# TITLE PAGE

## Decreased telomere length in children with cartilage-hair hypoplasia.

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## ABSTRACT

**Background**: Cartilage-hair hypoplasia (CHH) is an autosomal recessive chondrodysplasia caused by *RMRP* (RNA component of mitochondrial RNA processing endoribonuclease) gene mutations. Manifestations include short stature, variable immunodeficiency, anemia and increased risk of malignancies, all of which have been described also in telomere biology disorders. *RMRP* interacts with the telomerase reverse transcriptase (TERT) subunit, but the influence of *RMRP* mutations on telomere length is unknown. We measured relative telomere length (RTL) in patients with CHH, their first-degree relatives and healthy controls, and correlated RTL with clinical and laboratory features.

**Methods**: The study cohort included 48 CHH patients with homozygous (n=36) or compound heterozygous *RMRP* mutations (median age 38.2 years, range 6.0-70.8 years), 86 relatives (74 with a heterozygous *RMRP* mutation) and 94 unrelated healthy controls. We extracted DNA from peripheral blood, sequenced the *RMRP* gene and measured RTL by quantitative-PCR.

**Results**: Compared with age- and sex-matched healthy controls, median RTL was significantly shorter in CHH patients (n=40 pairs, 1.05 *vs* 1.21, p=0.017), but not in mutation carriers (n=48 pairs, 1.16 *vs* 1.10, p=0.224). RTL correlated significantly with age in *RMRP* mutation carriers (rho -0.482, p<0.001) and non-carriers (rho -0.498, p<0.001), but not in patients (rho -0.236, p=0.107). Especially children (<18 years) with CHH had shorter telomeres than controls (median RTL 1.12 *vs* 1.26, p=0.008). In patients with CHH, RTL showed no correlation with genotype, clinical or laboratory characteristics.

**Conclusions**: Telomere length was decreased in children with CHH. We found no correlation between RTL and clinical or laboratory parameters.



## KEY WORDS

Bone marrow failure, relative telomere length, *RMRP*, telomerase, telomere biology disorders.

## INTRODUCTION

Telomeres constitute the protective end-parts of human chromosomes and can contribute to the pathogenesis of aging and cancer. The phenotype of inherited telomere disorders includes bone marrow failure, malignancies, pulmonary fibrosis, liver cirrhosis, diabetes, and cardiovascular and gastrointestinal diseases [1]. Telomeres shorten with every cell division, but the loss can be compensated by telomerase-mediated elongation. The telomerase ribonucleoprotein complex consists of an RNA template, a catalytic reverse transcriptase subunit (TERT) and associated proteins that affect assembly, stability and recruitment of telomerase to telomeres (e.g. DKC, NOP10, NHP2, and GAR). In addition to telomere elongation, telomerase has also been associated with regulation of a number of cellular functions including survival, inflammation, apoptosis, transcription and metabolism [2].

Cartilage-hair hypoplasia (CHH, MIM #250250) is a rare autosomal recessive metaphyseal chondrodysplasia caused by biallelic mutations in the *RMRP* (RNA component of mitochondrial RNA processing endoribonuclease) gene [3]. The disease is over-represented in the Amish and Finnish populations [4,5]. Clinical features include severe disproportionate short stature, hair hypoplasia, variable immunodeficiency, anemia and increased risk of malignancies. Genotype-phenotype correlations are inconsistent and clinical manifestations vary even between siblings [6]. Mutation carriers remain asymptomatic [5,7].

Complex pathogenesis of CHH involves cell cycle impairment and altered regulation of genes associated with cell proliferation and differentiation [8,9]. In addition, formation of a ribonucleoprotein complex by *RMRP* and TERT has been confirmed [10]. This complex produces double-stranded RNAs and regulates *RMRP* expression.

Disorders of telomere maintenance, like dyskeratosis congenita (DC), share some clinical features with CHH, e.g. growth retardation, bone marrow failure leading to anemia and immunodeficiency, and increased incidence of malignancies [11]. Pulmonary fibrosis has also been linked to *TERT* mutations, both in idiopathic cases and in patients with DC [12]. Interestingly, we have recently reported fibrosis-like changes on high-resolution computed tomography of the lungs in patients with CHH [13].

Despite the known association between *RMRP* and TERT, it remains unknown whether *RMRP*

77 mutations have a significant impact on the telomere elongating functions of telomerase. In order to further
78 elucidate the pleiotropic consequences of *RMRP* mutations and the pathogenesis of CHH we evaluated
79 relative telomere lengths (RTL) in a large cohort of Finnish children and adults with CHH, their first-degree
80 relatives most of whom were heterozygous mutation carriers, and healthy controls. We also analyzed the
81 correlation of RTL with the patients' clinical and laboratory features.
82

## METHODS

Patients with genetically confirmed CHH were identified from the Finnish Chondrodysplasia Register and recruited to a study exploring clinical, genetic and immunological characteristics of CHH. Their first-degree relatives were contacted via the index persons. All individuals who agreed to participate, or their guardians, signed an informed consent. The study was approved by the Research Ethics Committee at Helsinki University Hospital, Finland.

