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Identification of Adult Patients With Classical Dyskeratosis Congenita or Cryptic Telomere Biology Disorder by Telomere Length Screening Using Age-modified Criteria

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ABSTRACT

Telomere biology disorders (TBD) result from premature telomere shortening due to pathogenic germline variants in telomere maintenance-associated genes. In adults, TBD are characterized by mono/oligosymptomatic clinical manifestations (cryptic TBD) contributing to severe underdiagnosis. We present a prospective multi-institutional cohort study where telomere length (TL) screening was performed in either newly diagnosed patients with aplastic anemia (AA) or if TBD was clinically suspected by the treating physician. TL of 262 samples was measured via flow-fluorescence in situ hybridization (FISH). TL was considered suspicious once below the 10th percentile of normal individuals (standard screening) or if below 6.5 kb in patients >40 years (extended screening). In cases with shortened TL, next generation sequencing (NGS) for TBD-associated genes was performed. The patients referred fell into 6 different screening categories: (1) AA/paroxysmal nocturnal hemoglobinuria, (2) unexplained cytopenia, (3) dyskeratosis congenita, (4) myelodysplastic syndrome/acute myeloid leukemia, (5) interstitial lung disease, and (6) others. Overall, TL was found to be shortened in 120 patients (n = 86 standard and n = 34 extended screening). In 17 of the 76 (22.4%) standard patients with sufficient material for NGS, a pathogenic/likely pathogenic TBD-associated gene variant was identified. Variants of uncertain significance were detected in 17 of 76 (22.4%) standard and 6 of 29 (20.7%) extended screened patients. Expectedly, mutations were mainly found in *TERT* and *TERC*. In conclusion, TL measured by flow-FISH represents a powerful functional in vivo screening for an underlying TBD and should be performed in every newly diagnosed patient with AA as well as other patients with clinical suspicion for an underlying TBD in both children and adults.

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INTRODUCTION

Telomeres are repetitive DNA structures located at the chromosome ends thereby protecting genetic integrity. Telomeres shorten with each cell division until a critical telomere length (TL) is reached, leading to replicative senescence or apoptosis.[1] In case of further proliferation past the respective telomere checkpoints, cells develop chromosomal instability potentially promoting tumor development. Physiologically, telomeres are re-elongated by the enzyme telomerase. Conversely, inactivating variants in genes coding for the telomerase complex cause enzyme dysfunction resulting in accelerated premature telomere shortening and subsequent organ failure.[2]

The telomere biology disorders (TBD) such as dyskeratosis congenita (DKC) typically manifest themselves during childhood or young adulthood and are paradigmatic to study the consequences of premature telomere shortening.[3] Besides the typical disease-defining clinical features—the mucocutaneous triad of abnormal skin pigmentation, nail dystrophy, and mucosal leukoplakia combined with bone marrow failure syndrome (BMFS)—DKC patients can show additional symptoms like interstitial lung disease (ILD) or liver disorder and are at substantially increased risk of hematologic and solid malignancies.[2,4,5] However, growing evidence of a yet significantly underdiagnosed proportion of late onset types of TBD first presenting during adulthood often with a monosymptomatic or oligosymptomatic phenotype led to the addition of the term cryptic for this subgroup of TBD patients. Clinically, patients with cryptic TBDs often lack the classical DKC triad, and the underlying inherited disorder is frequently hidden behind mostly aplastic anemia (AA) and related BMFS, ILD, chronic liver or gastrointestinal disorders,[2,4] therefore resulting in underdiagnosis particularly when the patients with this ultra-rare disease are not being taken care of in specialized centers. Consequently, correct diagnosis of TBD is of utmost importance, as prognosis, treatment, and management of complications are significantly affected by the limited replicative potential of cells across all affected organ systems.[6]

Genetic testing panels mostly include the known TBD-associated genes (*ACD, CTC1, DKC1, NAF1, NHP2, NOP10, PARN, POT1, RTEL1, STN1, TERC, TERT, TINF2, WRAP53, ZCCHC8*).[7] However, genetic testing fails to properly diagnose an underlying TBD in at least 20% of patients,[8] simply because not all TBD-causing genes are known to date as exemplified by the very recent discovery of alterations in candidate genes such as Apollo or *RPA1*.[9,10] In addition, the functional consequences of yet undefined variants found in TBD-genes can more accurately be determined once the in vivo read out, that is, shortened TL result, is available. In addition, rare families with cases of phenocopy (reviewed in Revy et al[11]), that is, where screened relatives from yet unknown patients with genetic TBD have short telomeres despite of being genetically normal, will obviously be missed by next generation sequencing (NGS) screening alone.

The accepted screening tool for patients with suspected heritable TBD is the measurement of TL in peripheral blood (PB) leukocytes using flow-fluorescence in situ hybridization (FISH).[12–16] The method is based on the relation of patients TL measured by quantitative FISH to age-adjusted controls. Recent guidelines recommend genetic testing for patients with TL below the 10th percentile,[2,17] for example, recommendations for diagnosis and treatment of AA of the German Society of Hematology and Medical Oncology (DGHO) published in Onkopedia, a collection of national diagnostic and treatment guidelines for hematologic disorders frequently used in German-speaking countries.[18] Although successfully established for the screening of pediatric cohorts for cases with classical DKC, the continuous physiological age-dependent TL decline in normal cells significantly narrows down the diagnostic window between normal and the functionally critical level of telomere shortening. Thereby, the age percentile-based approach is somewhat limited particularly in older adults.[19]

Data on TL screening strategies aimed at improving the detection of adult cryptic TBD patients in a multicenter real-life setting are scarce. So far, institutional registries focus on patients with known TBD or on selected disease entities like AA or ILD.[20,21] Here, we provide a comprehensive multicenter, multidisciplinary, and international approach to routine TL screening with flow-FISH in patients with AA and AA/paroxysmal nocturnal hemoglobinuria (PNH) syndromes as well as other disorders suspected to result from an underlying TBD. In this study, we report the detection rate and the genetic landscape of TBD-associated variants according to the individual screening categories in patients with short telomeres and aim to improve the current age-dependent screening algorithms in adults.

