

## Dyskeratosis Congenita links telomere attrition to age-related systemic energetics

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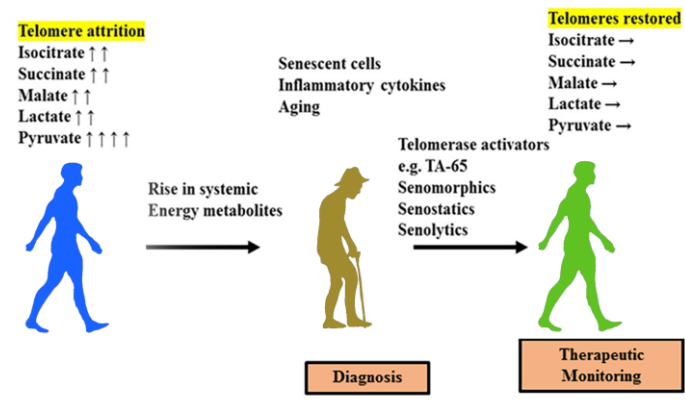
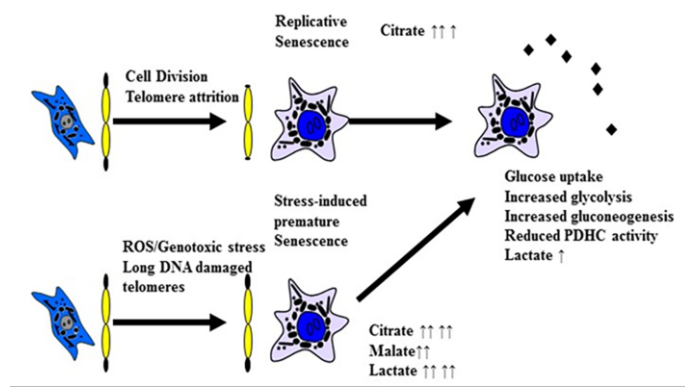
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## Abstract

Underlying mechanisms of plasma metabolite signatures of human ageing and age-related diseases are not clear but telomere attrition and dysfunction are central to both. Dyskeratosis Congenita (DC) is associated with mutations in the telomerase enzyme complex (*TERT*, *TERC*, and *DKC1*) and progressive telomere attrition. We analyzed the effect of telomere attrition on senescence associated metabolites in fibroblast conditioned media and DC patient plasma. Samples were analyzed by gas chromatography/ mass spectrometry and liquid chromatography/ mass spectrometry. We showed extracellular citrate was repressed by canonical telomerase function *in vitro* and associated with DC leukocyte telomere attrition *in vivo*; leading to the hypothesis that altered citrate metabolism detects telomere dysfunction. However, elevated citrate and senescence factors only weakly distinguished DC patients from controls, whereas elevated levels of other tricarboxylic acid cycle metabolites, lactate and especially pyruvate distinguished them with high significance. The DC plasma signature most resembled that of patients with loss of function pyruvate dehydrogenase complex mutations and that of older subjects but significantly not those of type 2 diabetes, lactic acidosis, or elevated mitochondrial reactive oxygen species (1-3). Additionally, our data are consistent with further metabolism of citrate and lactate in the liver and kidneys. Citrate uptake in certain organs modulates age-related disease in mice and our data has similarities with age-related disease signatures in humans. Our results have implications for the role of telomere dysfunction in human ageing in addition to its early diagnosis and the monitoring of anti-senescence therapeutics, especially those designed to improve telomere function.

**Key Words:** metabolism; telomeres; human ageing; cellular senescence; citrate.

# Graphical abstract

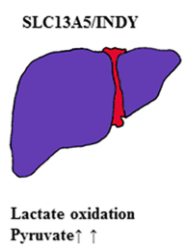


### DC plasma

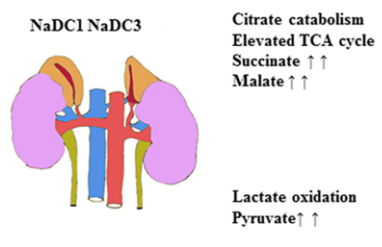
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- Aconitate ↑
- Isocitrate ↑↑
- Oxaloacetate ↓
- Succinate ↑↑
- Malate ↑↑
- IL-6
- IL-1
- Alpha ketoglutarate →
- Urate →
- 7-methylguanine →
- C-mannosyltryptophan →

- Lactate ↑↑
- Pyruvate ↑↑↑↑
- Low Lactate/Pyruvate Ratio

### Liver metabolism



### Kidney metabolism



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SCRIPT

## Introduction

Cellular senescence is a dynamic process induced by a variety of cellular stresses, including irreparable DNA double strand breaks (IrrDSBs). IrrDSBs accumulate at telomeres following telomerase deficiency following telomere attrition (4) or in non-dividing cells due to inadequate DNA repair (5, 6). IrrDSBs are resolved by telomerase in dividing cells (7, 8) but not so readily in non-dividing cells.

Telomeres shorten in most human tissues with chronological age (9). Additionally, age-adjusted leukocyte telomere length (AALTL) is associated with poor health, especially cardiovascular disease (10), and is reversible upon positive changes in lifestyle (11) or telomerase activators (12).

In mice, telomere dysfunction induced by complete *Tert* or *Terc* deletion results in IrrDSBs, impaired mitochondrial biogenesis and function, decreased gluconeogenesis, cardiomyopathy, and increased production of reactive oxygen species (ROS). These phenotypes are countered by increased telomerase function (13) and excessively long telomeres (13, 14), to benefit lifespan and health span. However, the extent of telomere attrition in dividing human cells with age is less dramatic than in *tert*<sup>-/-</sup> or *terc*<sup>-/-</sup> mice.

Telomere dysfunction is associated with senescence in human disease (15) but its influence on human metabolism and in particular the plasma biomarkers of age-related diseases, is not clear. To investigate this, we took advantage of Dyskeratosis Congenita (DC) patient plasma samples. DC patients possess heterozygous (*TERT/TERC*) or hemizygous (*DKC1*) rather than homozygous loss of function mutations, and DC is phenotypically closer to the *terc*<sup>+/-</sup> mouse than the *tert*<sup>-/-</sup> or *terc*<sup>-/-</sup> mouse (16).

