abusers were tested with an independent samples t-test. Pearson's correlation coefficient (Pearson's rho) was used for correlation coefficient calculations. A value of P<0.05 was set for the acceptance of the null hypotheses.

**Results**

A total of 16 participants, both heavy cannabis and opiate users and 70 healthy individuals participated in the current study. From each participant, we collected images from different metaphases, where the specific binding of the PNA probe in the telomeric ends is clearly visible. The intensity of the signal of all telomeric measurements, in all metaphases of each participant, in combination with the intensity of cell line will be used for the determination of telomere length (Fig. 1).

The mean age of the drug abusers was 36.7±8.9 years old, ranging from 24 to 63 years, whereas the control group adjusted with this group (P=0.136) had a mean age of 41.2±11.2 years. The same adjustment was found when the participants were divided into 10-year age groups (P=0.287). In addition, sex distribution differed significantly since only 1 female was present (6.25%) in the drug abusers group (P<0.001; Table I).

The prevalence of drug abuse is depicted in Fig. 3. All participants had a systematic abuse of cannabis (100%), while 93.3, 73.3, 40.0 and 20.0% of the abusers reported a systematic use of opiates, benzodiazepines, cocaine and amphetamines, respectively. Fig. 4 represents the years of chronic abuse of the reported drugs. More specifically, cannabis abusers exhibited a mean of 14.1±6.2, opiate abusers a mean of 11.6±7.1 years, cocaine abusers a mean of 9.0±4 years, benzodiazepine abusers a mean of 9.6±9.0 years, and amphetamines abusers a mean of 6.0±1.7 years.

Bar charts of quartiles per the 5 percentile increment between the drug abusers and the control group are shown in Fig. 5. In Table II, the representation of a comparison between the quartiles of TL from the whole and from the short telomeres (TLS) (<20% of TL) between the drug abusers and the controls is presented. Additionally, in Fig. 5, it can be seen that although the pattern of increase was similar between the 2 groups, there was a definite distance, a statistically significant difference in the TL (P<0.001).
Moreover, Table II indicates that at all examined quartiles, the TL and TLS were estimated to be significantly higher in the controls than in the drug abusers (P<0.001). In particular, for the median TLS, which is a marker of biological age, the control groups exhibited a mean of median 7,155±1,353 bases, higher than that of the drug abusers (4,450±2,121 bases).

Finally, the years of chronic abuse of cannabis and opiates did not significantly correlate with the median TLS. The estimated correlation values between the median TLS with the years of cannabis abuse were (rs=-0.030, P=0.912) and median TLS (rs=-0.399, P=0.158) with opiate abuse (Fig. 6).

Discussion

In the current study, we examined the association between the length of telomeric repeats of the DNA and the actual biological age of each individual. It was found that there was a statistically significant difference in telomere length between abusers and the control group (healthy individuals). This led us to the conclusion that the actual biological age of the abusers was far greater than their chronological age, which possibly classifies these psychotropic substances (opiates and cannabinoids) into exogenous factors that lead to the premature biological aging of the organism.

More specifically, with regard to the statistical distribution of telomere length, the distribution of the abusers had the same pattern with that of the controls (Fig. 5), although a statistically significant reduction in the median value of the telomere length of the abusers was observed. In addition, we can evaluate the reduction in telomere length by the fact that it has been proven bibliographically that short telomeres play a major role in the reduction of telomere length. As the number of shorter telomeres increases the total telomere length decreases and this is related to cellular aging (17-22).

Furthermore, it is important to mention the association between telomere length and the years of drug abuse. As illustrated in Fig. 6, a specific, yet not significant trend was observed between telomere length and the years of both cannabis and opiates abuse. More specific, the mean TL of the drug users appeared to decrease as the year of abuse increased, and this result was more evident in the opiate abusers. Depending on previous findings, which demonstrated a downward trend as the years of opiate abuse increased (23), there is a clear need for further investigation of this issue. The non-association of the

### Table I. Demographic data of the participants in the study and the control group.

<table>
<thead>
<tr>
<th>Participant group</th>
<th>Control (n=70)</th>
<th>Drug abusers (n=16)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agea (years)</td>
<td>Mean 41.2</td>
<td>Mean 36.7</td>
<td>0.136</td>
</tr>
<tr>
<td>Age groupsb</td>
<td>n 10</td>
<td>n 3</td>
<td>0.287</td>
</tr>
<tr>
<td>20-30 years</td>
<td>76.9</td>
<td>23.1</td>
<td></td>
</tr>
<tr>
<td>30-40 years</td>
<td>72.7</td>
<td>27.3</td>
<td></td>
</tr>
<tr>
<td>40-50 years</td>
<td>89.7</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td>60-70 years</td>
<td>90.9</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Sex (male)</td>
<td>68.8</td>
<td>31.2</td>
<td></td>
</tr>
</tbody>
</table>

aData were analyzed using an independent samples t-test; bData were analyzed using Pearson's Chi-square test.