Clinical and laboratory data were retrieved retrospectively from the patient hospital records, as well as prospectively during study visits. Apart from CHH-related clinical features we evaluated also factors that could theoretically influence telomere length, such as smoking, obesity (body mass index Z-score) and hormone or immunosuppressive therapy. We also selected clinical features that could emerge from telomere impairment (immunodeficiency, malignancies, short stature), as well as the need for repeated blood transfusions and immunoglobulin replacement therapy. Since growth failure in CHH is progressive, we used age- and sex-specific growth data for CHH [14] to classify patients as having mild, moderate or severe growth failure, as described previously [15]. In the majority of patients, blood samples were drawn simultaneously for telomere measurement and for analysis of immunologic parameters characterizing the degree of bone marrow deficiency and/or immunodeficiency: hemoglobin, red blood cells, leukocytes, neutrophils, lymphocytes, CD3+, CD4+, CD8+, CD19+, CD16/56+ cell counts, immunoglobulin A, M and G levels, as well as Epstein-Barr virus (EBV) viral load and antibodies.

**Control group**. First-degree relatives of the patients (n=86), all without features of CHH, included 37 parents, 38 siblings and 11 children. Altogether, 74 of them were confirmed to be heterozygous *RMRP* mutation carriers while 12 were negative for *RMRP* mutations. The control group consisted of *RMRP* mutation-negative individuals: 1) siblings of the patients (n=12) and 2) individuals who had participated in our previous studies involving healthy children and adults (n=94). The data available on controls included age, sex, ethnic background (all of Finnish origin) and overall health (all healthy). Parts of the statistical analyses were performed in a case-control setting, and age- and sex-matched controls were selected from the control group for each patient or mutation carrier aiming at age difference of no more than 12 months.

**DNA extraction and RMRP sequencing**. Peripheral blood samples were collected for all study participants. DNA was extracted with 5 Prime Archive Pure DNA Blood kit according manufacturer's

instructions (5 Prime GmbH, Hilden; Germany). All samples were sequenced for *RMRP* to confirm the genotype in patients with CHH and to confirm or exclude heterozygous mutations in the patients' unaffected relatives and the healthy controls. For patients and their relatives, blood samples were collected prospectively, whereas readily available DNA (extracted with the same methods) was used for healthy controls. Primers for *RMRP* (GRCh37/hg19) were designed with Primer3 v.0.4.0 (http://frodo.wi.mit.edu/primer3/) for the gene, with a minimum of 60 bases of flanking regions adjacent to the coding region. PCR amplification was performed with DreamTaq (ThermoScientific, Waltham, MA, USA). The DNA fragments were then visualized with Midon Green Advanced DNA Stain (NIPPON Genetics, GmbH, Europe) on a 1.2% agarose gel, purified with ExoSAP (USB, Cleveland, OH, USA) and labeled with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). After bidirectional sequencing with an ABI3730 sequencer (Applied Biosystems), chromatograms were analyzed with Sequencher v5.0 (Gene Codes Corporation, Ann Arbor, MI, USA) using genomic NG_017041.1 and RNA reference sequence NR_003051.3. Primer sequences and detailed PCR protocols are available upon request.

**Telomere length measurement**. Telomere length was determined by the quantitative-PCR method described by Cawthon [16], with minor modifications [17]. Briefly, sample DNA was analyzed in triplicate wells in a separate Telomere (TEL) and a single copy gene (HBG) reaction on the ABI7900HT instrument (Applied Biosystems), at two separate times. TEL/HBG (T/S) values were calculated by the $2^{-\Delta Ct}$ method, where $\Delta Ct$ = average CtTEL-average CtHBG. RTL were generated by dividing samples T/S value with the T/S value of a reference CCRF-CEM cell line DNA included in all runs.

**Statistical analysis**. Subject's age on the day of blood sampling was used for the analyses. To evaluate RTL correlations, we classified individuals to categories of short, average or long RTL for age and used both, RTL itself and RTL category, in the analysis. These categories were determined based on RTL and age data from the healthy controls. Individuals with RTL > 0.5 standard deviation (SD) from the regression line for age *vs* RTL were classified as having long RTL, those with RTL < -0.5 SD as having short RTL, and the remaining as having average RTL (*Supplementary Figure 1*). We applied the Mann-Whitney test for categorical and the Spearman's coefficient (rho) for continuous variables. Linear or logistic regression analysis was used for multivariate models. A p value <0.05 was considered significant. Statistical analyses were performed with the IBM SPSS software.