MATERIALS AND METHODS

Patients and sample characteristics

Analysis comprised the first 262 consecutive patients referred to our site for TL screening from a variety of clinical institutions of German-speaking countries included into the Aachen TBD registry from October 2014 to October 2017. Patients from over 63 participating hematology centers in Germany, Switzerland, and Austria were included in the registry either according to the recommendations for diagnosis and treatment of AA of the DGHO[18] or if the local treating physician suspected an underlying TBD or other type of inherited BMFS. All patients signed written informed consent for registry inclusion to collect demographic and clinical data as well as informed consent for genetic analyses (EK206/09, University Hospital Aachen). PB samples were processed within 48 hours for flow-FISH analysis and DNA extraction for NGS.

Flow-FISH

Flow-FISH was carried out as described previously.[16,22,23] Briefly, vital sterile frozen mononuclear cells from PB were used for the flow-FISH analysis of TL. Samples were prepared for cell denaturation and mixed with a fluorescein isothiocyanate (FITC)-labeled telomere-specific (CCCTAA) 3-peptide nucleic acid FISH probe (Eurogentec, Liège, Belgium) for DNA hybridization followed by DNA counterstaining with LDS 751 (Sigma). Bovine thymocytes were used as internal controls. All measurements were carried out in triplicates. TL of bovine thymocytes was used to convert the TL of lymphocytes and granulocytes into kilobases (kb). Healthy controls and percentiles were used for age adaptation of TL for flow-FISH as described previously.[16]

Targeted amplicon sequencing

For NGS, performed at the NGS center of the Aachen TBD registry, the MiSeq Illumina platform was used as previously described.[24] Library preparation was done using the TruSeq Amplicon kit (Illumina). Genetic variants in telomere maintenance genes were screened using a custom-designed panel containing the entire coding sequences of *CTC1, DKC1, NHP2, NOP10, RTEL1, TERC, TERT, USB1,* and exon 6 of *TINF2*.[23,24]

Sequencing was done 250 base-pair paired-end and raw data were analyzed with Illumina RTA software on board. For alignment and variant calling, the SeqNext module (SeqPilot software version 4.3.0, JSI medical systems GmbH, Ettenheim, Germany) was used. The runs showed good quality with a Q30 score of >85% of all called bases. A minimal coverage of 30× was reached in 93%–97%, dependent on the sample quality. Since we focused on germline variants, cutoff levels of variant allele frequency of >30% or ≥10 absolute variant reads were chosen. Eight patients had commercial NGS screening.

All variants, except those in *TERC*, were classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines for the interpretation of sequence variants: class 3 (variant of uncertain significance [VUS]), class 4 (likely

pathogenic), and class 5 (pathogenic).[25] Since *TERC* is a non-coding RNA, many of the ACMG criteria supporting pathogenicity are not applicable. Therefore, for the classification of *TERC* variants, we added shortened TL as a moderate pathogenicity criterion.

Statistical analysis

The data were collected via Microsoft Excel 2007 and GraphPad Prism (GraphPad Software version 9.0.0, La Jolla, CA, USA). Results are expressed as median including range.

RESULTS

Characteristics of the registry cohort

A total of 262 patients were consecutively included in the registry from October 2014 to October 2017 (Figure 1). Based on the initially assigned underlying diagnosis by the participating physicians, we divided the patients into 6 diagnostic screening categories: (1) patients with AA or PNH or AA/PNH overlap syndrome; (2) unexplained cytopenia; (3) DKC/TBD/screening family members of known DKC patients; (4) myelodysplastic syndrome (MDS)/acute myeloid leukemia (AML); (5) ILD; and (6) other. Detailed information on the initially assigned diagnosis in category 6 is given in Suppl. Table S1.

The largest category comprised AA/PNH cases, representing 114 patients (43.5%) of the entire cohort, including 94 patients with AA, 10 with classical PNH, and 10 with AA/PNH overlap syndrome (Table 1). The categories unexplained cytopenia, DKC, MDS/AML, ILD, and other represented 40 (15.3%), 43 (16.4%), 26 (9.9%), 14 (5.3%), and 25 (9.5%) patients, respectively. Eight (20%) patients in the unexplained cytopenia category had single-lineage cytopenia, in 14 (35%) patients, 2 lineages were affected, and 18 (45%) patients had pancytopenia.

For the entire cohort, median age at inclusion was 38.3 years (range, 0.5–87.5). Median age for patients with AA/PNH, unexplained cytopenia, DKC, AML/MDS, ILD, or other was 37.7 years (range, 8.5–87.5), 42.3 years (range, 0.5–67.9), 33.7 years (range 0.5–86.7), 52.0 years (range 2.2–79.4), 71.2 years (range 51.5–84.8) and 28.5 years (range 3.1–66.8), respectively. There were 57 (50.0%) men in category AA/PNH as well as 26 (65.0%), 21 (48.8%), 11 (42.3%), 6 (42.9%), and 9 (36.0%) in the categories unexplained cytopenia, DKC, MDS/AML, ILD, and other, respectively (Table 1).

