Mitochondrial DNA Poly-C length heteroplasmy as a marker for risk of critical COVID-19

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Abstract

Mitochondria play a central role in the innate and acquired response against viral infections. Common mtDNA variants have been associated with severe COVID-19 and mtDNA depletion. A poly C length variation has been associated with mtDNA instability and increased risk for several diseases. We studied 482 patients who required treatment in the intensive care unit and age matched population controls. The 16184-16193 poly-C and 514-523 CA-repeats were determined by fluorescent capillary electrophoresis and Sanger sequencing of PCR fragments. We found a significantly higher frequency of 16184-16193 mtDNA poly-C heteroplasmy in patients aged [?]60 compared to patients aged >60 years. Poly-C heteroplasmy did not differ between the age control groups. Poly-C heteroplasmy was associated with the presence of the 16223 T allele, that was associated with the risk of critical COVID-19 at [?]60 years. In Conclusion, heteroplasmy in the poly-C tract of the mtDNA control region might be a marker for critical COVID-19. The 16184-16193 heteroplasmy was linked to the 16223 T allele, that was significantly increased among patients aged [?]60 years. This finding requires validation in other cohorts and to determine the functional link between length variation in the mitochondrial DNA control sequence and risk of severe SARS-CoV-2 disease.

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Mitochondria play a central role in the innate and acquired response against viral infections. Common mtDNA variants have been associated with severe COVID-19 and mtDNA depletion. A poly C length variation has been associated with mtDNA instability and increased risk for several diseases. We studied 482 patients who required treatment in the intensive care unit and age matched population controls. The 16184-16193 poly-C and 514-523 CA-repeats were determined by fluorescent capillary electrophoresis and Sanger sequencing of PCR fragments. We found a significantly higher frequency of 16184-16193 mtDNA poly-C heteroplasmy in patients aged [?]60 compared to patients aged >60 years. Poly-C heteroplasmy did not differ between the age control groups. Poly-C heteroplasmy was associated with the presence of the 16223 T allele, that was associated with the risk of critical COVID-19 at [?]60 years.

In Conclusion, heteroplasmy in the poly-C tract of the mtDNA control region might be a marker for critical COVID-19. The 16184-16193 heteroplasmy was linked to the 16223 T allele, that was significantly increased among patients aged [?]60 years. This finding requires validation in other cohorts and to determine the functional link between length variation in the mitochondrial DNA control sequence and risk of severe SARS-CoV-2 disease.

Key words: COVID-19; mitochondria; haplogroups; heteroplasmy; ageing

1. Introduction

Mitochondria contain their own genome, a circular double-strand DNA molecule (mtDNA) of 16,569-bp that encodes for proteins of the mitochondrial respiratory chain and mitochondrial tRNAs and rRNAs. Because mtDNA is inherited from the mother, germ-line mutations in the mtDNA are associated with rare maternal inherited diseases. The mtDNA is prone to acquire nucleotide changes that accumulate with age or under exposure to environmental toxics [**Lee** et al., 1998;**Wallace** 2010; **Bratic and Larsson** , 2013; **Ziada**et al., 2019].

In addition to rare pathogenic variants the mtDNA contains many common variants that originated in individuals from particular populations and spread with worldwide migrations. Specific combinations of these variants classify the mitochondrial haplogroups, with frequencies that are characteristic of each human population [**Wilson** and Allard, 2004; **Torroni et al., 2006; Brotherton et al., 2013]** . For instance, haplogroup H is defined by 7028C (among other nucleotide changes) and is the most common among Europeans while is absent among individuals of African or East Asian ascent. These mtDNA haplogroups are transmitted from mother to offspring and their worlwide distribution permitted to trace the migration of humans outside Africa, raising the concept of a *mitochondrial Eve* [**Pakendorf andStoneking** , 2005].