## RESULTS

**Patient characteristics**. Altogether, 48 Finnish patients (31 females, 17 males) with CHH participated in the study. Their median age was 38.2 years (range 6.0-70.8 years). Sanger sequencing of the *RMRP* gene showed that most of the patients (75%, 36/48) were homozygous for the g.70A>G mutation (rs199476103, now referred to as g.71A>G) while 12 patients (25%) were compound heterozygous for g.70A>G and either g.262G>T mutation (rs727502774, now referred to as g.263G>T) (n=11) or a 10-nucleotide duplication at position -13 (TACTCTGTGA, rs727502775) (n=1).

*Tables 1 and 2* present patients' clinical and laboratory data. Nine patients had been diagnosed with malignancies, including basal cell carcinoma (n=6), B-cell lymphoma (n=2), uterus carcinoma (n=1) and vocal cord carcinoma (n=1). In the two patients who had survived lymphoma, no data were available on EBV status in lymphoma samples. No EBV-associated diseases were reported in our patients. Blood EBV viral load was undetectable by PCR in 10 individuals and 34/41 (83%) patients tested positive for serum antibodies to EBV. Two patients had required repeated red blood cell transfusions for anemia and another four patients had been treated with immunoglobulin replacement therapy. Some individuals reported history of smoking (n=9), intake of inhaled corticosteroids for physician-diagnosed asthma (n=15) or growth hormone treatment in childhood (n=3).

*Table 1.* Clinical features of 47 study subjects with cartilage-hair hypoplasia.

| **Clinical feature** | | **Number of patients** | **Proportion of patients** |
|---|---|---|---|
| Growth deficiency | | | |
| | Severe | 3/47 | 6% |
| | Moderate | 17/47 | 36% |
| | Mild | 27/47 | 58% |
| History of | | | |
| | Pneumonia | 10/47 | 19% |
| | Rhinosinusitis | 27/47 | 57% |
| | Otitis media | 34/47 | 72% |
| | Warts | 15/47 | 32% |
| | Hospitalization for varicella | 5/34 | 15% |
| | Smoking | 9/47 | 19% |
| Normal susceptibility to infections* | | 15/47 | 32% |
| Combined immunodeficiency** | | 25/47 | 53% |
| History of | | | |
| | Malignancies | 9/47 | 19% |
| | Repeated blood transfusions | 2/47 | 4% |
| | Immunoglobulin therapy | 4/47 | 8% |
| | Immunosuppressive therapy | 2/47 | 4% |
| | Growth hormone treatment | 3/47 | 6% |
| | Therapy with inhaled glucocorticoids | 15/47 | 32% |
| Bronchiectasis | | 8/30 | 27% |
| Fibrosis-like lung changes | | 5/30 | 17% |

* Normal susceptibility to infections was defined as occasional uncomplicated RTI or otitis media/rhinosinusitis not requiring surgical interventions, absence of pneumonias and sepsis and varicella not requiring hospitalization.
** Patients with warts, recurrent HSV infections, varicella requiring hospitalization or malignancy were considered to have combined immunodeficiency.

*Table 2.* Laboratory characteristics of study subjects with cartilage-hair hypoplasia. Cell counts are shown in cells $x10^9$/l with the exception of red blood cells ($x10^{12}$/l). Hemoglobin and immunoglobulin levels are in g/l. Normal values in adults represent local laboratory reference values.

| **Laboratory parameter** | **Normal values in adults** | **Patients tested (n)** | **Results, median (range)** | **Patients with decreased counts/levels, n (%)** |
|---|---|---|---|---|
| Red blood cells | F 3.90-5.20, M 4.25-5.70 | 47 | 4.32 (2.69-5.34) | 5 (11%) |
| Hemoglobin | F 117-155, M 134-167 | 47 | 136 (102-161) | 3 (6%) |
| White blood cells | 3.4-8.2 | 47 | 6.0 (1.2-12.0) | 4 (9%) |
| Absolute neutrophil count | 1.5-6.7 | 47 | 1.76 (0.28-8.40) | 3 (6%) |
| Absolute lymphocyte count | 1.3-3.6 | 47 | 1.31 (0.26-3.25) | 26 (55%) |
| CD3+ cells | 0.85-2.28 | 46 | 0.93 (0.16-3.01) | 19 (41%) |
| CD4+ cells | 0.458-1.406 | 46 | 0.548 (0.118-1.312) | 17 (37%) |
| CD8+ cells | 0.24-0.98 | 46 | 0.30 (0.04-1.72) | 19 (41%) |
| CD19+ cells | 0.12-0.43 | 46 | 0.12 (0.00-0.34) | 30 (65%) |
| CD16/56+ cells | 0.08-0.57 | 46 | 0.18 (0.05-0.55) | 3 (7%) |
| Immunoglobulin A | 0.52-4.84 | 45 | 1.9 (0.4-7.5) | 4 (9%) |
| Immunoglobulin M | 0.36-2.84 | 46 | 0.9 (0.2-3.1) | 4 (9%) |
| Immunoglobulin G | 6.8-15.0 | 44 | 10.7 (4.2-15.7) | 1 (2%) |

