

Nucleotide sugars correlate with leukocyte telomere length as part of a dyskeratosis congenita metabolomic plasma signature

Dyskeratosis congenita (DC) is a genetically well-characterized telomeropathy.^{1,2} There is increasing evidence that senescence is intrinsically linked with changes in metabolism compared to non-senescent cells.³ We have previously reported that plasma lactate, pyruvate and certain tricarboxylic acid cycle metabolites discriminate DC patients from controls with high significance, independently of glucose levels, chronological age, sex, and disease status.⁴ Here, we present a broader profiling analysis using an LC-MS-MS-based targeted metabolomics strategy, with no overlap in terms of detected metabolites with our earlier study. While targeted metabolomics is, by definition, limited to detecting only metabolites that are known a priori, it provides high-quality⁵ data that are well suited to clinical and translational research.

We analyzed a set of plasma samples taken from 29 DC patients and 30 matched controls. The study was approved by the London-City and East Research Ethics Committee (reference 07/Q0603/5). The cohorts were age-matched, with a mean of 38±17 (standard deviation [SD]) years for the control samples, and 37±17 for the patient samples (P value, Welch's t test, 0.81). Further details of the patients are given as supplementary files by James *et al.*⁴ We used a combined amine derivatization and ion-pairing chromatography analytical strategy, which gives broad metabolome coverage including highly polar metabolites.⁶

In total, we detected 146 metabolites, following filtering for quality. Thirty-one of these were significantly different at a significance cutoff of $P < 0.01$; 16 and 11 of these remained significant following correction for false discovery rate (1%), and Bonferroni correction, respectively. In particular, the nucleotide sugars uridine diphosphate glucose (UDPG) and adenosine diphosphate ribose (ADPR) combined highly significant P values with the largest fold changes of any of the metabolites (Figure 1A) (it should be noted that UDP-glucose was not chromatographically distinguished from the structural isomer UDP-galactose by our analytical method, and therefore the signal may contain contributions from both metabolites; similarly, 2-phosphoglycerate and 3-phosphoglycerate were also not chromatographically separated.) Inspection of the individual metabolites showed, as expected, clear differences between the two groups (Figure 1B). Furthermore, an unsupervised multivariate analysis (principal components analysis [PCA]) showed a clear difference between the healthy and DC patient samples along PC1, and a supervised linear discriminant analysis (LDA) was an excellent classifier, with an area under the receiver operating characteristic (ROC) curve of 0.98 (Figure 2A, B). Indeed, selecting as few as three metabolite

biomarkers was sufficient to give near-complete separation of the two groups (Figure 1D). We believe that there is significant potential to use metabolite analysis to help inform diagnosis of DC.

The DC signature was independent of age and sex. We constructed logistic regression models with disease status as the dependent variable, and age + sex + metabolite as independent variables. These showed that the P values (from effect likelihood ratio tests) were essentially unaffected by age and sex, generally lying along $x=y$ when comparing the two models (i.e., logistic regression against DC alone, vs. DC + age + sex). The only metabolite with a substantial difference between the two was UDPG, and this was actually more significant with the additional variables (age and sex) than without. For the top nine metabolites (i.e., those still significant after Bonferroni correction) all metabolites still had $P < 0.0001$ with respect to DC status.

We also analyzed the effect of sample age (i.e., length of storage in -80°C freezer, not patient age) on metabolites. The control samples consisted of two groups: "old" (i.e., collected roughly contemporaneously with the DC samples), and "new" (collected more recently). The results were highly comparable whether analyzing "old" control samples only against DC, or whether analyzing the combined old + new control group against DC (*data not shown*). In addition, we also analyzed the effect of sample age for the control samples alone. Most metabolites were highly stable (as shown by lack of significant change with respect to sample age), and furthermore, comparing the significant metabolites with and without the new sample age group showed that the pattern of separation according to DC was basically unchanged. We therefore excluded just two metabolites, where the effect of the sample age was similar to the effect of DC (iminodiacetic acid, and cysteinylglycine). The nucleotide sugars showed no evidence for association with sample age ($P=0.39$ and $P=0.33$ for UDPG and ADPR, respectively).