Mitochondrial DNA variants/haplogroups have been associated with differences in physiological processes such as energy production, ageing, regulation of apoptosis or pathogen immune-mediated responses [**Gomez-Duran** et al., 2010; **Chen et al., 2012;Kenney et al., 2014; Krzywanski et al., 2016; Friedrich** et al., 2022]. As a consequence, these haplogroups have been associated with adaptation to exercise or susceptibility to develop several traits such as diabetes, cardiovascular disease, or infectious diseases [Castro et al., 2007; **Yonova-Doing** et al., 2022]. In reference to infections these variants might be associated with the risk of sepsis or the severity of HIV and herpex disease, among others [**Hendrickson et al., 2008; Yang et al., 2008; Hart et al., 2013; Levinson et al, 2016**]. Haplogroups might also play a role in the risk for severe COVID-19, the disease caused by SARS-CoV-2 [**Wu** et al., 2021; **Dirican et al., 2022; Vazquez-Coto et al., 2022; Kumari** et al. 2023].

Each cell has a variable number of mitochondria and each mitochondria contains several copies of the mtDNA. For a particular nucleotide position, the mitochondria from each individual may exhibit the same variant (homoplasmy) or different alleles (heteroplasmy) [**Santos** et al., 2008; **Li** et al., 2010;**Klutsch** et al., 2011]. Heteroplasmy is commonly inherited from the mother and for disease-related variants the degree of heteroplasmy in the different tissues determines the extent of the symptoms. Rare highly penetrant

mutations cause monogenic disorders that often affect the nervous system, muscles, heart, and endocrine organs, and many healthy individuals carry low levels of heteroplasmy (<1% of mtDNA with the mutation) either inherited from the mother or acquired. An increased burden of heteroplasmy contributes to increased risk for diseases such as MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes), diabetes mellitus, and others [**Avital** et al., 2012; **Folmes** et al., 2013; **Chae** et al., 2020].

Heteroplasmy is common in poly-cytosine tracts in the mtDNA control region [**Bendall** et al., 1995; **Lagerstrom-Fermer** et al., 2001; **Shin** et al., 2006; **Zhao** et al., 2010;**Mueller** et al., 2011; **Shen** et al., 2015]. One of these is located between nucleotides 16184-16193, that contains the origin for replication of the mtDNA heavy (H) chain. These poly-C tracts are prone to length instability that would increase the risk of mtDNA loss and impairment of mitochondrial regulated processes [**Chiaratti** et al., 2022]. Among others, the T16189C mtDNA polymorphism increases the risk for poly-C instability and has been associated with diabetes, cancer, and coronary artery disease (CAD), among other diseases [**Zhao** et al., 2010; **Mueller** et al., 2011; **Shen** et al., 2015]. Length heteroplasmy might be at low level in resting while increases in cells subjected to extensive division, such as the immune cells under chronic inflammation. Increased heteroplasmy might thus represent a marker of a deleterious immune-response that would increase the risk of developing severe infectious diseases [**Stefano** et al., 2022; **Ren** et al., 2020; **Elesela** et al., 2021; **Li** et al., 2021].

Due to the overactivation of the immune system among individuals infected by SARS-CoV-2 we hypothesised that blood leukocytes from patients with severe COVID-19 might exhibit a different profile of poly-C heteroplasmy. To address this issue we characterised the mtDNA region containing the 16184-16193 poly-C tract in patients with critical COVID-19 and age-matched population controls.

2. Patients and Methods.

Study participants. We obtained the demographic and clinical data of 482 COVID-19 patients (age range 24-95 years) who required admission in the intensive care unit (ICU). The study was approved by the Ethics Committee of Principado de Asturias (Oviedo, Spain; approval id project ISCIII-PI21/00971), and all the patients (or their next of kin) gave informed consent to participate in the study. These patients were hospitalised between March-2020 and December-2021, a period with four SARS-CoV-2 pandemic waves in our community. We did not determine the SARS-CoV-2 variant in all the patients, but the study period was characterised by the Wuhan (pandemic waves 1-2), alpha (wave 3) and delta (wave 4) variants.

The inclusion criteria was a severe pneumonia in need for ICU admission with SARS-CoV-2 confirmed by PCR test, and exclusion criteria were age <18, respiratory failure due to condition other than COVID-19, or refusal to participate. These patients were followed till disease remission with hospital discharge or death. Body-mass index (BMI) and preexisting cardiovascular comorbidities (hypertension, diabetes, hypercholesterolemia) were obtained from the clinical history at ICU admission. Based on previous reports we compared COVID-19 patients aged [?]60 and >60 years [**Nakanishi** et al., 2021]. At the time of inclusion, none of the patients had a recorded diagnostic of mitochondrial disease.