F females, M males, n number

**Telomere length**. RTL measurement was performed by quantitative PCR on altogether 228 samples from patients (n=48), first-degree relatives (n=86) and healthy unrelated controls (n=94). Sanger sequencing detected 74 carriers for *RMRP* mutations among the CHH patients' unaffected relatives (86%). *Table 3* demonstrates the characteristics of the participants according to the *RMRP* mutation status. There was a significant negative correlation between RTL and age in mutation carriers (rho -0.482, $p<0.001$) and non-carriers (rho -0.498, $p<0.001$), but not in patients (rho -0.236, $p=0.107$) (*Figure 1)*. RTL was not influenced by sex in any age group.

*Table 3.* Characteristics of patients with cartilage-hair hypoplasia, asymptomatic *RMRP* mutation carriers and non-carriers.

| | | **Patients with CHH** | ***RMRP* mutation carriers** | **Mutation-negative individuals** |
|---|---|---|---|---|
| **Size of group (n)** | | 48 | 74 | 106 |
| **Sex, F/M (%)** | | 31/17 (65%/35%) | 39/35 (53%/47%) | 64/42 (60%/40%) |
| **Median age, years** | | 38.2 (6.0-70.8) | 48.8 (5.0-70.8) | 37.1 (6.0-70.8) |
| **Number of subjects aged:** | | | | |
| | **5.0-18.0 years** | 9 (19%) | 17 (23%) | 19 (18%) |
| | **18.1-40.0 years** | 17 (35%) | 12 (16%) | 40 (38%) |
| | **40.1-70.8 years** | 22 (46%) | 45 (61%) | 47 (44%) |
| **Mutation type (n, %):** | | | | n/a |
| | **homozygote, g.70A>G** | 36 (75%) | none | |
| | **compound heterozygote** | 12 (25%) | none | |
| | **heterozygote, g.70>G** | none | 58 (78%) | |
| | **heterozygote, other*** | none | 16 (22%) | |
| **Relation to patients (n, %):** | | n/a | | |
| | **parents** | | 37 (50%) | none |
| | **siblings** | | 26 (35%) | 12 (11%) |
| | **children** | | 11 (15%) | none |
| | **unrelated** | | none | 94 (89%) |

CHH cartilage-hair hypoplasia, F female, M male, n number of subjects, n/a not applicable, *RMRP* RNA component of mitochondrial RNA processing endoribonuclease
* other mutations included g.262G>T or a 10-nucleotide duplication at position -13 (TACTCTGTGA).

**RTL in patients and mutation carriers**. The proportion of CHH patients with short RTL for age was significantly higher (52%, 25/48) than among mutation carriers (20%, 15/74, $p<0.001$) or healthy non-carriers (29%, 31/106, $p=0.011$) (*Table 4*). In the sub-analysis by age group, almost all children with CHH had short telomeres for age (89%, 8/9). Further, two thirds of patients aged 18.1-40.0 years (65%, 11/17) and one fourth of those aged over 40.1 years (27%, 6/22) had short telomeres. Compared with *RMRP* mutation-negative individuals, the proportion of patients with short RTL was significantly higher in children ($p=0.016$) and adults up to 40.0 years of age ($p=0.047$), but not in older individuals ($p=0.769$) (*Table 4*).

*Table 4*. Relative telomere length as median (range) and as short, average or long for age in various age groups of patients with cartilage-hair hypoplasia, *RMRP* mutation carriers and non-carriers. Higher proportion of patients demonstrated RTL short for age (bold) compared with mutation carriers (p<0.001) or healthy non-carriers (p=0.011). Compared with *RMRP* mutation-negative individuals, the proportion of patients with short RTL was significantly higher in children (p=0.016) and adults up to 40.0 years of age (p=0.047), but not in older individuals (p=0.769).

| **Study group** | **Age group (years)** | **Number of subjects** | **Median RTL (range)** | **Subjects with short RTL, n (%)** | **Subjects with average RTL, n (%)** | **Subjects with long RTL, n (%)** |
|---|---|---|---|---|---|---|
| *Patients* | All ages | 48 | 1.07 (0.70-1.81) | **25 (52%)** | 11 (23%) | 12 (25%) |
| | 6.0-18.0 | 9 | 1.12 (0.88-1.31) | **8 (89%)** | 1 (11%) | 0 (0%) |
| | 18.1-40.0 | 17 | 1.08 (0.91-1.72) | **11 (65%)** | 2 (11.5%) | 4 (23.5%) |
| | 40.1-70.8 | 22 | 1.06 (0.70-1.81) | 6 (27%) | 8 (36.5%) | 8 (36.5%) |
| *RMRP mutation carriers* | All ages | 74 | 1.16 (0.88-1.78) | 15 (20%) | 32 (43%) | 27 (37%) |
| | 5.0-18.0 | 17 | 1.30 (0.95-1.77) | 7 (41%) | 6 (35%) | 4 (24%) |
| | 18.1-40.0 | 12 | 1.20 (0.99-1.78) | 3 (25%) | 5 (42%) | 4 (33%) |
| | 40.1-70.8 | 45 | 1.09 (0.88-1.45) | 5 (11%) | 21 (47%) | 19 (42%) |
| *RMRP mutation-negative subjects* | All ages | 106 | 1.16 (0.71-2.05) | 31 (29%) | 45 (43%) | 30 (28%) |
| | 6.0-18.0 | 19 | 1.25 (0.86-2.05) | 7 (37%) | 5 (26%) | 7 (37%) |
| | 18.1-40.0 | 40 | 1.22 (0.88-1.78) | 14 (35%) | 17 (43%) | 9 (22%) |
| | 40.1-70.8 | 47 | 1.07 (0.71-1.45) | 11 (23%) | 22 (47%) | 14 (30%) |