DC patients display premature aging phenotypes and short telomeres in their tissues *in vivo* (17, 18) and premature senescence *in vitro* (19). DC patients are a useful study group for the investigation of the effects of telomere dysfunction and senescence on human metabolism because

the mechanism of premature senescence is characterised and DC patients do not usually have confounding conditions such as type 2 diabetes (T2D) at an early age, which would affect metabolism.

Telomere dysfunction culminates in cellular senescence, which is accompanied by the accumulation of an array of extracellular proteins and metabolites, known as the senescence-associated secretory phenotype (SASP (20)) and the extracellular senescence metabolome (ESM (21)), respectively. Some metabolites of the ESM are associated with chronological ageing in humans (22, 23), but the underlying mechanisms are largely unknown. Several anti-senescence approaches are now being considered to ameliorate age-related diseases, including senolytic drugs (24), which have shown some promise in clinical trials. However, the non-invasive detection of senescent cells and/or telomere attrition in human disease is challenging owing to the small number of senescent cells and/or dysfunctional telomeres. SASP proteins are not generally detectable, their detectability varies in different disease states (25, 26) and it is acknowledged that additional plasma biomarkers are needed (reviewed in (24)).

The ESM metabolite citrate is particularly interesting because its uptake has been implicated in ageing, caloric restriction, type 2 diabetes, blood pressure, heart rate, memory, adipocyte inflammation, epigenetic regulation and cancer (reviewed in (27)) and so we initially investigated this metabolite.

## **Materials and methods**

### **Cell characterization, senescence induction by ionizing radiation and collection of conditioned medium**

The cell culture methods, the senescence-associated  $\beta$  galactosidase assay and conditioned medium collection have been described previously (21). Glucose was measured using the Glucose Assay Kit, (Abcam, Cambridge, MA), following the manufacturers instructions. BJ and NHOF-1 cells

have been described previously (21) . BJ cells are normal newborn dermal fibroblasts (28) and NHOF-1 cells are normal oral fibroblasts derived from the buccal mucosa (29). Both BJ cells and NHOF-1 cells have low levels of p16<sup>INK4A</sup> (30) and can be immortalized by telomerase (28) see also Figure1. BJ and NHOF-1 cell line panels were tested for mycoplasma using a Lonza MycoAlert™ Mycoplasma Detection Kit and found to be negative.

### **Retroviral infection of BJ and NHOF-1 cells**

pBABE retroviral vectors on the puromycin-resistant backbone expressing *TERT* and *TERT-HA* (31) or the empty vector were obtained from Addgene Europe, Teddington, Middlesex, UK. Retroviral particles were produced by transfection of phoenix A cells and used to infect target cells. Transduced cells were selected with puromycin.

### **Patients, normal subjects, plasma collection and ethics**

Ethical approval for the collection of normal and DC plasma samples and the metabolomics analysis was granted by the London-City and East Research Ethics Committee (certificate number 07/Q0603/5). Blood (2-10mL) was collected by venipuncture using a 19-gauge butterfly needle into EDTA vacutainers from non-starved healthy volunteers and DC patients. Within 2 hours, blood was centrifuged at 1300g for 15 minutes at 4°C to pellet the blood cells. Following this, the plasma supernatant was transferred to a 1.5 mL Eppendorf centrifuge tube and centrifuged again at 20,600g for 2 minutes at 4°C. Plasma was then transferred to a fresh Eppendorf centrifuge tube and snap frozen in a dry ice/ethanol bath for at least 15 minutes before storage at -80°C.

Patient symptoms, white blood cell counts, platelet counts, hemoglobin and mean red blood cell corpuscular volume were all recorded at the time of diagnosis and in subsequent clinical assessment when samples were collected.

## **AALTL measurement**

Whole blood telomere lengths were measured using the monochrome multiplex quantitative PCR (qPCR) method (32).

## **Telomere length, *hTERT* transcript and telomerase activity measurement**

For the absolute telomere length analysis, a qPCR method was performed as described in (33). Telomerase activity using qPCR was performed using the TRAP protocol (34) and primer sequences as described. The quantification of fully spliced *hTERT* and GAPDH mRNA was described previously (35). RQ data analysis was initially performed using the SDS 2.4 software (Applied Biosystems; Thermo Fisher Scientific, Inc.) then plotted as bar charts in Prism.

## **Enzyme-Linked Immunosorbent Assay (ELISA)**

To measure the cytokines (IL-1 $\alpha$ , and IL-6), a sandwich ELISA method (Quantikine<sup>®</sup> ELISA Immunoassay, R&D Systems, Abingdon, UK) was employed following the manufacturer's protocol. The detection limits were 3.9-250 pg/ml IL-1 $\alpha$  and 3.13- 300 pg/ml for IL-6.

## **Metabolomic analysis**

### **(a) Unbiased metabolomic screen**

The details of the unbiased metabolic screen have been published previously (21).

### **(b) Targeted measurement of extracellular citrate by gas chromatography/mass spectrometry (GC-MS).**

The methods for targeted GC-MS analysis have been described previously (30).

### **(c) Metabolite extraction and targeted analysis by liquid chromatography/mass spectrometry**

For the analysis of organic acids from plasma, samples stored at -80°C were thawed at room temperature and 100  $\mu$ l aliquots treated with methanol (400  $\mu$ l) to deproteinize the samples. The



sample order was randomized before extraction. The samples were vortexed briefly, kept on ice for at least 60 min, then centrifuged (7378 g) for 10 min at 4°C. Multiple aliquots of the supernatant were collected and transferred to new microcentrifuge tubes. For the pooled QC sample, 75 µl of each sample supernatant was mixed in a new tube. Supernatants were dried in a centrifugal vacuum concentrator (Savant Speedvac, Thermo), and kept at -80°C until analysis.