All the participants were of European ancestry from the region of Asturias (Northern Spain, total population 1 million). The controls (N=363) were individuals from the general population recruited with the main purpose of defining the DNA variant frequencies. These controls were not serologicaly studied (presence of anti-SARS-CoV-2 antibodies) to exclude previous asymptomatic infection. In order to exclude the posibility of age-bias in the genotype frequencies we compared patients and controls within the same age-range.

Genetic analysis. Patients and controls were studied for mtDNA single nucleotide polymorphisms (SNPs) C7028T and C16223T by Sanger sequencing of PCR fragments. The 7028 polymorphism differentiates the major European haplogroup H (7028C) from no-H (7028T), while 16223 differentiates the ancestral African macro-haplogroup N (16223T) from the out-of-Africa macro-haplogroup R (16223C) (*www.mitomap.org)* . This method allowed to determine the presence of heteroplasmy at the 7028 and 16223 nucleotides.

To define the degree of length heteroplasmy at the 16184-16193 poly C tract we per-

formed a fluorescent capillary electrophoresis of PCR fragments generated with primers FAM-5'CTGCCAGCCACCATGAATATTGTACGG and 5'GTGGCTTTG GAGTTGCAGTTGATGTGTGA (annealing at 65oC). The forward primer was labelled with FAM and fragments were thus visualised as fluorescent peaks after capillary electrophoresis (**Figure 1A**). Homoplasmic fragments corresponded to single peaks while the presence of additional peaks was considered as heteroplasmy. We also characterised the degree of heteroplasmy at the CA-repeat between nucleotides 514-523 by fluorescent capillary electrophoresis of PCR fragments with primers fam-5' CACTTTTAACAGTCACCCCCCAACTAAC and 5'TTCGGGGTATGGGGTTA GCAGCG (annealing at 65oC) (**Figure 1B**). To discrimitate true from PCR stutter peaks (characteristic of amplicons containing nucleotide repeats) we condidered heteroplasmy when the additional peaks had a heigh >10% relative to the main fluorescent peak **[Walsh et al., 2016]** . The accuracy of this method was validated by sequencing PCR fragments with different degrees of heteroplasmy (**suppl figure**).

Statistical analysis. All the values were collected in an excell file. The statistical analysis was performed with the R-software (*www.r-project.org*). Logistic regression (linear generalized model, LGM) was used to compare mean values and frequencies between the groups.

3. Results.

In **table 1** we show the main characteristics of the critical COVID-19 patients aged [?]60 years or older. We found a higher frequency of male in the two age groups. As expected the frequency of hypertensives, diabetics, and hyperlipaemics was higher among the patients >60 years. There were a total of 103 deaths (**table 1**). The frequency of mtDNA 7028C that characterises haplogroup H was significantly lower in the [?]60 years patients (38% vs 48%). The frequency of 16223T was significantly higher in the [?]60 years group (18% vs 10%). The 7028C and 16223T did not differ between the two age control groups (**table 2**). Thus, we concluded that the difference between pagtients aged [?]60 and >60 years would not be a consequence of a survival-effect in the general population, and these mtDNA markers could thus represent risk factors for age-dependent critical COVID-19.

Control region length heteroplasmy. Fluorescent capillary electrophoresis of the amplified fragments showed multiple peaks (heteroplasmy) at the 16184-16193 poly-C tract significantly more frequent among the [?]60 years patients compared to age matched controls (19% vs 9%; p=0.002). Heteroplasmy was also more common among the [?]60 years compared to the older patients (19% vs 10%; p=0.02), without differences between patients and controls >60 years (**table 2**). We found a maximum frequency of length heteroplasmy among patients aged <50 years, suggesting that this mitochondrial genomic event was significantly associated with critical COVID-19 at younger age (**Figure 2; suppl. Table 1**).

The 16184-16193 poly-C tract that increases length heteroplasmy has been associated with several multifactorial disorders, including metabolic and cardiovascular. Because critical COVID-19 was significantly associated with hypertension, diabetes, dyslipidaemia, and male sex, we determined whether length heteroplasmy was higher among patients with these conditions. We did not find statistical differences between the groups (**suppl. table 2**). We also compared death and survivors, without significant difference. Moreover, after correcting by multiple variables advanced age was the only significantly associated with the risk of death (p<0.001).