n number, *RMRP* RNA component of mitochondrial RNA processing endoribonuclease, RTL relative telomere length.

We were able to find from the control group age- and sex-matched controls with no *RMRP* mutations for 40 patients (12 males, 28 females, median age 37.5 years) for case-control analyses. RTL was significantly shorter in patients (median RTL 1.05) compared with the controls (median RTL 1.21, p=0.017). Children aged ≤18 years accounted for this difference in RTL (median 1.09 in patients *vs* 1.25 in controls, p=0.015, n=8 pairs), while only a trend for shorter RTL was observed in those aged >18 and ≤40 years (median 1.07 *vs* 1.26 in controls, p=0.069, n=14 pairs) and no difference was detected in the age group >40 years (median RTL 1.03 *vs* 1.08 in controls, p=0.443, n=18 pairs) (*Figure 2*).

When all study samples were included in analyses, a significant difference in RTL between patients with CHH and healthy subjects (including *RMRP* mutation carriers and non-carriers) was also observed in children (6.0-18.0 years of age, median RTL 1.12 in nine patients *vs* 1.26 in 36 controls, p=0.008) (*Figure 3*).

No significant difference was detected in the older age-groups, although young adults with CHH demonstrated a tendency for shorter telomeres (median RTL 1.08 in 17 patients *vs* 1.22 in 52 controls, p=0.082).

We compared RTL in 48 *RMRP* mutation carriers with age- and sex-matched non-carrier controls, and observed no difference in the median RTL between these two groups (1.16 *vs* 1.10, p=0.224) (data not shown).

**RTL and CHH-related characteristics and morbidity**. In the patient cohort, RTL and classified RTL (short, average or long for age) showed no correlation with the type of *RMRP* mutation, sex, history of blood transfusions, immunoglobulin substitution, hormone or immunosuppressive therapy, obesity or history of smoking. Various infectious manifestations separately or in combinations, fibrosis-like lung changes, history of malignancies, the severity of growth failure and analyzed laboratory parameters did not correlate with RTL itself or RTL category (*Supplementary Table 1*). In two patients who had survived lymphoma, RTL was average and long for age respectively.

## DISCUSSION

Our study demonstrates shorter telomeres in DNA from peripheral blood in children with CHH. The interpretation of our findings necessitates further studies to determine whether shorter telomeres are the cause or the consequence in CHH pathology. Decreased telomere length may reflect the increased number of cell divisions required to compensate for the impaired cell cycle and increased apoptosis reported in CHH [18]. Alternatively, telomerase defects can represent primary pathologic mechanism contributing to stem cell exhaustion, including bone marrow failure present in some patients with CHH. Our findings confirm the significance of *RMRP* in telomerase function.

RTL correlated significantly with age in *RMRP* mutation carriers and non-carriers, but not in patients with CHH, which can be explained by shorter telomeres in pediatric patients. Individuals with CHH have increased mortality in childhood and young adulthood from infections and malignancies [19,20]. A high proportion of our patients (46%, 22/48) were over 40 years of age and thus represent a selected population of less affected patients who escaped fatal complications in early life. Therefore, normal telomere length in adults with CHH can indicate a survival advantage. The absence of correlations between telomere length and clinical and laboratory characteristics can derive from the small sample size. Also, RTL is a rough estimate of telomere structure and represents only a part of the complex telomere biology.

Interestingly, some DC mutation carriers demonstrate telomere shortening while remaining asymptomatic and inheritance of shorter telomeres probably induces more severe disease in subsequent generations [21]. The finding of normal telomeres in *RMRP* mutation carriers contradicts the data from families with DC. More research is needed to test pedigrees where CHH has been diagnosed in more than one generation. If the shorter telomere length is indeed inherited, this may result in more severe disease in the affected offspring of patients with CHH in future generations.