While there were some associations between metabolites and other, potentially confounding clinical parameters (available for DC patients only), only ornithine of the highly significant metabolites showed a weak relationship with platelet count (*Online Supplementary Table S2*; $P=0.04$ and MCV $P=0.006$). We also carried out an analysis of sub-groups; if patients with lung disease, liver disease, or skin symptoms are removed from the analysis, then the nine most significant metabolites all remain highly significant, indicating that the differences are not just down to these potential confounders (Table 1). Several metabolites correlated with age-associated leukocyte

telomere length (AATL) (available for 18 of the DC group of patients only; *Online Supplementary Table S1*). While we do not have data on other cell/tissue types, all tissues analyzed in DC have shorter age-associated telomere lengths (AATL) than normal.⁷ Furthermore, in *TERT* and *TERC* knockout mice,

postmitotic tissues inherit shorter telomeres and display telomere dysfunction and progeroid phenotypes,⁸ but leukocyte AATL may still be an indicator of systemic telomere dysfunction. In particular, the sugar nucleotides UDPG and ADPR were again distinguished from all other metabolites by

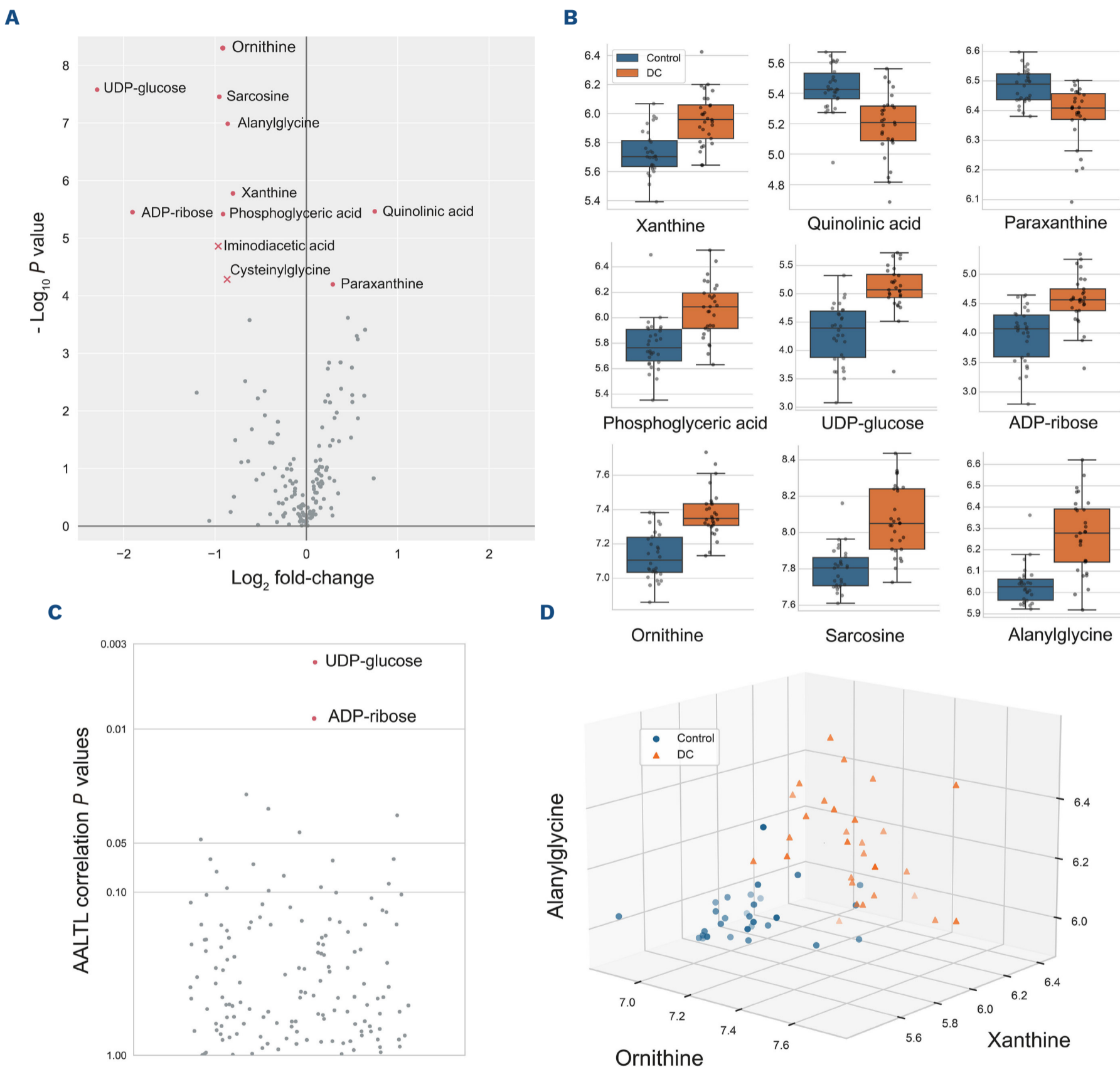


Figure 1. Individual metabolites discriminate dyskeratosis congenita patient from control samples with high accuracy. (A) Volcano plot shows fold-change (abscissa) against P value (t test, log-transformed data). Eleven metabolites are significant following Bonferroni correction and are named on the plot, although two (cysteinylglycine, iminodiacetic acid - points marked by crosses) are not considered further because of potential analytical problems. The nucleotide sugars ADP-ribose and UDP-glucose are distinguished from all other metabolites by the magnitude of the fold-change. (B) Differences between healthy and dyskeratosis congenita (DC) patient groups for individual metabolites (only metabolites significant following Bonferroni correction are shown here). (C) The nucleotide sugars ADP-ribose and UDP-glucose are distinguished from all other metabolites on the basis of their correlation with age-adjusted leukocyte telomere length (AATL; data for DC patients only): P value from correlation shown on Y axis (X axis does not represent any data, and is used purely to prevent point overlap: the points are ordered randomly.) (D) Three metabolites are sufficient to provide near-complete separation of disease classes.

