Leukocyte telomere length in relation to pancreatic cancer risk: a prospective study.

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The authors declare no conflict of interest.

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Abstract

**Background:** Several studies have examined leukocyte telomere length (LTL) as a possible predictor for cancer at various organ sites. The hypothesis originally motivating many of these studies was that shorter telomeres would be associated with an increase in cancer risk, the results of epidemiologic studies have been inconsistent, however, and suggested positive, negative, or null associations. Two studies have addressed the association of LTL in relation to pancreatic cancer risk and the results are contrasting.

**Methods:** we measured LTL in a prospective study of 331 pancreatic cancer cases and 331 controls in the context of the European Prospective Investigation into Cancer and Nutrition (EPIC).

**Results:** We observed that the mean LTL was higher in cases (0.59±0.20) than in controls (0.57±0.17), although this difference was not statistically significant (p=0.07), and a basic logistic regression model showed no association of LTL with pancreas cancer risk. When adjusting for levels of HbA1c and C-Peptide, however, there was a weakly positive association between longer LTL and pancreatic cancer risk, OR=1.13 (1.01-1.27). Additional analyses by cubic spline regression suggested a possible non-linear relationship between RTL and pancreatic cancer risk (P=0.022), with a statistically non-significant increase in risk at very low LTL, as well as a significant increase at high LTL.

**Conclusion:** Taken together, the results from our study do not support LTL as a uniform and strong predictor of pancreatic cancer.

**Impact:** The results of this manuscript can provide insights into telomere dynamics and highlight the complex relationship between LTL and pancreatic cancer risk.
Introduction

Pancreatic cancer is one of the leading causes of cancer deaths in the European Union and in the USA, with a five-year relative survival of less than 5% (1, 2). Established risk factors for pancreas cancer are cigarette smoking, heavy alcohol consumption, pre-existing diabetes mellitus (diagnosed at least 3 years before diagnosis of pancreatic cancer), obesity, chronic pancreatitis and family history of pancreatic cancer (3, 4). In addition, elevated fasting blood glucose levels (also within the non-diabetic range) and a small number of genetic polymorphic variants have been related to an increased risk (3, 5-11). Overall, however, these known risk factors can account for only a modest proportion of pancreas cancer occurrences, and taken together they can provide only a very weak prediction of an individual’s pancreas cancer risk. The identification of further and stronger risk predictors, e.g. in the form of integrative biomarkers of biologic response to various exposures, could help identify higher-risk individuals who would benefit from targeted prevention measures.

Several recent studies have examined leukocyte telomere length (LTL) as a possible predictor for cancer at various organ sites (12), including the pancreas (13, 14). The hypothesis originally motivating many of these studies was that shorter telomeres would be associated with an increase in cancer risk, since LTL has been found to be inversely related to a number of cancer risk factors, including age, smoking, diabetes, hyperinsulinemia, low grade chronic inflammation and exposure to environmental air pollution (15-17). The results of epidemiologic studies have been inconsistent, however, and suggested positive, negative, or null associations (12).
Interestingly, considerable evidence on molecular cancer biology indicates that tumor development may be generally related to telomere dysfunction and activation of telomerase, the enzyme that lengthens telomeres (18-20). Genetic studies have shown that the telomerase reverse transcriptase (TERT) gene, an enzyme that is fundamental for the accurate de novo synthesis of telomeric ends, is one of the few identified risk loci for pancreatic cancer (8) as well as other tumor types (21). There is evidence pointing to a strong correlation in telomere length (TL) across somatic tissues and suggesting that the variability across cell and tissue types is remarkably lower than the inter-individual variability of LTL (22-24), and natural rates of telomere shortening with age appear to follow a pattern of remarkable synchrony across different somatic tissue types (25). These observations identify LTL as a good proxy for overall telomere length and therefore a possibly useful marker for cancer risk.

So far, only two studies have addressed the association of leukocyte telomere length in relation to pancreatic cancer risk and the results are contrasting (13, 14). To further clarify the association between LTL and pancreatic cancer development we performed a case-control study of 331 cases and 331 matched control subjects nested within the EPIC cohort.
Materials and Methods

Study population

The European Prospective Investigation into Cancer (EPIC) is a large prospective cohort study including more than 500,000 men and women, recruited between 1992 and 2000 in 23 centers in 10 European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden and the UK). The study was approved by the local ethical review boards and all participants provided informed consent. Detailed descriptions of study design, population and methods used were described previously (26). Incident cancer cases were identified by population cancer registries (Denmark, Italy, the Netherlands, Spain, Sweden and the UK) or by a combination of health insurance and/or active follow-up with systematic verification of clinical and pathology records for cases identified through insurance data or self-reports (France, Germany, Greece).