Cell immortalization and subsequent development of cancer can emerge from abnormal telomere maintenance [22]. While DC and CHH are both characterized by increased risk of malignancies, the types of tumors developing in patients with these disorders differ. The most common malignancies in individuals with DC include head and neck squamous cell carcinomas, while in subjects with CHH, non-Hodgkin lymphomas and basal cell carcinomas predominate [20,23]. Accordingly, in the light of present data, immunodeficiency

rather than the telomere length is the nominator in predisposition to malignancy in CHH patients. Immunodeficiency in patients with CHH may predispose them to EBV-associated lymphoproliferative disorders. Unfortunately, no data were available on EBV status in lymphoma samples from the two patients in our cohort. The majority of our patients had detectable serum antibodies to EBV but EBV DNA was not detected by PCR in peripheral blood. EBV causes telomere dysfunction in the infected cells [24, 25] and it is possible that this is relevant in CHH. Therefore, further research on alteration in telomere functions and the role of EBV in CHH patients with malignancies would be warranted.

The results of our study have important clinical implications. Patients with CHH can require hematopoietic stem cell transplantation and defective telomere biology should be taken into account when choosing conditioning regimen. Also, bone marrow failure in patients with DC has been successfully treated with androgens (probably due to telomerase up-regulation) and this treatment option may be considered in selected patients with CHH [26-28]. Furthermore, telomere length measurement may guide diagnostic process in individuals with immunodeficiency of unknown etiology and CHH should be included in the differential diagnosis of children with short telomeres.

We recognize strengths and limitations in our study. This is the first study to evaluate telomere length in patients with CHH. The high prevalence of the disease in our population provided us with a unique opportunity to recruit a large cohort of patients and their unaffected relatives with a homogenous genetic and ethnic background and with a wide age range; in rare diseases such an approach is seldom possible. The drawbacks of our study include insufficient clinical data from relatives of patients with CHH and healthy controls. Thus, it was impossible to analyze the influence of e.g. smoking or medications on RTL. However, in the patient group, these factors did not affect telomere length and none of the patients aged <18 years reported smoking. The number of healthy controls was rather small, which increases the risk of bias and due to the inter-individual variability of telomere length our data may not be applicable to particular individuals with CHH. Also, no data were available on metabolic profile (glucose, insulin and lipid profile, blood pressure) of our patients, hindering the evaluation of its relationship with RTL. Another limitation is the use of RTL, where mean telomere length of all chromosome ends is estimated. Telomere length distributions and critically short individual telomere ends cannot be detected by this method.

Our results suggest that telomere length is abnormal in children with CHH. Further studies are required to explore functional consequences of altered telomere maintenance and possible clinical

implications of these findings. Longitudinal follow-up of patients with CHH is necessary to establish the significance of telomere length as a predictor of disease severity and mortality.

## CONTRIBUTORSHIP STATEMENT

Study design: PK, MT, GR and OM. Study conduct: SK, MP, SD. Data collection: SK. Data analysis: SK, SD, ML. Data interpretation: SK, SD, MT, GR, OM. Drafting manuscript: SK. Revising manuscript content: All authors. Approving final version of manuscript: All authors.

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## COMPETING INTERESTS

The authors declare no conflicts of interest.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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## LEGENDS TO FIGURES

*Figure 1*. Correlation of relative telomere length with age in 228 samples from patients with cartilage-hair hypoplasia, their relatives and healthy controls. Every dot corresponds to a measurement from a single individual. RTL correlated significantly with age in *RMRP* mutation carriers (rho -0.482, p<0.001) and non-carriers (rho -0.498, p<0.001), but not in patients (rho -0.236, p=0.107).