Samples were reconstituted for analysis in 75 µl of a solution containing stable isotope labelled (SIL) internal standards, made up in LC-MS grade water at 1 µg/mL. The SIL standards were L-malic acid-<sup>13</sup>C<sub>4</sub>, succinic acid-<sup>13</sup>C<sub>4</sub>, sodium lactate-<sup>13</sup>C<sub>3</sub>, citric acid (1,5,6-carboxyl-<sup>13</sup>C<sub>3</sub>) and L-phenyl-d<sub>5</sub>-alanine (this last used to monitor the consistency of 29 injection volumes, but not otherwise used to normalize the data). The reconstituted samples were then centrifuged and transferred to LC-MS vials.

The samples were analysed by ion-pairing chromatography-mass spectrometry, as described fully in (36), with additional transitions added for the SIL standards, as listed in (37), using a XEVO TQ-S tandem mass spectrometer coupled to a Acquity UPLC binary solvent manager equipped with a CTC autosampler (Waters Ltd, Wilmslow, UK). Briefly, data were acquired with electrospray ionization in negative mode, and the chromatography used a Waters HSS T3 column (1.8 µm, 2.1 x 100 mm) with a binary solvent system of 10mM tributylamine + 15mM acetic acid in water (solvent A), and 80% methanol + 20% isopropanol (solvent B) to 100%. The sample run order was randomized. Before analysis, injections of double blanks (water) and single blanks (SIL standard mix) were performed to ensure system stability, and to identify carryover/contaminant peaks. A pooled QC sample was injected at the beginning of the run and then once every 10th injection throughout the run, to assess instrument stability across the entire analytical run. The compounds monitored were pyruvate, lactate, succinate, oxaloacetate, malate, 2-oxoglutarate, 7-methyl guanosine, urate, aconitate, isocitrate, citrate, α-C-mannosyltryptophan, plus the SIL standards as described above.

The LC-MS data were processed using Skyline (MacCoss Lab, (38)). Blanks and QC samples were used to exclude contamination and low quality samples, with acceptance criteria of <0.3 relative standard deviation (RSD) for QC samples, and <1% peak area (of QC) in blanks. Metabolite concentrations were expressed as the ratio relative to the values for the SIL internal standards for each metabolite; except for those metabolites without a SIL standard available (pyruvate, isocitrate,  $\alpha$ -C-mannosyltryptophan, urate, 7-methylguanosine, oxaloacetate, aconitate), for which relative count data were used.

## **Statistical Methods**

### **(a) Multivariate analysis**

Two-way hierarchical clustering was performed using standardized data and Ward's method of linkages for the control and DC groups separately. The heat map represents the compound concentrations. Principal components analysis was carried out using auto-scaled and mean-centered data. The first four PCs were inspected for associations with disease status, and the first 10 PCs used as input for Fisher's linear discriminant analysis.

### **(b) False discovery rates (FDRs)**

FDRs of 0.05 were determined using an adaptive linear step-up procedure (39).

### **(c) Other statistical methods**

Cell culture and unbiased metabolomics screen data were analyzed by Student's unpaired T test. Raw and normalized plasma metabolomics data were analyzed by the Wilcoxon-Mann-Whitney and Mann-Whitney U tests and corrected in the latter case for false discovery rate. Linear regression analysis was conducted by using the Excel data analysis package and the graphs prepared in Excel.

## Results

### **Citrate is regulated by telomere function and the canonical function of telomerase, independently from inflammatory cytokines *in vitro***

Extracellular citrate (EC) and interleukin 6 (IL-6) both increased in senescent fibroblasts. The catalytic subunit of telomerase, *TERT*, reduced levels of EC (Figure 1A, 1B) in parallel with the frequency of senescence-associated beta galactosidase (Figure 1C, 1D) in both BJ fibroblasts and NHOF-1 cells, but the empty vector and *TERT-HA* (extrachromosomal telomerase functions only) did not (see also Figure 1E). The *TERT-HA* construct was made by Meyerson and colleagues (40) and turned out to be very useful because it does not lengthen telomeres or immortalize cells (31) even when ectopically expressed making it a useful control for *TERT* over expression and extrachromosomal (non-canonical) *TERT* functions (41).

Only the *TERT* transgene was able to increase telomere length (**Figures 1F, 1G**) despite the fact that *TERT-HA* was expressed (**Figure 1H, 1I**) and induced telomerase activity in BJ cells (**Figure 1J**). These data demonstrate that EC is regulated by the canonical function of telomerase and links telomere function to EC accumulation in senescent fibroblasts. Neither *TERT* nor *TERT-HA* reduced IL-6 levels in BJ cells (**Figure 1K**) under the *in vitro* conditions described here indicating that EC is regulated independently from the inflammatory cytokines. These *in vitro* results gave rise to the hypothesis that plasma citrate is upregulated *in vivo* by telomere attrition.

## **Citrate, malate and lactate are upregulated in senescent cells induced by IrrDSBs**

EC is upregulated following replicative senescence of oral fibroblasts (21). However, IrrDSB-induced senescence (IrrDSBsen), also induces telomere dysfunction along with EC, malate and lactate and a depletion of pyruvate (all extracellular; see eFigure 1 in the supplement) consistent with a shift of senescent cell metabolism towards glycolysis.

## **DC patients**

The clinical and genetic characteristics of the DC patients are described in (eTable 1 in the supplement), and the age and gender of all DC and control subjects in eTable 2 in the supplement.

## **Upregulation of plasma citrate in DC patients detected by gas chromatography/mass spectrometry (GC-MS)**

First, we analysed the citrate levels DC samples (n = 28) and controls (n = 12) using the GC-MS platform (Figure 2 top left). There was no significant difference in citrate levels when non-starved plasma and starved serum samples were compared, suggesting that nutritional status did not affect plasma citrate levels. (eFigure 2A in the supplement; P=0.60). Plasma citrate levels were resistant to haemolysis (eFigure 2B in the supplement) and stable at room temperature for 24h so citrate is a highly stable plasma metabolite in healthy controls. Citrate showed a strong trend for upregulation in DC patients when compared to controls (Figure 2 top left) but the effect was of only borderline significance (P=0.06) except when only patients with more severe aplastic anaemia symptoms were considered (P=0.008). Asymptomatic DC patients in this study set were not significantly different from controls (P=0.74).