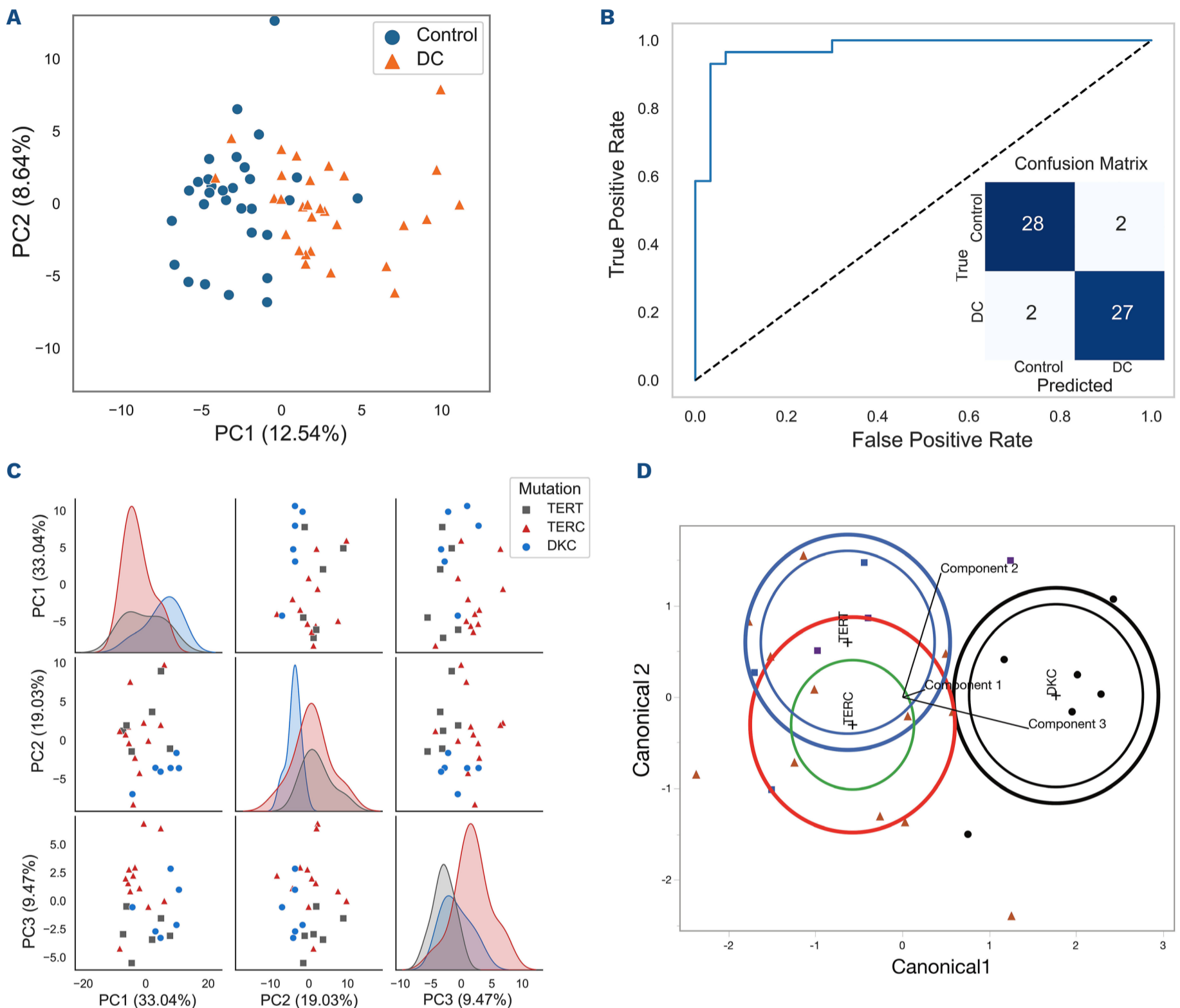


Figure 2. Multivariate analyses distinguish both disease from control, and mutational sub-groups. (A) Unsupervised principal components analysis (PCA) of scores plot shows clear difference between dyskeratosis congenita (DC) patient and control samples along PC1. (B) Supervised linear discriminant analysis (LDA; following initial dimension reduction to first 10 PC by PCA) demonstrates excellent classification into groups (area under receiver operating characteristic curve =0.98). (C) Unsupervised PCA following single-sample pathway analysis (SSPA-PCA) demonstrates potential metabolic pathway differences between disease mutation loci: PC1-3 are shown. (D) Supervised LDA shows classification of disease sub-types on the basis of metabolic pathway differences, following dimension reduction by SSPA-PCA (PC1-3).

their high correlation with AALTL (Figure 1C; Pearson's $R=-0.65$ and $R=-0.60$, respectively). This gives us confidence that the associations for these two compounds are real and biologically meaningful, in that we see within-group quantitative effects of telomere length, and not just differences between control and patient samples. In addition, in order to check that this association between UDPG and ADPR with AALTL was not confounded by other clinical parameters (white blood cell count, platelet count, mean corpuscular volume,