Cases subjects were selected according to ICD-10 (www.who.int/classifications/icd/en/) and included all invasive exocrine pancreatic cancers (ICD-10 codes 25.0–25.3, 25.7–25.9). Exclusion criteria were the occurrence of other malignant tumors preceding the diagnosis of pancreatic cancer, except for non-melanoma skin cancer. By the end of December 2006, 638 incident cases of pancreatic cancer were identified, of which 578 were primary exocrine pancreatic tumors. For 435 of the 578 case subjects with primary exocrine pancreas cancer a DNA sample was available, and, for each of these 435 cases, one control participant, alive and free of cancer at time of diagnosis of the index case, was selected using incidence density sampling. Matching characteristics were study center, sex, age at enrolment (±6 months), date at entry in the cohort, time between blood sampling and time of last consumption of food and drink (<3, 3–6,
≥6 h). A total of 104 case-control pairs were discarded from the statistical analysis because of the poor quality of the LTL measurements, according to control criteria for our quantitative real-time PCR assay. Thus a total of 331 matched case-control pairs were left for statistical analyses. Among these 331 case-control sets, 245 cases of pancreas cancer (74%) were microscopically confirmed, and for the remaining 26% diagnosis had been confirmed by clinical symptoms, imaging results and/or physical examination.

**Sample preparation**

Blood samples were collected, at baseline recruitment and fractionated into serum, plasma, erythrocytes and buffy coat from which DNA was extracted. For in seven of the EPIC countries (France, Germany, Greece, Italy, the Netherlands, Spain and the UK) aliquots were stored in liquid nitrogen (−196°C) in a central biorepository. In the other three countries, aliquots were stored only locally, in liquid nitrogen vapor. For the present study DNA was extracted from buffy coat on an Autopure robotic work station (Qiagen, Hilden, Germany) with Puregene chemistry (Qiagen, Hilden, Germany). The DNA of cases and control subjects were extracted using the same techniques, at the same time and by the same personnel.

**Telomere length measurement**

The experimental procedures were carried out at the Harvard School of Public Health laboratories. The average relative telomere length as represented by the telomere repeat copy number to single gene copy number (T/S) ratio was determined by quantitative real-time PCR using a modification of Cawthon's (27) using an Applied Biosystems 7900HT Thermocycler in a
384-well format. All samples for both the telomere and single-copy gene (36B4) reactions were performed in triplicate on different plates and the threshold value for both reactions was set to 0.5. In addition to the samples, each 384-well plate contained a 10-point standard curve from 1.25ng to 50ng using pooled buffy-coat derived genomic DNA. Blinded quality control samples were interspersed throughout the dataset in order to assess inter-plate and intra-plate variability of threshold cycle (Ct) values. The T/S ratio (-ΔCt) for each sample was calculated by subtracting the average 36B4 Ct value from the average telomere Ct value. The relative T/S ratio (-ΔΔCt) was determined by subtracting the T/S ratio value of the 5ng standard curve point from the T/S ratio of each unknown sample. The order of DNAs from cases and controls was randomized on PCR plates in order to ensure that an equal number of cases and controls could be analyzed simultaneously.

**Measurement of other biomarkers**

Measures of C-peptide and glycated hemoglobin (HbA1c) on cases and controls of this study have been performed in a previously published study (28). Briefly, serum or plasma samples from cases and individually matched controls from one center were analyzed within the same analytical batch. HbA1c was measured with the BioRad Variant Haemoglobin Analyzer, while C-peptide was analyzed by a double-antibody radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX, USA).

**Statistical analysis**
Case-control comparisons of the baseline distributions of selected variables that are putatively associated with pancreatic cancer risk, such as type two diabetes (T2D) status, alcohol consumption and smoking status, were made using the t-test for continuous variables and the chi-square test for categorical variables. LTL measurements were log-transformed to obtain a variable with an approximately normal distribution, and we compared the geometric mean LTL values between cases and controls and at different levels of baseline variables in order to identify any possible association between LTL and the variables used in the study. We calculated Pearson correlation coefficients to examine the associations of (log) LTL with blood levels of C-peptide and glycated hemoglobin (HbA1c) that are two pancreatic risk factors.

Conditional logistic regression (matching variables age, gender, country of origin) was used to calculate ORs and the corresponding 95% CIs for the associations between LTL and pancreatic cancer risk. LTL was analyzed both as a continuous (log-transformed) variable and as a categorical variable, using quartiles according to its distribution in control subjects. Tests for statistical trend of pancreas cancer risk with increasing LTL were performed with LTL as a continuous variable as well as for quartile categories.

In addition to linear models, we assessed the risk association with restricted cubic spline regression with 4 knots using the LGTPHCURV9 macro (29).