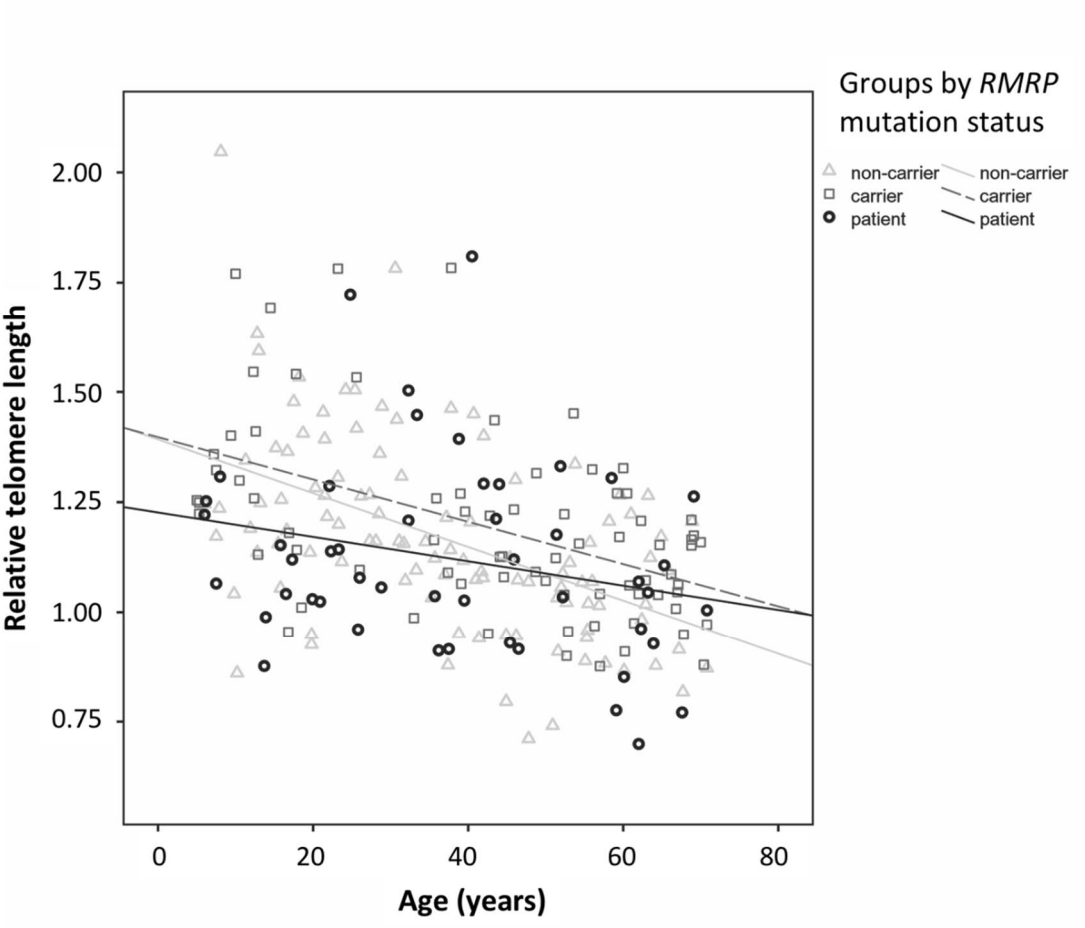



*Figure 2*. Comparison of relative telomere length (RTL) in a case-control setting in 40 patients with cartilage-hair hypoplasia (median RTL 1.05) and 40 age- and gender-matched healthy controls negative for *RMRP* mutation (median RTL 1.21, p=0.017). RTL was significantly shorter in children with CHH (median 1.09 *vs* 1.25 in controls, p=0.015, n=8 pairs). Adults with CHH showed a trend for shorter RTL in the age group >18 and ≤40 years (median 1.07 *vs* 1.26 in controls, p=0.069, n=14 pairs), while no difference was detected in the age group >40 years (median RTL 1.03 *vs* 1.08 in controls, p=0.443, n=18 pairs).