## DC patients show specific changes in energy metabolism

As plasma citrate is tightly regulated *in vivo*, we considered the possibility that citrate might be further metabolised *in vivo* to other energy metabolites. We used targeted liquid chromatography/mass spectrometry (LC-MS) on an overlapping but distinct sample set to measure energy metabolites and a subset of ESM metabolites (21) in DC patient (n=29) and control (n =30) plasma (Figure 2; eFigure 3 in the supplement). The metabolites were selected because they are altered in age-related diseases *in vivo* (23, 42-46).

The TCA cycle metabolites isocitrate (P=0.0007), malate (P=0.0005), succinate (P=0.008) and to a lesser extent oxaloacetic acid (P=0.06) aconitate (P=0.03) and citrate (P=0.08) were elevated in DC samples, but other TCA cycle metabolites such as alpha ketoglutarate (AKG: P=0.39) were not significantly altered. Lactate (P=0.0003) and pyruvate (P=0.0000007) levels were consistently elevated in DC patients relative to controls (Figure 2; supplementary eFigure 3; supplementary eTable 3A) and were significant after correction for false discovery rate (eFigure 3B in the supplement). Significantly, glucose levels were within the normal range in DC plasma and the lactate:pyruvate ratio (LPR) was lower than normal (P=0.005) arguing against lactic acidosis and T2D.

The *DKC1* gene is X-linked and the DC patient set had a preponderance of males. However, all changes remained when only males were considered (Figure 2; eTable 3 in the supplement), in patient subsets with *TERC* or *TERT* mutations/variants and in the case of pyruvate, *DKC1* as well (eTable 3 in the supplement), arguing against a role for non-canonical functions of *TERC* and *TERT*. With the exception of succinate, all changes were significant in weakly symptomatic patients (eTable 3 in the supplement).

## **A subset of TCA metabolites, lactate and pyruvate clearly distinguish DC patients from control subjects with high specificity and selectivity**

Receiver operator characteristic (ROC) curves are shown in Figure 3 and clearly show the TCA cycle metabolites isocitrate (area under the curve (AUC) = 0.76), malate (AUC = 0.77), succinate (AUC = 0.75), lactate (AUC = 0.77) gave acceptable discrimination between controls and DC; pyruvate (AUC = 0.88) gave a value that was excellent bordering on outstanding. Thus, high plasma pyruvate alone could indicate systemic telomere attrition.

## **Relationship of DC profile to leukocyte age-associated telomere loss (LAATL)**

Linear regression analysis (Figure 4A; eTable 4 in the supplement) showed that only citrate (P=0.01) and malate (P=0.03) levels correlated with LAATL. In the smaller GC-MS data set only citrate correlated with LAATL in the DC subgroup with *TERC* mutations by linear regression analysis (P=0.006).

In addition, as with telomerase-deficient mice (16), LAATL decreases with each generation (47). We therefore examined the DC profile in individual family members from four DC families harbouring the same *TERT* or *TERC* mutation/ variant (Figure 4B). Although the numbers were small, in three families, the offspring had higher levels of citrate and malate than their parents/ aunts. In two families, lower LAATLs were also associated with higher IL-6. These data support the inverse relationship between these metabolites and LAATL.

## **DC patients show a shift in energy metabolism indicative of increased citrate catabolism**

Unsupervised multivariate analyses also showed clear differences between the control and DC groups. The two groups were largely separated by principal component analysis within the first two components (eFigure 4 in the supplement). There was no association with patient age for the

first four principal components ( $P > 0.05$  for all). The between-metabolite correlations were also different in the two groups, as showed by both clustered heat-maps of the Pearson correlations (Figure 5A), and two-way hierarchical clustering of the two groups (Figure 5B). Unsurprisingly, plasma TCA metabolites citrate, isocitrate and aconitate clustered together in normal subjects whilst lactate, pyruvate and malate formed a separate cluster. However, in DC plasma, citrate, aconitate and isocitrate became much more strongly associated with malate and succinate indicating a shift in systemic metabolism in DC patients. In addition, isocitrate and malate became more strongly associated with lactate in DC patient plasma. Linear regression analysis (eTable 5 in the supplement) and scatter plot matrices (eFigures 5A and 5B in the supplement) further supported these conclusions. Furthermore, supervised linear discriminant analysis (LDA) correctly classified 24 out of 30 control and 24 out of 28 DC patient samples; however as the AUC (0.90) was not significantly different from the best single metabolite AUC values (95% confidence intervals 0.87-0.99, 5000 bootstraps), we did not explore the use of LDA further.

#### **DC patients show elevated levels of energy metabolites in the absence of high levels of SASP and other ESM factors**

We did not have access to tissue biopsies of DC patients so to estimate the amount of cellular senescence we measured several SASP and ESM factors. Interleukin 1 alpha (IL-1 $\alpha$ ) and IL-6 levels were measured in some of the samples. IL-6 levels were generally undetectable in control subjects, as expected, but were also very low in most of the DC samples and not significantly different from controls ( $P=0.09$ ). IL-1 $\alpha$  levels were below detection limits in all but one of the DC patients (Figure 2; supplementary eFigure 2; supplementary eTable 3A).

In addition, we tested ESM metabolites, previously associated with ageing (23), mortality (43, 44) and age-related disease (43, 44). The plasma concentrations of the ESM metabolites urate, 7-methyl guanine (7-MG) and C-mannosyl tryptophan were not significantly different from controls

(Figure 2; supplementary eFigure 2; supplementary eTable 3). The data indicate a low level of cellular senescence in DC patients.