hemoglobin, presence of skin symptoms), we analyzed the data by regression against telomere length and each other parameter in turn, i.e., metabolite concentration = $f(\text{AALTL} + \text{parameter} + \text{AALTL} \times \text{parameter})$. The AALTL term was significant ($P<0.05$) for all ten additional models, i.e., five clinical parameters and two metabolites (Table 1). Importantly, all the nine highly significant metabolites remained highly significant when only the *TERC*-mutated group were considered (*Online Supplementary Table S1*) arguing

Table 1. Metabolites most significantly associated with dyskeratosis congenita and with telomere length.

Metabolite	<i>P</i> values for metabolites calculated for DC sub-groups				<i>P</i> values for metabolite association with telomere length, including other clinical factors as possible confounders					
	No lung disease N=23	No liver disease N=24	Neither liver nor lung disease N=22	No skin symptoms N=14	None	White blood cell count	Platelet count	Mean corpuscular volume	Hemoglobin	Presence of skin symptoms
ADP-ribose ^a	2.1x10 ⁻⁶	2.6x10 ⁻⁵	2.2x10 ⁻⁶	0.01	0.0088	0.0036	0.014	0.0087	0.0061	0.0033
Alanylglycine ^a	1.5x10 ⁻⁶	1.2x10 ⁻⁷	2.7x10 ⁻⁷	0.002	-	-	-	-	-	-
Ornithine ^a	1.9x10 ⁻⁷	4.4x10 ⁻⁷	7.9x10 ⁻⁷	9.9x10 ⁻⁶	-	-	-	-	-	-
Paraxanthine ^b	0.0002	0.0002	0.0005	0.008	-	-	-	-	-	-
Phosphoglycerate ^a	3.7x10 ⁻⁶	1.7x 10 ⁻⁶	2.1x10 ⁻⁶	0.003	-	-	-	-	-	-
Quinolinate ^b	2.5x10 ⁻⁶	1x10 ⁻⁵	6.2x10 ⁻⁶	0.004	-	-	-	-	-	-
Sarcosine ^a	7.8x10 ⁻⁸	3.2x10 ⁻⁷	1.1x10 ⁻⁷	0.0008	-	-	-	-	-	-
UDP-glucose ^a	7.3x10 ⁻⁹	7.9x10 ⁻⁹	7.7x10 ⁻⁹	2.3x10 ⁻⁶	0.0039	0.0049	0.0095	0.0087	0.0061	0.0046
Xanthine ^a	1x10 ⁻⁵	2.1x10 ⁻⁵	2.9x10 ⁻⁵	7.9x10 ⁻⁶	-	-	-	-	-	-