Smoking status, diabetes alcohol consumption and, additionally, levels of C-peptide and HbA1c that were suggested as possible pancreatic risk factors by a previous publication (28) were examined as possible confounding factors. Since only the latter two showed a statistically significant association with LTL they were included as additional, continuous variables in the logistic regression model.
Stratified analyses by follow-up time since the date of blood donation (≤ or > 5 years) were conducted to assess the potential for reverse causation biases, and to assess whether the association of LTL with risk changed with increasing duration of prospective follow-up. The 5-year cut-point for these stratified analyses was motivated by a previously published study on LTL measures and pancreatic cancer risk (13). As an exploratory analysis, we examined the effects of removing all diabetic individuals from our analyses, since T2D is associated with both pancreatic cancer and LTL.

All analyses were performed using SAS software (Version 9.2, SAS Institute Inc. Cary, North Carolina).
Results

We measured the relative telomere length in 331 pancreatic cancer cases and in 331 matched control subjects recruited in the context of the prospective EPIC study. The distribution of relevant baseline characteristics of cases and controls is shown in Table 1. For none of the selected variables, except smoking, did baseline distributions show significant differences between cases and control subjects. Current smoking, however, was reported more frequently among the cancer cases. Furthermore, a higher proportion of cases than of controls had diabetes mellitus, as indicated by self-report or by elevated HbA1c levels.

Regarding the distribution of LTL measures across different strata of risk covariates (Table 2), age was inversely related to LTL, among the cases as well as among cases and controls combined. Among the cases only, smoking and higher levels of alcohol consumption both also showed suggestive inverse relationships with LTL, although these were not statistically significant (p = 0.07-0.08). There was no association of LTL with gender. HbA1c and C-peptide levels both showed weak but statistically significant inverse associations with LTL (coefficient of correlation = -0.07 [p=0.049] and -0.13 [p<0.001], respectively; Table 3) and therefore were used as adjusting variables in the logistic regression.

We observed that the mean LTL was higher in cases (0.59±0.20) than in controls (0.57±0.17) (Table 1), although in conditional logistic regression models adjusting for gender, age and country of origin (matching variables) this difference was not statistically significant (p=0.07). Analyzing LTL as a categorical variable, conditional logistic regression models showed no significant association of pancreatic cancer with LTL (OR=1.18 [95% CI 0.71-1.97] for highest vs. lowest quartile) (Table 4). When adjusting for levels of HbA1c and C-Peptide, however, there
was a weakly positive association between longer LTL and pancreatic cancer risk (for LTL as a continuous variable, OR=1.13 [1.01-1.27]; p=0.028; for top vs. bottom quartile, OR=1.38 [0.78-2.41]; p=0.08). These latter results remained practically unchanged when individuals with T2D were excluded from the statistical analyses (for LTL as continuous variable, OR=1.15 [1.01-1.30], p=0.026; for top vs. bottom quartiles OR=1.49 [0.80-2.76], p=0.10) (Table 4). Analysis adjusting for smoking showed results that did not materially differ from those found without this adjustment (Supplementary Table 1), and the same was true for analyses restricting to histologically confirmed pancreatic cancer cases (Supplementary Table 2). We additionally performed a cubic spline regression which suggested a possible non-linear relationship between RTL and pancreatic cancer risk (P=0.022) (Supplementary Figure 1), with a statistically non-significant increase in risk at low LTL levels, and statistically significant increase at high levels of LTL. Considering cases that had either developed pancreatic cancer up to 5 years after blood donation (N=171) or after a time interval of more than 5 years we observed no statistically significant heterogeneity of the association of LTL with risk (data not shown).
Discussion

Telomeres are highly specialized structures that have a key role in various cellular processes such as control of chromosomal stability, regulation of cell growth (30-32) and the proper segregation of chromosomes to daughter cells (33). Associations of LTL with multiple cancer-related risk factors, plus the correlation of TL across different tissue types, including blood cells, in humans and animals suggests that LTL levels may reflect TL in tissues where tumors may later develop and serve as a proxy risk marker for cancer.

Cross-sectional analyses of our data did not show any major association between LTL and smoking status or alcohol consumption overall, contrary to previous reports (34-36). Among the cancer cases, however, alcohol consumption and smoking did appear to be associated with borderline significant reductions in LTL, in line with associations previously reported both among cancer patients and healthy individuals (34-37). Likewise, our data also confirmed an inverse relationship between LTL and age (tables 1, 2 and 3).