### **DC profile and SASP factors**

Only citrate ( $P=0.01$  when analysed by GC-MS), isocitrate ( $P=0.02$ ), and 7-MG ( $P=0.008$ ) correlated with plasma IL-6 levels (eTable 6 in the supplement), indicating that the energy metabolite changes were largely independent of IL-6.

### **DC profile and clinical parameters, age and gender**

In both the GC-MS and LC-MS data sets there was no significant relationship between any of the above changes with any clinical indicators of aplastic anaemia, gender, donor age or different control batches and with the exception of oxaloacetic acid and urate repeat samples where available varied by less than 25% (1-22%). (eTables 7-10 in the supplement). Low dose Danazol treatment in a small patient subset, had no effect on the results, succinate excepted (eTable 11 in the supplement).

### **Discussion**

The ESM metabolites citrate and malate correlated with LAATL but other metabolites were better at separating DC patients from controls, indicating further, or tissue specific effects of telomere attrition on metabolism. DC plasma metabolites were not associated with any particular disease phenotype, and so are unlikely to be a consequence of disease. Trivial explanations such as cell lysis and exosome release are inconsistent with the normal levels of the cytoplasmic metabolites urate and AKG in DC plasma.



Significantly, virtually all aspects of the DC profile increase with chronological age and age-related disease (22, 23, 27, 42, 48). High isocitrate, aconitate and malate are associated with frailty, cardiovascular disease and mortality in humans (42, 45, 46). Therefore, the plasma energy alterations are not limited to DC. AKG, which extends lifespan and protects against frailty and inflammaging in mice (49), was not significantly altered, consistent with recent studies of aged or frail humans (22, 42, 45, 48). However, plasma AKG increases in centenarians (48) and although this may reflect further telomere attrition or age-related disease, it could reflect an adaptive mechanism in long-lived individuals.

Although it is difficult to speculate on mechanistic details from plasma metabolite levels the low LPR and normal levels of urate, distinguish DC from T2D, most respiratory chain disorders and all forms of acidosis (1-3). The high levels of isocitrate and aconitate in DC are opposite to those in humans deficient in the ROS-sensitive (50) mitochondrial aconitase (ACO2) (51), arguing against an increase in mitochondrial ROS in DC. The DC profile most closely resembles pyruvate dehydrogenase complex (PDHC) deficiency (3) and a shift towards glycolysis.

Only citrate and malate levels correlated with LAATL yet isocitrate, succinate, lactate and pyruvate were consistently elevated in DC plasma. Telomere attrition may have different direct consequences on metabolism in other cell types such as the liver.

An alternative hypothesis is that elevated plasma citrate in DC patients enters the liver and kidneys to drive the TCA cycle to cause altered metabolism. A recent study conducted in pigs offers some support for this hypothesis. Sampling of arterial and venous blood in pigs showed that citrate taken up by the kidneys contributes to the TCA cycle to produce malate and succinate (52), which accumulate in kidney tissue (52). This could explain the higher plasma levels of these and other TCA metabolites in DC plasma and with chronological age.

Lactate and pyruvate levels are also associated with chronological ageing in humans (22, 23, 48) as well as DC. Lactate is oxidised predominantly in the liver and kidneys to pyruvate (52) to regulate its levels and could produce the low lactate:pyruvate ratio and high pyruvate observed in the DC patient plasma.

Tert <sup>-/-</sup> and Terc <sup>-/-</sup> mice telomeres often lack detectable telomere DNA and form end-to-end chromosomal fusions reminiscent of crisis and cancer. However, a recent study of a large cohort of DC leukocytes employing Single Telomere Length Analysis (STELA) showed no evidence of telomere fusions such as those found in cellular crisis or cancer despite the fact that some telomere lengths are very short (53). This suggests that the observed phenotypes in DC are mediated independently of telomere fusions, perhaps by unpaired DNA double strand breaks, which are known to occur following telomere attrition and replicative senescence (4).

Regardless of the underlying mechanism, the systemic accumulation of energy metabolites in DC and ageing subjects would likely accelerate ageing and cellular senescence as caloric restriction does the reverse (54).

The most important aspect of the data described here is that a subset of plasma metabolites (isocitrate, malate, succinate, lactate and pyruvate) outperformed SASP factors in distinguishing DC patients from controls. Such metabolites may be valuable indicators of telomere dysfunction in humans and consequently therapies designed to reverse it (12, 55).

## Conclusions

In summary, although mechanistic details are still to be elucidated, plasma metabolomics may have considerable utility in the monitoring of telomere dysfunction in human disease, in regenerative medicine and anti-ageing therapies. In addition, combining metabolomics with defined human mutations may shed light on the underlying mechanisms of ageing and contribute to the early diagnosis of age-related diseases.

## Funding

The work was supported by the Dunhill Medical Trust (grant number R452/1115) and Barts and the London Charity (grant number MRD&U0004) and European Union H2020, grant number 633589. Karen-Ng Lee Peng received a Ph.D. scholarship (Hadiah Latihan Persekutuan) from the Malaysian Ministry of Education.

## Acknowledgments

We thank Professor Tom Vulliamy for performing the telomere length analysis of the human leukocytes, Dr. Harriet Allan and Professor Tim Warner for assistance with blood samples from normal volunteers, Dr. Amy Lewis for technical advice and Mr. Lindsay Parkinson for graphical design. We would also like to thank Dr. Fabian Flores-Borja for valuable discussions regarding the metabolism of citrate and Professors Andrew Silver and Julie Adam (University of Oxford) for critical reading of the manuscript.

## Author contributions

E.N.J and M.B performed the Gas Chromatography/ Mass Spectrometry analysis of the conditioned medium and plasma samples; V.S-K performed the Liquid Chromatography/ Mass Spectrometry analysis of plasma samples; M.M, E.K.P and J.B performed the statistical analysis of all the data; E.K.P and J.B prepared the figures; K-N.L.P performed the ELISA assays; T.R performed the qPCR analysis of *TERT* and telomerase on the fibroblast lines; S.M performed the telomere length analysis. I.D provided the patient samples, the telomere length data and all the clinical data in addition to obtaining ethical approval for the study; E.K.P conceived the study, obtained funding, performed most of the *in vitro* experiments and wrote the initial draft of the manuscript; E.K.P, M.M, I.D and J.B analysed the data and finalised the writing of the manuscript.