TL measurement via flow-FISH as a screening parameter to identify patients with TBD-associated gene variants

In order to analyze the applicability of flow-FISH screening in a multicenter, clinical routine setting to trigger subsequent NGS analysis, flow-FISH was performed in all patients (n = 262,

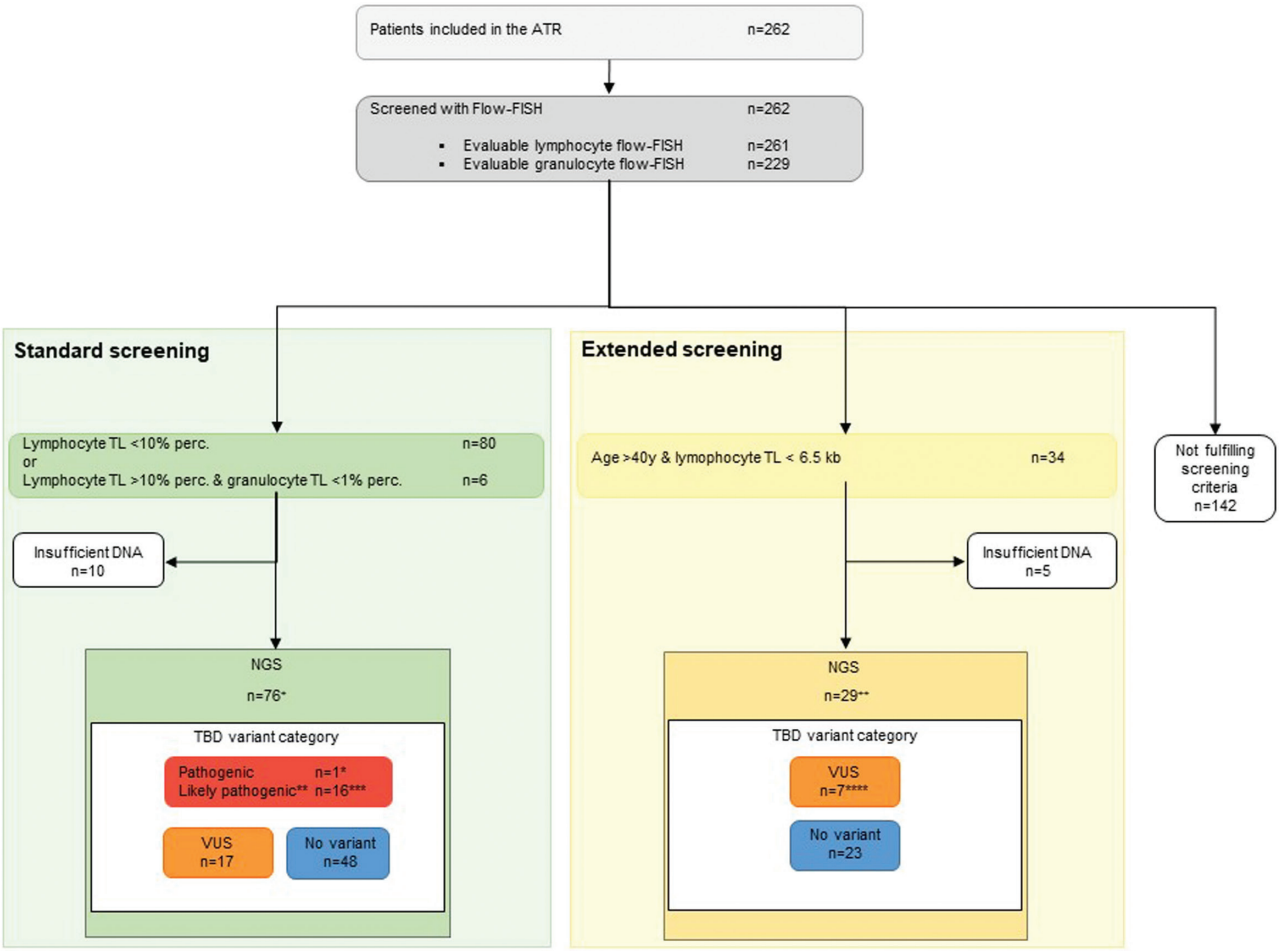

Figure 1. Flow-chart of screening work-up. All patients included in the Aachen TBD registry (ATR) were screened with flow-FISH. Telomeres were considered critically short when (1) lymphocyte TL was <10th percentile or if lymphocyte TL was >10th but granulocyte TL less than first percentile (standard screening) or (2) age was >40 y and lymphocyte TL <6.5 kb (extended screening). Patients with critically short TL underwent NGS with analysis of TBD-related genes. +n = 7 patients had commercial NGS. ++n = 1 patient had commercial NGS. *Patient had 1 more variant (VUS). **Likely pathogenic includes *TERC* variants that were not classified according to the (ACMG) criteria (see Methods section). ***Three patients had >1 variant (VUS). ****One patient had 2 variants. ACMG = American College of Medical Genetics and Genomics; kb = kilobase; NGS = next generation sequencing; TBD = telomere biology disorder; TL = telomere length; VUS = variant of uncertain significance.