### Table II. Descriptive statistics of TL and TLS quartiles (expressed as bases) between the drug abusers and the control group.

<table>
<thead>
<tr>
<th>Quartile</th>
<th>Control (n=70)</th>
<th>Drug abusers (n=16)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL</td>
<td>Mean 8,656</td>
<td>Mean 5,631</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median</td>
<td>1,0469</td>
<td>6,901</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3rd</td>
<td>12,630</td>
<td>8,506</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TLS</td>
<td>Mean 6,270</td>
<td>Mean 3,818</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median</td>
<td>7,155</td>
<td>4,450</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3rd</td>
<td>7,750</td>
<td>4,961</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

TL, telomere length of the whole chromosomes; TLS, telomere length from the short telomeres (TL <20th percentile).
years of abuse and TL could possibly be affected by the limited number of participants.

Over the past few years, research on telomere length has intensified, with the majority of scientists considering telomere length in relation to various diseases, such as cancer, diabetes and various cardiovascular diseases (24-35). In addition, there are some studies which have associated various psychotropic substances, such as anxiolytics, antidepressants and hypnotics with TLS (36-47). These investigations, revealed in a more general manner, the association of telomere length with the above-mentioned factors, although they did not associate it with biological age specifically.

The findings of this study are in accordance with those of the study by Imam et al which provided an association between drug abuse and telomere length shortening (48). In the study by Yang et al, was shown that heroin and diazepam abusers displayed shorter long telomere lengths (LTLs) compared to those who used other drugs, probably due to the increase in oxidative stress. Furthermore, they discovered a significant negative correlation between the LTL and the length of time before relapse (23). In the same study, no significant difference in telomere length was depicted between drug abusers using intravenous injection and those using other methods (e.g., smoking) (23).

It has been shown that oxidative stress is one of the major factors that lead to the reduction in telomere length with increasing age. An increase in oxidative stress results in a decreased telomerase activity (49,50), a reduced cell

Figure 2. An indicative diagram of BIOTEL usage.

Figure 3. Prevalence of chronic abuse of cannabis, opiates, cocaine, benzodiazepines (Benzos) and amphetamines.

Figure 4. Duration of abuse for each reported drug. Benzos, benzodiazepines.
proliferation and increased apoptosis (18,20). This has been confirmed by the fact that when oxidative stress is suppressed by various factors, such as an increased concentration of vitamin C, the reduction in telomere length is limited (51). Thus, it is very likely that the reduction in telomere length in drug abusers occurs due to the high levels of oxidative stress generated by the psychotropic substances.

In conclusion, the present study demonstrates that there is an association between telomere length and drug abuse. Specifically, it was found that in the drug abusers, the decrease in telomere was more evident compared to the healthy controls, indicating that drug abuse does play a role in the shortening of telomeres. Although usually age is proportional to the years of chronic abuse, there is no clear evidence of telomere shortening with the duration of self-referred chronic abuse. However, further studies are warranted to confirm our findings, using larger samples sizes of drug abusers.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors’ contributions

EV, MT, DT, DAS and AT were involved in the design and conception of the study. EV, MT, DN, DT and K Kan were involved in blood sampling. EV, KT, PF, K Kal, GV and SP were performed the analysis of the blood samples following the Q-FISH protocol. EV, MT, KT, PF, DT and AA wrote the manuscript. EV and AA were involved in the statistical analysis of the results and to designing the figures and tables. EV, MT, KT, PF, DT, AA, DAS and AT were involved in the proofreading and editing if the manuscript. All authors have taken the responsibility for publishing this study and all authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

AT is affiliated with Toxplus S.A. and that this company also provided funding for this study. The information and views set out in this study are those of the authors and do not necessarily
reflect the official opinion of Toxplus S.A. Toxplus S.A. may
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the journal, but had no personal involvement in the reviewing
process, or any influence in terms of adjudicating on the final
decision, for this article. The other authors declare that there
are no conflicts of interest.

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