### **Conflict of interest statement**

Maria Mycielska is a co-inventor on the Patent Application no. EP15767532.3 and US2020/408741 (status patent pending) and US2017/0241981 (patent issued) “The plasma membrane citrate transporter for use in the diagnosis and treatment of cancer” owned by the University Hospital Regensburg. The other authors declare no conflict of interest.

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**Figure 1 Ectopic *TERT* but not *TERT-HA* expression suppresses extracellular citrate levels in parallel with senescence bypass.**

**A.** and **B.** The effect of ectopic *TERT* expression on citrate levels in BJ cells (**A.**) and NHOF-1 oral fibroblasts (**B.**) (n=3). Data are means +/- standard deviation. \*  $p < 0.05$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ . **C.** and **D.** The percentage of senescent cells in **A.** and **B.** as assessed by SA- $\beta$ Gal **C.** n =3 and **D.** is derived from one experiment. Symbols are the same as for **A.** and **B.** **E.** Typical images of late passage SA- $\beta$ Gal-stained BJ cells transduced with the empty PURO vector, the *TERT-HA* construct (extrachromosomal *TERT* functions only) and *TERT*; black scale bar = 100 $\mu$ m. **F.** and **G.** The telomere lengths of the cells analysed in **A.** and **B.** at the point of senescence showing a clear increase in telomere length in NHOF-1 cells expressing *TERT*. Data is from two replicate runs of the same samples. **H.** and **I.** *TERT* mRNA levels in the cells from **A.** and **B.** at the point of senescence. Showing high levels of *TERT* and *TERT-HA* mRNA in both BJ and NHOF-1 cells. PC3 mRNA is the positive control. **J.** Telomerase activity in the BJ cells from **A.** The data are derived from one experiment in **A.** PC3 is the positive control and heat-treated PC3 extract is the negative control. **K.** IL-6 levels in the BJ cells from **A.** (n=3). Data are means +/- standard deviation. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

**Figure 2 Citrate, glucose, IL-6 and normalised metabolite levels in DC patient and control subject plasma.**

The far left hand panel in the top row shows citrate levels in DC (n= 28) and normal control (n=12) subjects as assessed by GC-MS. DC patients with mild aplastic anaemia (0-1 abnormal clinical indicators n=10), severe (3-4 clinical indicators n=12) or a combination of severe and moderate (2-4 clinical indicators n=18) are also shown to assess the effect of aplastic anaemia on disease severity. Each point represents the average of between one and three determinations all performed at the same time. Glucose levels are in mM in a subset of the DC patients (n=26) and control subjects (n=20). IL-6 levels are in pg/mL in a subset of the DC patients (n =15) and control subjects (n= 21). All remaining panels show the LC-MS metabolite levels normalised to the average of the control subject levels in DC (n=29) and control (n=30). All P values were determined by the Wilcoxon-Mann Whitney Rank Test and/or Welch's Test and both methods gave similar P values.

**Figure 3 ROC curves of TCA metabolites, lactate and pyruvate in DC patient and control subject plasma.**

The data show ROC curves of the normalised LC-MS metabolite levels shown in Figure 2 but identical data was obtained from non-normalised values. The area under the curve (AUC) and the P values are given on each graph. An AUC of more than 0.70 is considered good and a value of more than 0.80 is considered excellent.

**Figure 4 Citrate is associated with DC leukocyte telomere loss and a shift in systemic energy metabolism.**

**A.** Linear regression analysis showing a statistically significant association between age-adjusted telomere reduction (LAATL) and plasma citrate (left panel) and malate (right panel) in DC patients ( n=19). **B.** Non-normalised data from four DC families showing that DC patients with shorter telomeres than their older relatives carrying the same mutation show a trend for increased plasma citrate (left panel) and malate (right panel). The values over the bars indicate the level of age-adjusted telomere reduction.

**Figure 5 Citrate becomes more associated with malate and succinate in DC**

**A.** Clustered heat map of correlations derived from normalised metabolite levels in normal subjects (left panel) and DC plasma (right panel) showing the relationship of each metabolite to citrate and showing an increased association of citrate with isocitrate, aconitate and especially malate and succinate. **B.** Cluster analysis of the data in **A.**