We also determined the frequencies of the control region CAn-repeat. We did not find significant differences between patients and controls in the two age-groups. Heteroplasmy at this region was found in 3-6% of the study cohorts, without significant difference between the groups.

Poly-C heteroplasmy was linked to 16223 T. We found a higher frequency of poly-C heteroplasmy among patients 16223 T compared to 16223 C, in the two age groups (**suppl. table 3**). The 16223 T is the ancestral African allele and is present in less than 10% of current Europeans with the rare IXW haplogroups (**suppl. table 4**). This pointed to the possibility that the higher frequency of 16189 T and 162184-16193 poly-C heteroplasmy were a consequence of the linkage between 16223 T and 16189 C, a variant that increases the risk of poly-C instability (**suppl. table 5**).

4. Discussion.

The main finding of our study was a significantly increased frequency of length heteroplasmy at the poly-C tract in the mtDNA region that contains the replication start site of the H strand. This sequence, between nucleotides 16184-16193, contains a poly-C tract interrupted by C>T changes, and the presence of C could results in replication instability with multiple poly-C sequences.

The poly-C heteroplasmy might be inherited from the mother with cellular heterogeneity between different tissues in the same individual. In addition, the degree of heteroplasmy might increase with age or under exposure to environmental toxics, drugs, bacterial and viral infections, among others. An increased length heteroplasmy at this poly-C region has been reported among cancer, diabetes and coronary artery disease patients [**Zhao** et al., 2010; **Mueller** et al., 2011;**Shen** et al., 2015]. This associations might be explained by a mechanism that links the poly-C tract with an impaired replication of the mtDNA that could result in a reduction of the number of mtDNA copies and the loss of mitochondrial functions. In a study with blood samples from 837 healthy adults **Liou** et al. found that the number of mtDNA copies was significantly reduced among individuals with uninterrupted poly-C [**Liou** et al, 2010]. Thus, variants that increased the amount of length heteroplasmy might cause alteration of mtDNA copy number in human blood cells compared to T-interrupted tracts [**Amo** et al., 2017]. Interestingly, length heteroplasmy at the control region was also associated with significantly lower copy number of mtDNA in leukocytes from breast cancer patients [**Zhao et al., 2010**].

The number of mitochondria and mtDNA copies vary between cells and tissues and might be critical for a proper physiological response to situations that require a high energy demand and other mitochondrial-mediated responses [**Stefano** et al., 2022;**He** et al., 2010; **Stefano** et al., 2017]. A high degree of heteroplasmy might impairs the maintenance of ATP levels in response to strong physiological demand, such as the required by the immunological cells after infections [**Koshiba** 2013;**Moore and Ting** , 2008; **Schilf** et al., 2021;**Shenoy** 2020]. The effect of heteroplasmy in mtDNA copy number and mitochondrial function has been validated in mouse strains with different mtDNA variants [**Hu et al., 2019**]. These engineered mice showed a non-random segregation of mtDNA copy numbers that suggested a pressure to reduce the degree of heteroplasmy when this was detrimental for covering the cellular demands. Situations that increase the degree of heteroplasmy, such as aging, exposure to environmental compounds, infections, etc, might result in an impaired capacity to respond through mitochondrial pathways, increasing the risk for disease.

Our study was based on whole blood cells and would thus be representative of the degree of heteroplasmy among leukocytes in a disease (COVID-19) characterised by an exacerbated inflammatory response with over-activation of immune-cells to respond to the infection. These cells are thus under strong metabolic demand that would require an increase in the number of mitochondria and mtDNA copies. It is tempting to speculate that a similar dynamic of mtDNA length heteroplasmy occurs in other cells and tissues such as lung, brain, heart. This possibility might be investigated in the context of persistent (long-COVID) symptoms.