The metabolites most significantly associated with dyskeratosis congenita (DC) and with telomere length remain significant, even when excluding patients with lung disease, liver disease, or skin symptoms (for all 9 metabolites significant after Bonferroni correction), or allowing for the other clinical parameters listed in this table (for the nucleotide sugars UDP-glucose and ADP-ribose only). ^aMetabolites increased in DC. ^bMetabolites decreased in DC.

strongly against proposed extrachromosomal functions of telomerase affecting the metabolite levels. Analysis of the metabolite data showed no clear effect of mutation locus (see the *Online Supplementary Appendix; Online Supplementary Figure S1*). However, single sample pathway analysis (SSPA) has recently been introduced as a novel approach for analysis of metabolomic data: the data are transformed from the metabolite space into a pathway space, which can provide additional power for revealing biological phenotypes.⁹ An unsupervised multivariate analysis (PCA) of the SSPA-transformed data shows, indeed, that the phenotypic separation that we see is subtly different from that of the raw data, and potentially very interesting. For example, the main separation between control and DC samples dropped down from PC1 to PC4 ($P=7.2 \times 10^{-7}$, *t* test); and there was a very clear difference between males and females on PC2 ($P=1.9 \times 10^{-6}$, *t* test). Therefore, we also carried out SSPA-PCA on the DC data alone, in order to look for possible differences between mutation subtypes. There were some possible differences between subtypes, as shown by an unsupervised analysis (Figure 2C); using the supervised LDA (with an initial dimension reduction step to the first 3 PC), then there was a clearer separation between DKC against the *TERC/TERT* mutation groups, which were overlapping (Figure 2D; area under the ROC curve for DKC group =0.98).

Several of the metabolic alterations detected in DC have potential significance for the role of telomeres in human aging and age-related conditions. It is tempting to look at the changes in ADPR, in particular, and conjecture a link to poly (ADP-ribose) polymerase activity. Five of the ADP-ribose transferase (ART) family have clear roles in genome maintenance and are involved in telomere biology.¹⁰ Perhaps the changes in free ADPR are simply reflecting

changes in overall ADP ribosylation. This, however, ignores the fact that UDPG is altered similarly to ADPR, both in terms of direction and amount of change, and therefore we think the changes in nucleotide sugars are more likely to be indicative of a broader biochemical change - for instance, a change in the balance between anabolism and catabolism.

Of further interest it has recently been reported that serum taurine levels decline in parallel with human chronological age and that taurine protects against telomere loss in fish but does not affect telomerase activity in fish or mice.¹¹ There were no changes in plasma taurine or hypotaurine levels in DC patients *versus* controls, suggesting telomere disruption is unlikely to drive taurine depletion in adult humans. Aspartate and sarcosine accumulation are associated with sarcopenia,¹² which is linked to frailty; if we also consider metabolites significant at a false discovery rate (FDR) of 5% (*Online Supplementary Table S2*), this then also covers β -hydroxyisovalerate, which is depleted in sarcopenia.¹³ Hydroxylysine accumulation is associated with collagen degradation,¹⁴ which is implicated in wrinkles; ascorbate is also essential for collagen production, and this is also significantly different, although actually increased in the DC group. Most interesting of all the depletion of four neuroprotective metabolites (hydroxykynurenine,¹⁵ kynurenine,¹⁵ N-acetyl-leucine,¹⁶ norvaline and paraxanthine¹⁷) as well as paradoxically three neurotoxic ones (N-acetyl-iso-leucine, quinolate¹⁵ and quinolate carboxylate¹⁵) was observed in DC patient plasma, indicating a possible link between telomere dysfunction and neuronal function. Many of these metabolites have been linked with other telomeropathies such as ataxia telangiectasia,¹⁶ in addition to Alzheimer disease, Parkinson disease and amyotrophic lateral sclerosis.

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Disclosures

No conflicts of interest to disclose.

Contributions

YL and VSK carried out analytical data acquisition and processing. YL, EKP and JGB carried out statistical analysis. ID was responsible for coordinating clinical sample collection. EKP, ELNJ, ID, and JGB were responsible for planning and conceptualizing the study. All authors read and approved the final draft.

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Data-sharing statement

The data are available from the corresponding author on request.