Figure 1

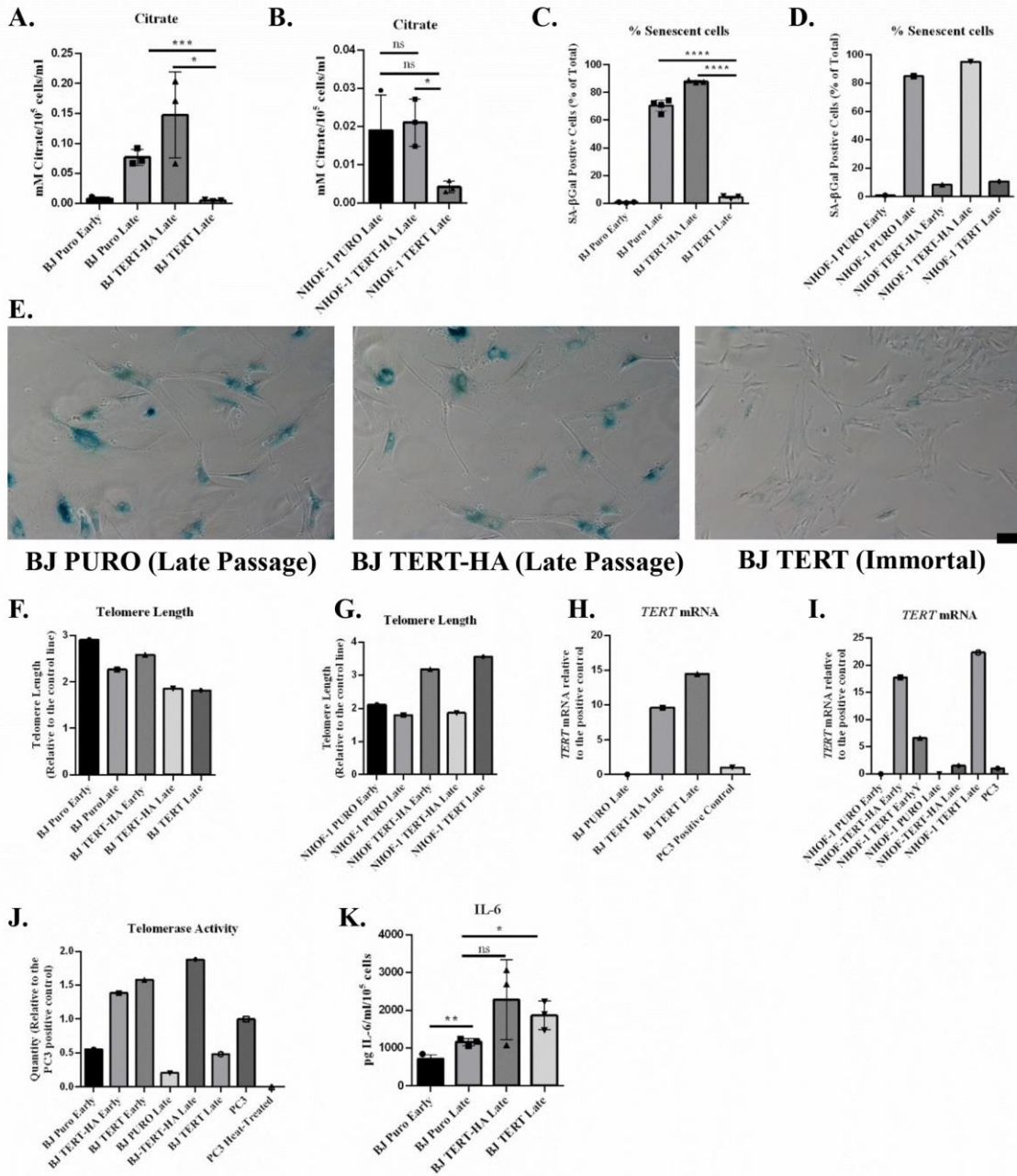


Figure 2

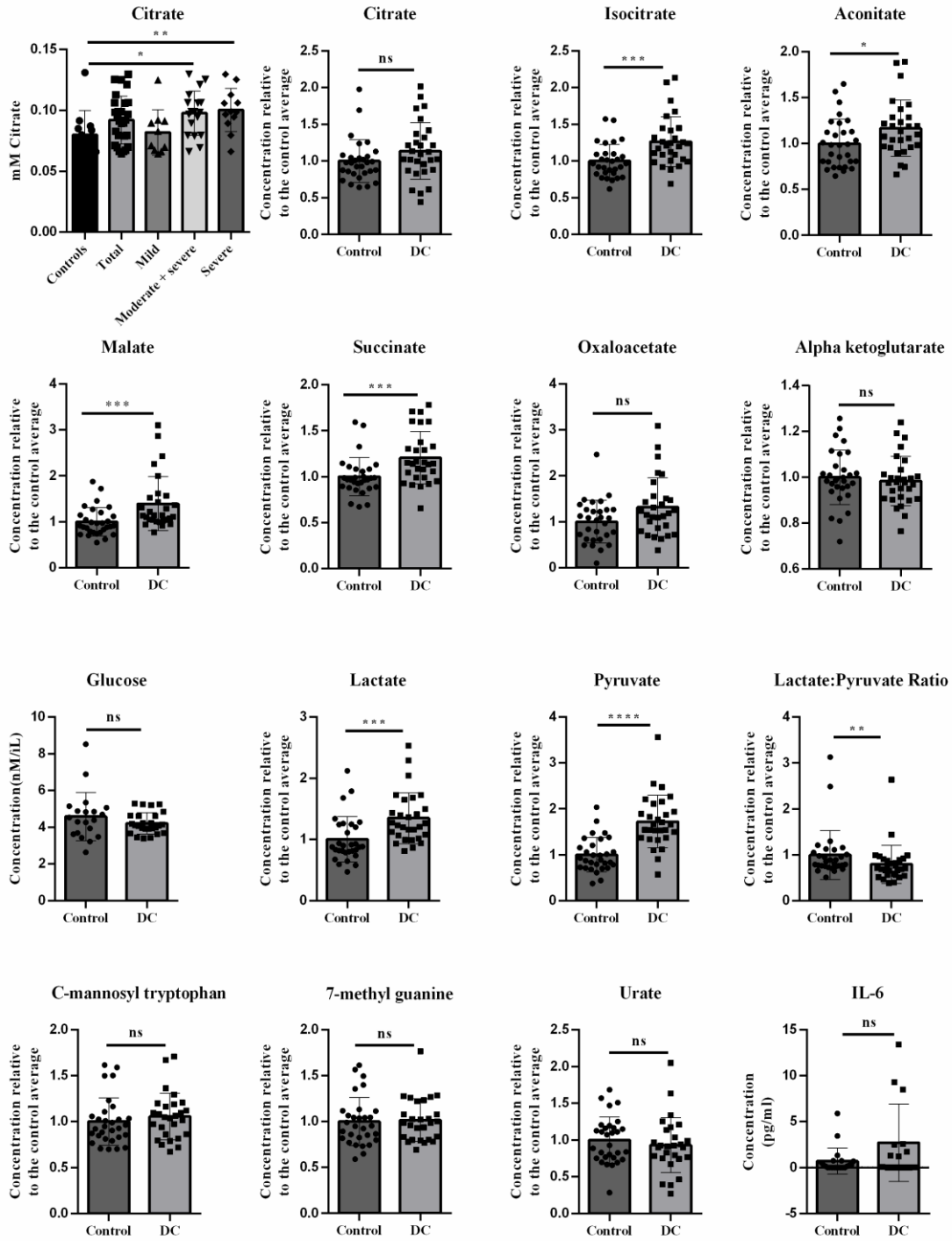




Figure 3