The role of mitochondria in the pathophysiology of acute and chronic inflammation has been extensively studied. Immune-competent cells such as monocytes, macrophages, antigen-presenting cells, are under programmed changes in mitochondrial bioenergetics in response to innate and adaptive immunological processes [**Angajala** et al., 2018;**Zuo** et al., 2019]. The degree of mtDNA length heteroplasmy might contribute to define the extent of these immune-mediated responses, making some individuals more susceptible to an impaired capacity to fight infection or to regulate the extent of the proinflammatory stimuli [**Lechuga-Vieco** et al., 2020;**Pollara** et al., 2018; **Zhu** et al., 2018]. Several studies have shown the capacity of viruses, including SARS-CoV-2, to hijack oxidative phosphorylation, ATP production, and other mitochondrial functions [**Stefano** et al., 2022;**Stefano** et al., 2020]. Key mitochondrial mediated processes might be also affected by viral proteins that reduced the antiviral innate immune responses, thus promoting the extent of infection and disease severity [**Chen** et al, 2007; **Yoshizumi** et al., 2014; **Choi** et al., 2018]. Some authors have suggested that SARS-CoV-2 infection and replication was improved by the takeover of mitochondrial processes, and viral proteins would suppress mitochondrial functions reducing the innate and adaptive immune responses

[**Singh** et al., 2020]. In this context, heteroplasmy at the poly-C tract might reduce the individual's capacity to exhibit a proper mitochondria mediated immune response increasing the risk for severe COVID-19.

A functional link between 16189C and increased risk for disease has been suggested by some authors. Park et al. identified 16189C as a risk factor for diabetes and used chromatin immunoprecipitation in cybrid cells to identify the mitochondrial single-stranded DNA-binding protein (mtSSB) as a candidate protein bound to the 16189 region [**Park** et al., 2008]. MtSSB has a lower binding affinity for the 16189C variant and because this nucleotide lies in the control region of mtDNA replication and transcription the variant might affect mtDNA replication and recovery after mitochondrial damage [**Takamatsu** et al., 2002; **Park** et al., 2008]. In this context, several studies have reported increased circulating mtDNA as a marker of COVID-19 severity and mortality [**Scozzi** et al., 2021; **Valdes-Aguayo** et al., 2022; **Streng** et al., 2022]. Studies to determine whether mtDNA variants and leght heteroplasmy were associated with reduced number of mtDNA copies and increased circulating mtDNA would be of special interest.

A limitation of our study was the absence of over-time comparison of the degree of poly-C heteroplasmy within the same individual. Heteroplasmy is commonly inherited and could thus be present in all the individual's cells, but it is well known that de degree of heteroplasmy might increase with age or in response to environmental exposures. It is possible that SARS-CoV-2 proteins that target the mitochondria enhance the miss-replication of mtDNA promoting impairment of innate and acquired immunity and increasing the risk for critical COVID-19. To verify this hypothesis it is necessary to determine the degree of heteroplasmy before and after infection.

Finally, the risk of developing severe COVID-19 has been associated with some mitochondrial haplogroups [**Wu** et al., 2021;**Vazquez-Coto** et al., 2022]. We previously reported a decreased frequency of the common European haplogroup H (7028 C) that could be protective for critical disease at younger age. In addition, 16223 T might be associated with an increased risk of severe disease. Interestingly, 16189 C is more common among individuals with haplogroups with 16223 T (such as the European X) than among haplogroup H (characterised by 16223 C) (**supplementary table**) [**Laricchia** et al., 2022]. Thus, the association of haplogroups with several diseases might be explained by its linkage to 16189 C and other variants that could increase the risk for poly-C length heteroplasmy.

In conclusion, we report an increased frequency of poly-C length heteroplasmy at the mtDNA control region that contains the replication start site of the H-strand among patients with critical COVID-19. Length heteroplasmy at this region has been associated with a higher risk of cancer, diabetes, coronary artery disease, and viral diseases, among others. The poly-C heteroplasmy could increase mtDNA instability and reduction of the mtDNA copies per cell, that might contribute to manifest an exacerbated inflammatory response and impaired activation of the innate and acquired immunological response to viral infection. Further research to confirm the association are necessary, as well as functional studies to uncover the linkage between mtDNA length heteroplasmy and viral disease.

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Competing interests. None of the authors have competing interests related to this work.

AUTHOR CONTRIBUTIONS . **Lead researchers** : EC, GMA, JG;**study design** : EC, GMA, JG; **patient assessment and data acquisition** : GMA, LAR, MGC, ECLL, JG; **database:** DVC, EC, GMA, JG; **genotyping:** DVC, CGL, MGF, LVC, EC; **data filtering and analysis:** EC, DVC; **statistical analysis:** EC, DVC;**analysis of results:** EC, DVC; **writing the manuscript:**EC; **revision of manuscript:** all the authors.