Table 1

Characteristics of the Registry Cohort, n = 262 Patients

Diagnosis Category	AA/PNH	Cytopenia	DKC	MDS/AML	ILD	Others
No of patients (%)	114 (43.5)	40 (15.3)	43 (16.4)	26 (9.9)	14 (5.3)	25 (9.5)
Median age at inclusion in years (range)	37.7 (8.5–87.5)	42.3 (0.5–67.9)	33.7 (0.5–86.7)	52.0 (2.2–79.4)	71.2 (51.5–84.8)	28.5 (3.1–66.8)
Sex; male, no (%)	57 (50.0)	26 (65.0)	21 (48.8)	11 (42.3)	6 (42.9)	9 (36.0)

AA = aplastic anemia; AML = acute myeloid leucemia; DKC = dyskeratosis congenita; ILD = interstital lung disease; MDS = myelodysplastic syndrome; No = number; PNH = paroxysmal nocturnal hemoglobinuria.

Figure 1). Of note, due to express delivery within 48 hours to improve preanalytics, lymphocyte flow-FISH was evaluable in 261 of 262 patients and granulocyte flow-FISH in 229 patients. Not unexpectedly given the underlying diagnoses at study, reasons for nonevaluability of granulocyte flow-FISH mostly were low cell counts. Insufficient overall cell number limited DNA-extraction for NGS in 15 patients.

First, we applied the recommended routine cutoff as published previously.[2,17] TL was interpreted as suspicious (critically short) when found to be below the 10th percentile in lymphocytes and/or below the first percentile in granulocytes (standard screening; Figure 1). TL was found below the 10th lymphocyte percentile in 80 patients and above the 10th lymphocyte but still below the first percentile in granulocytes in 6 patients (Figures 1 and 2A, Suppl. Figure S1).

In a second step, with the aim to increase diagnostic sensitivity, we applied an additional screening for patients aged >40 years (extended screening). Taken into account that age-adjusted telomere shortening leads to a decreased diagnostic window between normal telomere decline and critically short telomeres (estimated to become functionally important below an absolute TL of ~5 kb), the solely percentile-based cutoffs starts to become less stringent in patients aged ≥40 years.[19] With the aim to use the particular strength of flow-FISH in measuring the functional read out of altered telomere maintenance in vivo as a screening approach (ie, shortened TL) and thereby prioritizing on not to miss affected individuals and increase diagnostic sensitivity, we defined and investigated an additional extended absolute TL threshold in patients aged ≥40 years. Here, lymphocyte TL was interpreted as suspicious when it was below the absolute TL of 6.5 kb (Figures 1 and 2A). With this approach, 34 additional patients were identified. For comparison, 142 patients did not fall into the category of shortened TL either by the criteria for standard or extended screening and consequently had no NGS screening performed (Figures 1 and 2A).

Overall, based on the screening criteria for TL and availability of sufficient DNA, NGS was performed in 76 (standard screening) and 29 (extended screening) patients, respectively; that is, within the standard and extended screening, 10 and 5 patients had insufficient DNA, respectively. Eight patients (7 in standard and 1 in extended screening) had commercial NGS performed elsewhere and 97 had NGS performed locally. In total, 41 class 3–5 genetic variants were found in 36 patients (standard: 30; extended: 6; Figures 1 and 2A). Pathogenic and likely pathogenic variants were found in 17 standard screening patients and none in extended screening. In addition, 17 VUS were detected in standard and 7 in extended screening, respectively. Extended screening patients with VUS had ILD (n = 2) and 1 each had AA, unexplained cytopenia, MDS, or was a family member of a DKC patient. In 48 standard and 23 extended screening patients, no variant was detected.

Landscape of TBD-associated gene variants in the respective diagnostic categories

Genetic variant findings in relation to the initially assigned diagnosis of the local treating physician were analyzed (Figure 3). NGS was performed in 38 patients of category AA/PNH (including 2 with classical PNH and 5 with AA/PNH overlap). Pathogenic/likely pathogenic variants were found in 3 (7.9%) patients (2 *TERC* and 1 *RTEL1*). Within these, no variants were found in classical PNH and AA/PNH overlap patients. Besides, 7 (18.4%) VUS were found (3 *TERT*, 3 *RTEL1*, and *USB1*). One patient had each 1 pathogenic variant and 1 VUS. A total of 13 patients in the category unexplained cytopenia had NGS screening with a detection of one (7.7%) likely pathogenic variant (*TERT*). One patient had 2 VUS findings (*TERT*, *RTEL1*). In 26 screened patients of the category DKC, pathogenic/likely pathogenic variants were detected in 13 patients (50%; 7 *TERC*, 3 *TERT*, and 3 *NHP2*). Nine (34.6%) VUS were found (2 *TERT*, 2 *RTEL1*, 3 *CTC1*, and 2 *DKC1*). Two patients had each 1 likely pathogenic and 2 VUS, and 1 patient had 1 likely pathogenic and 1 VUS. In 13 screened patients of the AML/MDS category, 4 (30.8%) VUS were detected (3 *TERT* and 1 *CTC1*). In 9 patients of the ILD category, 2 (22.2%) had a VUS (2 *RTEL1*). In the screening category with other diagnoses, 6 patients were screened with no variant detected. Variant landscape class 3–5 within the respective diagnosis categories are shown in Suppl. Figure S2 for AA/PNH (A), unexplained cytopenia (B), DKC (C), and MDS/AML (D) with special indication of pathogenic and likely pathogenic variants, and detected gene variants are listed in Suppl. Table S2.

Landscape of TBD-associated gene variants by standard and extended screening

We observed that most of the pathogenic/likely pathogenic variants were found in the main components of the telomerase complex, *TERC* and *TERT*, and all were detected in standard screening patients (Figure 4A). By adding a moderate criterion of pathogenicity in context with shortened TL in case of *TERC* as described above, 9 *TERC* variants were identified. Four *TERT* variants were found, and 2 of the patients were relatives. In addition, 3 *NHP2* (all were relatives) and 1 *RTEL1* pathogenic/likely pathogenic variants were detected in this group (Figure 4A).