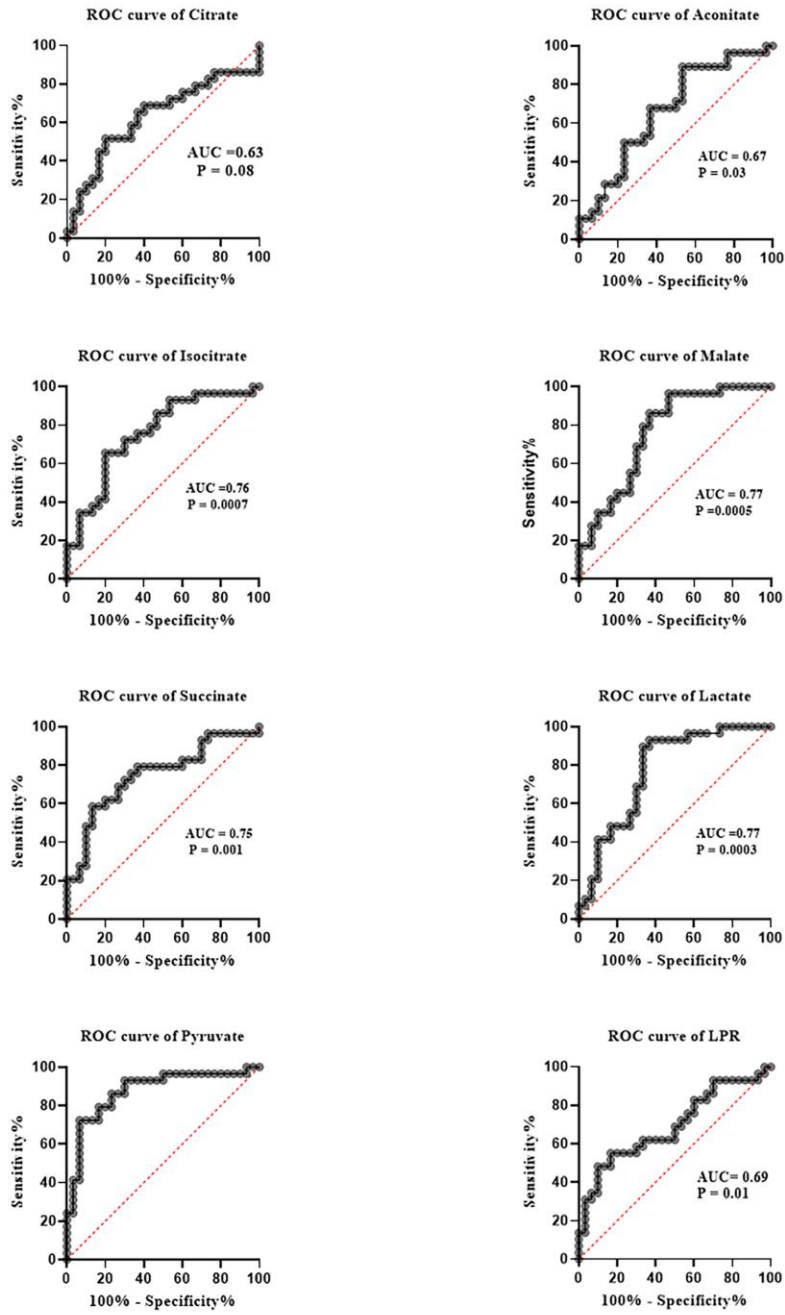
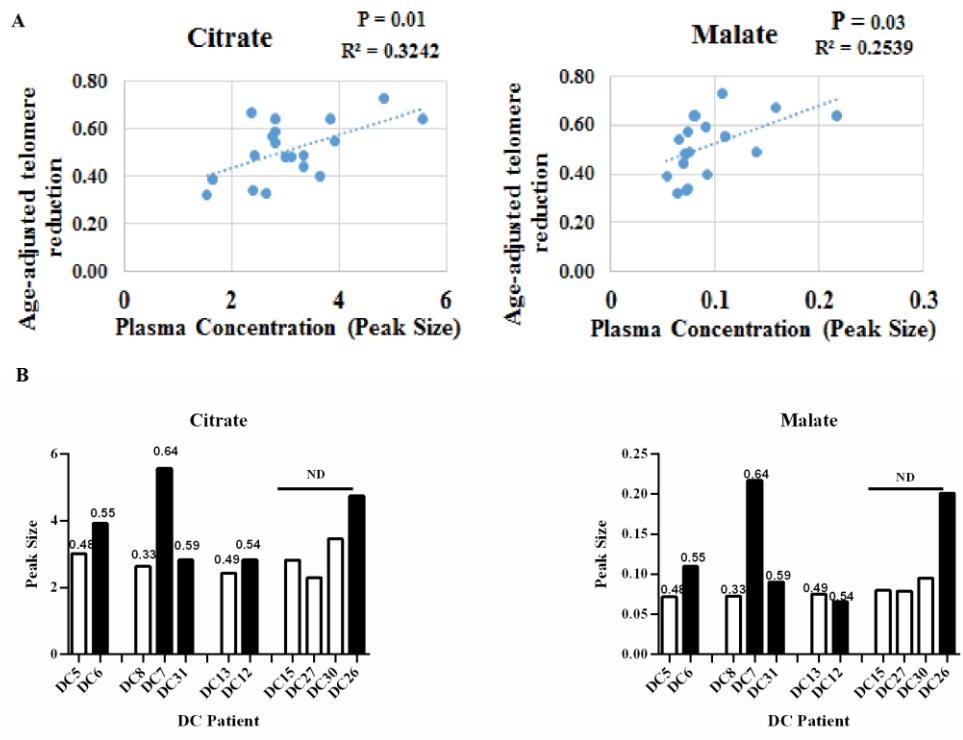


Figure 4



Accepted

Figure 5