ETHICS AND CONSENT. This study was approved by the clinical research ethics committee of Hospital Universitario Central Asturias (HUCA) (project approval id ISCIII-PI21/00971). All the participants or they next of kin gave written or verbal consent. Data were handled in observance of Spanish legislation on data

protection. The study complies with the principles of the Declaration of Helsinki ("Recommendations guiding doctors in biomedical research involving human subjects").

DATA AVAILABILITY STATEMENT . The data that support the findings of this study are available from the corresponding author upon reasonable request. An Excel file with the raw data would be available for meta-analysis research.

Table 1. Main values in the critical COVID-19 patients aged [?]60 years and older.

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	[?]60 years N=164	**>60 years N=318**	p-value
Male Female	118 (72%) 46 (28%)	235 (73%) 83 (27%)	0.85
Age Median years (range)	53 (18-60)	71 (61-92)	
BMI median (range)	29(19-54)	31(19-55)	0.01
BMI >30	69 (42%)	151 (47%)	0.01
Diabetes	21 (13%)	76 (24%)	0.005
Hypercholesterolemia	54 (33%)	162 (51%)	0.0004
Hypertension	66 (40%)	203 (64%)	<0.0001
Death	17 (10%)	86 (28%)	<0.0001

Table 2. Frequency of the mtDNA variants in the critical COVID-19 patients and population controls. All them were genotyped for the 7028 variant (C=haplogroup H) and the 16223 variant (T=haplogroup N; C=haplogroup R).

	[?]60 years	**[?]60 years**	**>60 years**	**>60 years**
	Covid-19 N=164	**Controls N=182**	**Covid-19 N=318**	**Controls N=181**
7028 C (H)	62 (38%)	86 (47%)	153 (48%)	82 (45%)
7028 T (no-H)	102 (62%)	96 (53%)	165 (52%)	99 (55%)
p-value (T) OR (95%CI)	p=0.11 OR=1.43 (0.92-2.22)	p=0.11 OR=1.43 (0.92-2.22)	p=0.19 OR=0.75 (0.49-1-15)	p=0.19 OR=0.75 (0.49-1-15)
16223 T (N)	27 (16%)	9 (5%)	31 (10%)	9 (5%)
16223 C (R)	133 (81%)	170 (93%)	283 (89%)	169 (93%)
16223 T/C Heteroplasmy	4 (3%)	3 (2%)	4 (1%)	3 (2%)
p-value (T) OR (95%CI)	p=0.002 OR=3.36 (1.49-7.54)	p=0.002 OR=3.36 (1.49-7.54)	p=0.39 OR=1.47 (0.60-3.59)	p=0.39 OR=1.47 (0.60-3.59)
514-523 CAn	21 (13%)	20 (11%)	32 (10%)	21 (12%)
CA_4				
CA_5	128 (79%)	140 (77%)	258 (81%)	145 (80%)
CA_6	6 (3%)	9 (5%)	12 (4%)	7 (4%)
CA_7	0	2 (1%)	4(1%)	4 (2%)
$CA_{heteroplasmy}$	9 (5%)	11 (6%)	12 (4%)	4 (3%)
16184-16193 Heteroplasmy	32 (19%)	16 (9%)	31 (10%)	24 (13%)
Homoplasmy	132 (71%) p=0.002 OR=2.67 (1.40-5.06)	176 (91%) p=0.002 OR=2.67 (1.40-5.06)	287 (90%) p=0.67 OR=0.89 (0.51-1.53)	157 (87%) p=0.67 OR=0.89 (0.51-1.53)

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Figure 1. Capillary electrophoresis of the 16184-16193 poly-C tract (A) and the 514 CAn repeat (B). Heteroplasmy (A2, A3, A4, B3): in addition to the main alleles all the fragments showed an additional peak known as DNA polymerase slippage product or stutter band. This peak would have a height <10% relative to the main peak (**85**). To confirm the accuracy of the capillary electrophoresis we confirmed the homoplasmy/heteroplasmy of PCR fragments by Sanger sequencing (see supplementary figures).

Figure 2. Frequencies of the poly-C lenght heteroplasmy and 16223 T patients in four age groups. Frequency of poly-C heteroplasmy and 16223 T were higher in the youngest (<50 years) group.