Totally 17 VUS were identified in standard screening patients: *TERT* in 7 patients, *RTEL1* in 4, *CTC1* in 4, and *DKC1* and *USB1* in 1 patient each (Figure 4B). Extended screening led to the detection of VUS in 4 patients in *RTEL1* and *TERT* in 2 and *DKC1* in 1 patient (Figure 4C). No pathogenic/likely pathogenic variant was found in this group.

DISCUSSION

TBD are clinically heterogeneous and challenging diseases in terms of correct and timely diagnosis, especially in cases of cryptic TBD not showing the classical clinical features of DKC. Given the tremendous functional biological reserve in TL in vertebrates, the clinical consequences of an accelerated loss of telomere repeats due to inherited altered telomere maintenance may initially be mild, and may require a certain degree of additional physiological aging for first onset of symptoms in the first

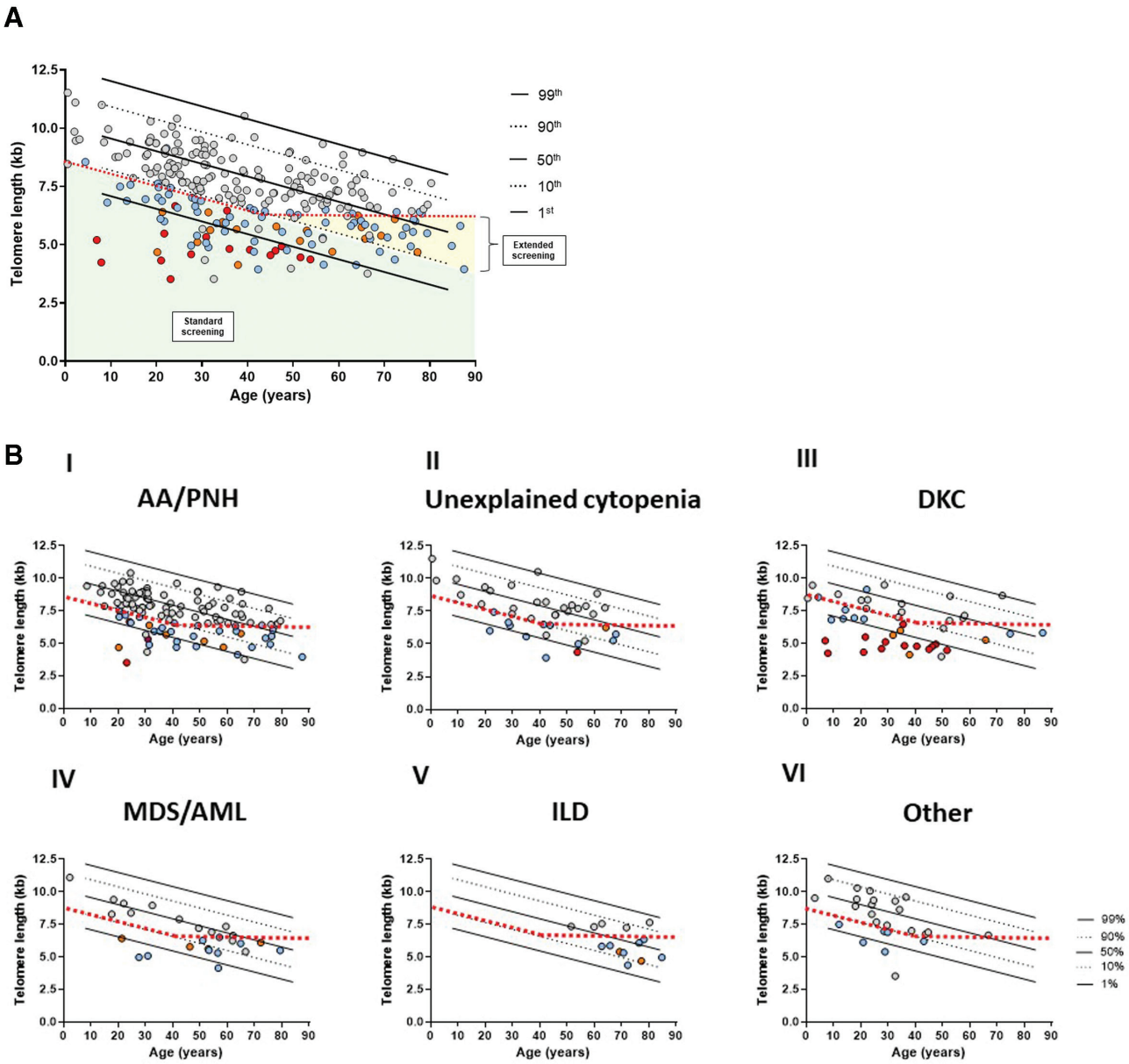

Figure 2. Telomere length measurement of the entire registry cohort. (A). Lymphocyte TL (given in kb) of 261 patients: first, 10th, 50th, 90th, and 99th percentiles are indicated. Blue, orange, and red dots show 105 patients with TL under 10th percentile (standard screening) or <6.5 kb if aged >40 y (extended screening), respectively, in whom NGS was performed. Of those, detection of variants is indicated as follows: Red dots indicate patients with pathogenic and likely pathogenic variants (n = 17), including *TERC* variants that were not classified according to the ACMG criteria (see Methods section). Orange dots represent patients with VUS (n = 24). Blue dots indicate no variant found in NGS. Gray dots indicate patients without NGS screening. Red dotted line indicates TL threshold as follows: <10th percentile until 40 y; <6.5 kb beyond 40 y. (B). Distribution of the TL (kb) within the respective diagnosis categories; AA/PNH (I), unexplained cytopenia (II), DKC (III), MDS/AML (IV), ILD (V), and other (VI). First, 10th, 90th, and 99th percentiles are indicated. Red dotted line indicates TL threshold as follows: <10th percentile until 40 y; <6.5 kb beyond 40 y. AA = aplastic anemia; ACMG = American College of Medical Genetics and Genomics; AML = acute myeloid leukemia; DKC = dyskeratosis congenital; ILD = interstitial lung disease; kb = kilobase; MDS = myelodysplastic syndrome; NGS = next generation sequencing; PNH = paroxysmal nocturnal hemoglobinuria; TL = telomere length; VUS = variants of uncertain significance.