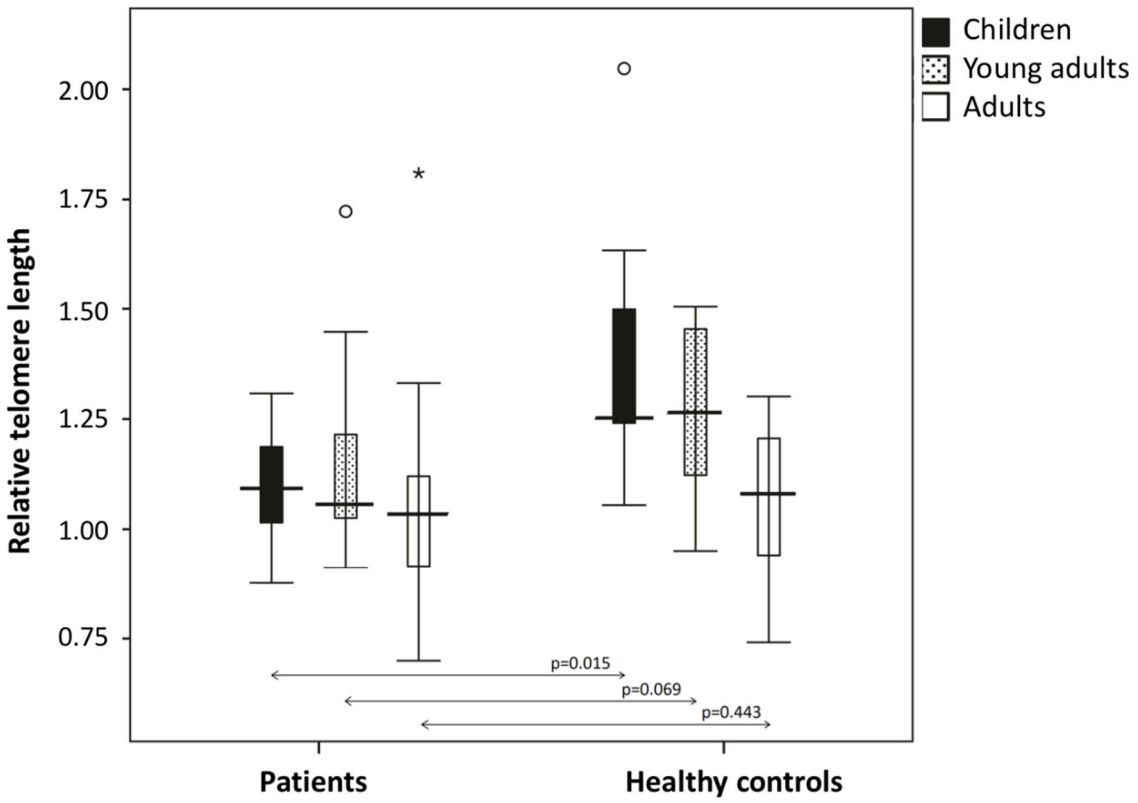
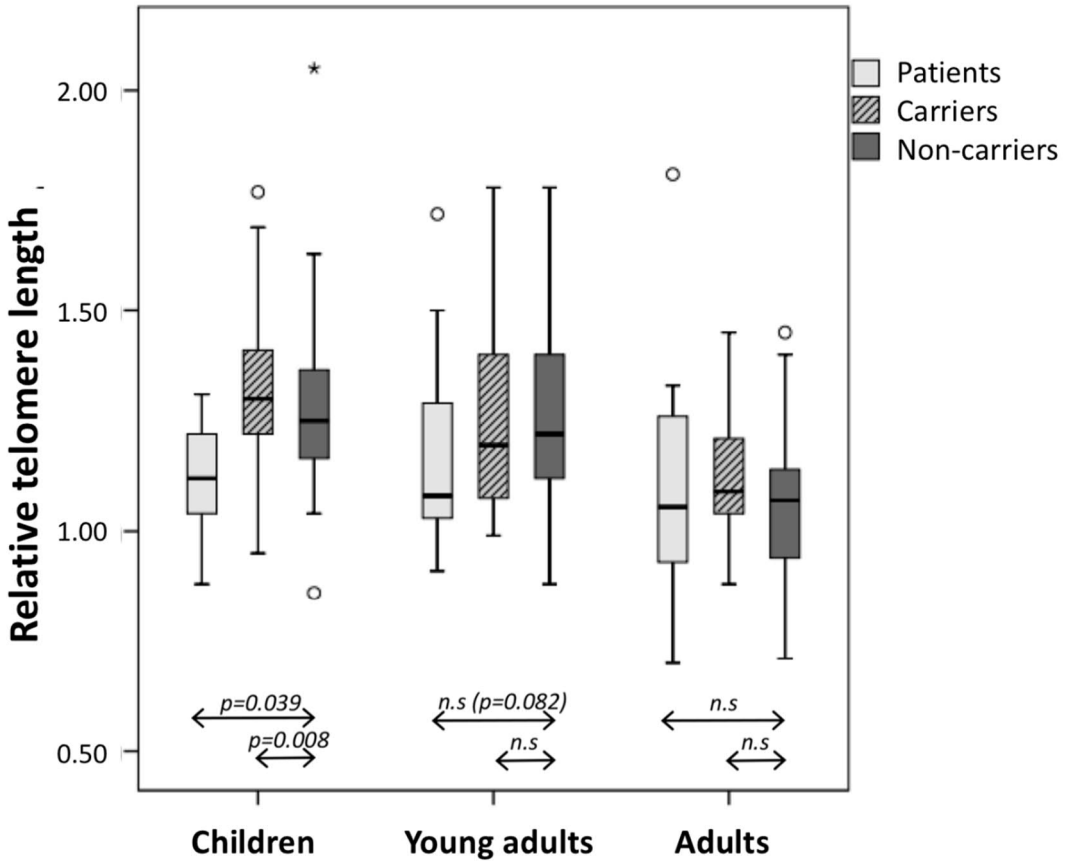



*Figure 3*. Comparison of relative telomere length (RTL) in different age groups of patients with cartilage-hair hypoplasia (CHH), *RMRP* mutation carriers and non-carriers. Children with CHH (aged ≤18 years) demonstrate significantly shorter RTL compared with asymptomatic mutation carriers and non-carriers. No difference in RTL was observed in young adults (aged >18 and ≤40 years) or adults (aged >40 years) with CHH compared with controls.


Relative telomere length
2.00
1.50
1.00
0.50
Patients
Carriers
Non-carriers
p=0.039
p=0.008
n.s (p=0.082)
n.s
n.s
n.s
Children
Young adults
Adults

438

439

## ABBREVIATIONS

CHH, cartilage-hair hypoplasia

DC, dyskeratosis congenita

EBV Epstein-Barr virus

rho, Spearman's correlation coefficient

*RMRP*, RNA component of mitochondrial RNA processing endoribonuclease

RTL, relative telomere length

SD standard deviation

TERT, telomerase reverse transcriptase