generation affected.[2,26] However, due to genetic anticipation being described for individual subtypes of TBD, onset of symptoms can be expected to be shifted toward younger age in successive generations affected (reviewed in Revy et al[11]). Moreover, various organ systems can be differentially affected in cryptic TBD because of the variety of genes involved as well as different ways of inheritance, incomplete penetrance, expression of affected genes, or somatic genetic rescue. In addition, environmental and life-style factors may have a heterogeneous impact on the replicative reserve of the different organ systems potentially affected. Therefore, patients with this group of diseases are not restricted to be seen by pediatricians or hematologists, but also pulmonologists, gastroenterologists, dermatologists, human geneticists, and other specialties as primary care physicians.[4,27,28] Systematic data collections on the incidence and different clinical presentations of TBDs are scarce, particularly with regards to cryptic and adult-onset TBD. Given the rarity of the disease, most of the literature on TBD is based on pediatric cohorts and/or single-expert center experience. Here, we present prospective real-life data using a comprehensive multicenter, multidisciplinary, and international approach covering different sectors of health systems of the German-speaking Europe. In

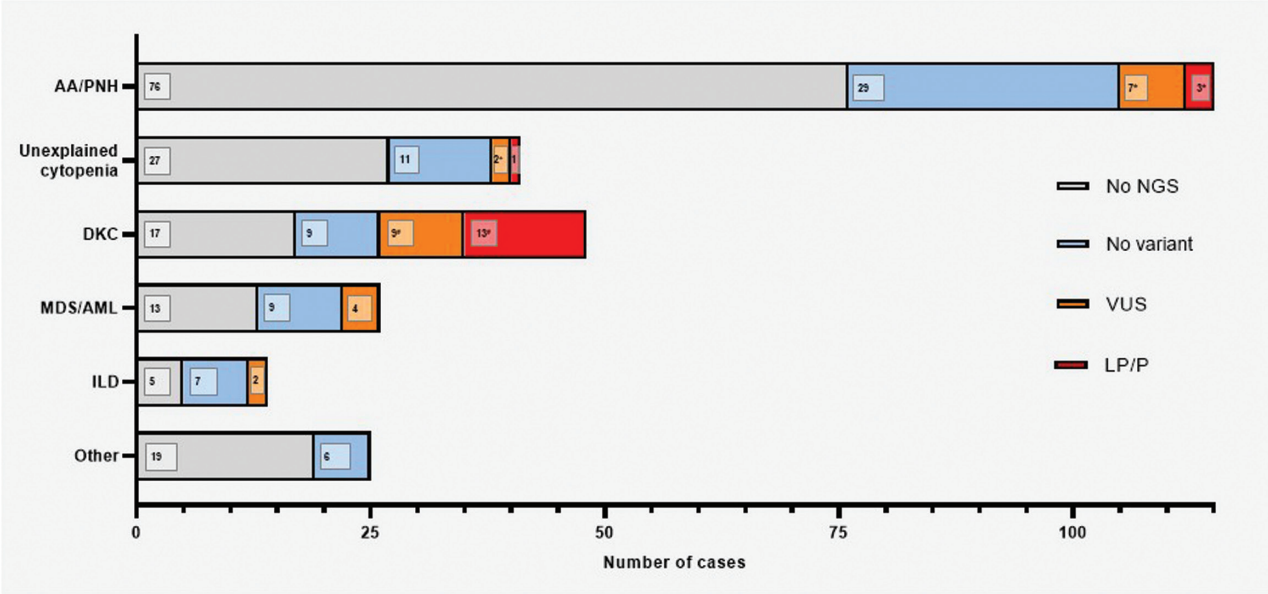

Figure 3. Absolute number of cases within the respective diagnosis categories. NGS screening is shown including patients not screened, because of not fulfilling the criteria. Absolute numbers of variants are shown after classification according to the ACMG. Likely pathogenic variants include *TERC* variants that were not classified according to the ACMG criteria (see Methods section). *One patient had each 1 pathogenic variant and 1 VUS. +One patient had 2 VUS. #Two patients had each 1 likely pathogenic variant and 2 VUS; 1 patient had 1 likely pathogenic variant and 1 VUS. AA = aplastic anemia; ACMG = American College of Medical Genetics and Genomics; AML = acute myeloid leukemia; DKC = dyskeratosis congenita; ILD = interstitial lung disease; LP = likely pathogenic; MDS = myelodysplastic syndrome; NGS = next generation sequencing; P = pathogenic; PNH = paroxysmal nocturnal hemoglobinuria; VUS = variant of uncertain significance.

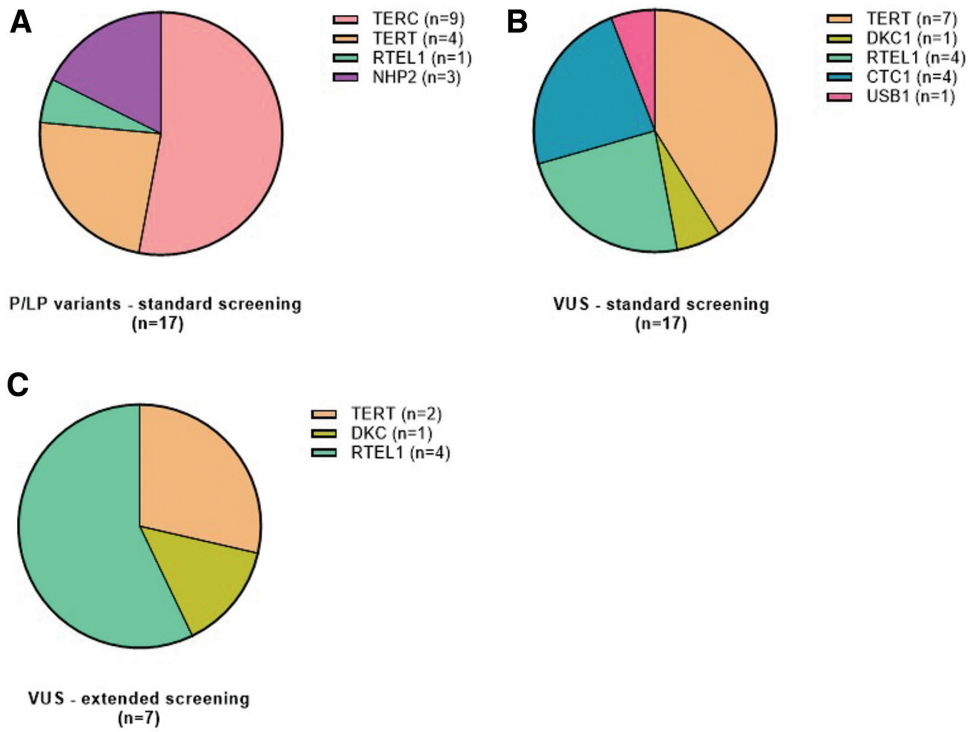

Figure 4. Distribution of detected genetic variants in TBD-associated genes. (A) Pathogenic/likely pathogenic variants in standard screening patients. (B) Variants of uncertain significance in standard screening patients. (C) Variants of uncertain significance in extended screening patients. LP = likely pathogenic; N = number; P = pathogenic; TBD = telomere biology disorder; VUS = variant of uncertain significance.

contrast to other studies, TL screening was either performed as part of the routine screening recommended for newly diagnosed adult BMFS[18] and/or because of clinically suspected TBD by the referring physician. The study included the evaluation of an extended, age-adapted TL-based screening algorithm that led to the identification of so far undetected, additional patients with TBD-associated gene variants.

Recent data emphasized the importance of the correct identification of TBD patients. In particular, TBD patients respond favorably to treatment with androgens and are considered to

respond to a significantly lesser degree to immunosuppressive therapy compared with immune-mediated acquired AA.[23,29] In case of allogeneic stem cell transplantation (allo-SCT), family members have to be adequately screened in the process of donor selection to avoid stem cell donation from asymptomatic carriers.[2] In addition, McReynolds et al[30] clearly demonstrated in a cohort of AA patients undergoing allo-SCT that patients with TBD show significantly impaired overall survival. Therefore, the development of adapted conditioning protocols is recommended to improve outcome for TBD patients receiving allo-SCT.[30] Supported by these strong observations and despite of the limited data on the frequency of underlying TBDs in these disease entities, screening for TL is recommended by recently established recommendations for diagnostic work-up in AA[31] and in ILD.[32]

Regarding BMFS, data show a frequency of underlying TBD of 1%–5% in AA.[30,33–35] For MDS/AML and ILD, a frequency of 1%–3%[33,34] and 3%–10%[28,36] has been reported, respectively. In the real-life cohort reported here, we identified pathogenic/likely pathogenic variants in 7.9% of patients with AA or AA/PNH syndrome. We did not identify pathogenic/likely pathogenic variants but VUS in 30.8% in the MDS/AML cohort and in 22.2% of the patients included in the ILD screening cohort. In the cohort with unexplained cytopenia, one pathogenic/likely pathogenic variant was found in 13 individuals. Because TL screening is strongly recommended in the German guidelines, our data provide an estimated incidence of TBD in patients with suspected AA. In contrast, we cannot draw definite conclusions about the absolute frequency of TBD within the other disease categories: heterogeneity in subpopulations clinically, patient selection (eg, monocentric expert center versus multicenter diagnostic referrals), inclusion criteria (eg, only newly diagnosed patients or also patients during the course of their disease), and age distributions as well as the restricted size of some previously published cohorts generally would allow comparisons between studies only with great caution.

Genetic testing following telomere screening is advised in patients with TL below the 10th percentile, while the most stringent and specific identification of individuals with telomerase or functionally related gene mutations is achieved if TL is below the 1st percentile.[37] Previous data showed that TL analysis using the 1st percentile above the age of >40 years becomes less specific.[19] In order to maximally reduce the risk of missing an underlying TBD in older patients in a screening assay such as flow-FISH, we chose an additional absolute TL threshold of 6.5 kb in patients >40 year to be evaluated here. As a consequence, 34 additional patients were identified and received NGS, in which we did not identify additional pathogenic/likely pathogenic variants. However, we detected additional 7 VUS in this extended screening group in addition to the 17 VUS in the standard screening cohort. Similar findings regarding VUS were reported by Ferrer et al[38] who had 31% with VUS in a cohort of 32 patients with suspected TBD. By using 3D molecular modeling and functional assays, they were able to reclassify 5 VUS identified patients into the pathogenic/likely pathogenic group. Overall, both the dramatic consequences of a proper TBD diagnosis for the affected patients and their families as well as the frequencies described here clearly underline the need for an increased clinical awareness for an underlying TBD as a particularly challenging scenario in these disease entities in adults. Notably, the genetic basis is only known for ~40%–60% of DKC/TBD patients.[39–41] Moreover, recent discoveries identified even candidate genes for patients with TBD features and normal TL (Apollo) or even increased TL (POT1).[10,42] Therefore, the decoding of previously unknown genomic alterations that interfere with telomere maintenance and telomeres/telomerases regulatory functions is of utmost importance to improve understanding of TBDs and close the gap of yet unknown TBD causes.

The genotypic findings reported here fit well to the expected numbers in an adult TBD cohort, although correlations between genotype and phenotype in TBDs are difficult. As mentioned above, numerous factors related to the clinical genetics of TBDs as well as technical reasons typically related to an ultra-rare disease such as small patient numbers or selection criteria in individual cohorts hinder the assignment of specific genes to specific clinical manifestations.[8] Typically, heterozygous variants in *TERT*, *TERC*, and *RTEL1* seem to be detected in cryptic TBD, manifesting as ILD or BMFS.[6,8,28,39,43] On the contrary, *DKC1* and *TINF2* are associated with a more severe phenotype that manifests at an earlier age, so one would not expect to find these variants enriched in an adult TBD cohort.[2] The large DKC cohort presented by Alter et al[44] comprises 197 patients with the most frequently involved variants detected in *DKC1*, *TERT*, *TERC*, *RTEL1*, and *TINF2*. This is in line with our observation in this mostly adult cohort, where *TERT*, *TERC*, and *RTEL1* were found to be the most affected genes (Figure 4).

The identified variants in *TERC* reported here are all very rare in the general population. Together with shortened TL and the very low number of benign variants in *TERC* in *ClinVar* database, which aggregates information about genomic variation and its relationship to human health, we classified the reported variants in *TERC* as likely pathogenic by adding a moderate criterion of pathogenicity in patients with short TL. Our motivation to add this additional criterion for the *TERC* variants is based on the fact that although a rare variant in *TERC* was identified in 9 patients, none of them could be classified as likely pathogenic/pathogenic with existing criteria because of a lack of fitting criteria for noncoding genes. In this respect, our approach proposes a new classification for variants in *TERC* that considers functional and in vivo shortened TL as a further moderate criterion of pathogenicity in addition to the established criteria.

Our study has some limitations. Despite reporting as one of the largest real-life cohorts to date, we probably identified only a subset of confirmed pathogenic variants in the respective diagnosis categories. The NGS panel was established on the basis of the standard established in 2017, so recently discovered genes in this highly dynamic field of research were not yet investigated.[45] In addition, follow-up functional studies could help to better classify and probably categorize VUS findings into pathogenic/likely pathogenic, although functional assays are not available for nearly half of TBD-associated variants indicating the need for further investigation.[38,41]

The screening algorithm evaluated here restricted the performance of NGS to the subpopulation of patients with abnormal TL. As stated above, it needs to be pointed out that also in case of TL >10th percentile and even in cases with elongated TL, a residual possibility for an underlying TBD and thereby a diagnostic gap remains. However, based on recently published data about the predictive value of TL screening for the identification of inherited BMFS,[2,46] we feel we can confirm this approach to be justified both in terms of the underlying biology of TBD but also regarding a rational cost-benefit ratio.[47]

Particular strength of our study resides in its prospective real-life, multicenter character, the screening by using the validated gold standard for TL determination, namely flow-FISH, and the implementation of the extended screening, which led to the identification of another 7 VUS in TBD-associated genes, although their clinical relevance needs to be further evaluated.

In conclusion, we show that prospective routine TL screening should be used to identify patients with suspected TBD for consecutive genetic work-up. In older adults, the addition of a fix TL cutoff might be more useful than mere percentile curves due to the concomitant reduction of the diagnostic window with increasing age, although this approach needs further investigation regarding its clinical consequences. Due to the rarity

and complexity of the disease without adequate knowledge of optimal treatment strategies, particularly in adults, systematic screening and inclusion in disease-specific registries, monitoring of organ complications, and treatment within clinical trials are highly recommended.[48]

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AUTHOR CONTRIBUTIONS

FB, MT, and THB did conceptualization. FB, MK, RM, and MV did methodology. MT, FB, MK, RM, and MV did validation. FB, MT, MK, and RM performed formal analysis. MT, MK, RM, IH, MV, KK, AM, UP, MR, PS, SC, BH, CMW, CH, JC, MH, MK, SK, JP, SI, IK, THB, and FB did data interpretation. THB and FB did investigation. THB contributed to resources. MT and FB did data curation. MT, FB, and THB did writing and original draft preparation. MT, MK, RM, IH, MV, KK, AM, UP, MR, PS, SC, BH, CMW, CH, JC, MH, MK, SK, JP, SI, IK, THB, and FB did writing and review and editing. MT, THB, and FB did visualization. THB and FB did supervision. THB and FB did project administration. All authors have read and agreed to the published version of the article.

DISCLOSURES

THB and FB have a long-ranging scientific collaboration with Repeat Dx., Vancouver, Canada. All the other authors have no conflicts of interest to disclose.

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