In recent years many studies have addressed the possible association between telomere length measured in the blood and cancer risk, but with inconsistent results. Some studies suggested a significant association between shorter LTL and increased risk while others reported the opposite, i.e. no association at all or even an association with the two extremes (very short and very long LTL) with increased risk of several cancer types (reviewed in (12)). One key factor that seems to make a difference is whether the studies were retrospective (i.e. the blood sample was taken after diagnosis, in a case control study) or prospective (the blood was taken before diagnosis, in a cohort study), with the retrospective studies generally showing stronger associations than the prospective studies (12). It is worth emphasizing that in several
recent publications, which include prospective studies, longer telomeres were found to be associated with an increase in cancer risk (12, 13, 38-45).

For pancreatic cancer only two studies on the relationship with LTL have been performed so far: one retrospective case-control study, which showed an inverse association between shorter LTL and pancreas cancer risk (14), and one prospective study that showed an association between longer LTL and increased pancreatic cancer risk (13). While our basic statistical analyses showed a null result, analyses adjusted for blood levels of C-peptide and HbA1c, two metabolic factors that are thought to be associated with pancreas cancer risk (28), suggested a modest positive association between longer LTL and increased pancreatic cancer risk. Using non-linear methods we observed a suggestive U-shape risk pattern between LTL and pancreatic cancer as already observed by Skinner and colleagues (14). Although several important risk factors for cancer, such as age and smoking, have been reported to be associated with shorter LTL, findings of a positive association between longer LTL and cancer risk are also plausible, in view of reports that short telomeres may induce cellular senescence whereas longer telomeres generally mark actively reproducing cells that are at an increased risk of acquiring tumor-causing mutations (46). In agreement with the hypothesis that longer telomere increase cancer risk a recent report by Robles-Espinoza and collaborators describes a mutation in the protection of telomeres (POT1) gene that predisposes to familial melanoma and that is strongly associated with longer telomeres (47). Taken together, our results and those from Skinner and colleagues suggest a possibly more complex, non-linear association of pancreas cancer risk with telomeres length, with increased risks both at long and very short telomeres. The association between short LTL and increased risk might represent the lifetime exposure to
pancreatic risk factors such as obesity and smoking that tend to reduce LTL, while the association between longer LTL and increased pancreatic cancer might indicate an increased capacity of cells to divide, and therefore a constitutional increased risk for the disease.

Clear strengths of our study are its prospective design, reducing by a certain extent the possibility of reverse causation which is certainly one of the critical points when looking for the relationship between LTL and cancer risk, as pointed out by various authors (12, 45, 48, 49) and the possibility to avoid possible confounders by using a matched analysis and adjusting for variables which are pancreatic cancer risk factors and/or possible LTL determinants, such as smoking and alcohol consumption, C-peptide and HbA1c levels. A possible limitation is the relatively small sample size and therefore we cannot exclude the possibility that we could have missed a true but weak association, although the previously reported studies on LTL and pancreas cancer risk were performed on populations of comparable size (13, 14). Moreover, due to DNA availability and quality we only included 331 of the 578 exocrine pancreatic cancer that were identified in the EPIC cohort till 2006 and, therefore, we cannot completely exclude that the cases selected are not representative of the whole case set. In addition the strength and the magnitude of the association are moderate and, we, also, conducted multiple tests. Considering all these limitations, our results must be taken with caution.

In conclusion, although it was originally hypothesized that shorter LTL should be predictive of increased risk for cancer, and in spite of support for this hypothesis from some epidemiologic studies (reviewed in (12)), recent and repeated observations by different epidemiologic studies have indicated longer LTL among individuals developing cancer (12, 13, 38-44). Our present
data seem to be in line with this latter hypothesis; however they do not support LTL as a uniform and strong predictor of pancreas cancer risk.

Acknowledgments

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References


<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n = 331)</th>
<th>Controls (n = 331)</th>
<th>p-value</th>
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<tr>
<td>Men, women (n)</td>
<td>150, 181</td>
<td>150, 181</td>
<td>Matched</td>
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<tr>
<td>Age at recruitment (years), mean (range)</td>
<td>57 (30 - 75)</td>
<td>57 (30 - 75)</td>
<td>Matched</td>
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<tr>
<td>Age at diagnosis (years), mean (range)</td>
<td>63 (37 - 82)</td>
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<tr>
<td>Follow-up (years), mean (range)</td>
<td>5.3 (0–13)</td>
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<td>Telomere length, mean+SD</td>
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<td>0.57±0.17</td>
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<td>0.56±0.17</td>
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<td>Women</td>
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<td>0.57±0.18</td>
<td>0.08</td>
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<td>99 (30)</td>
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<tr>
<td>Alcohol intake at recruitment (g/day), median range</td>
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<td></td>
<td></td>
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<tr>
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<td>10.56 (8.37-13.31)</td>
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<td>26 (8)</td>
<td>14 (4)</td>
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<td>HbA1c ≥6.5% (48 mmol/mol) (%)</td>
<td>40 (12)</td>
<td>21 (6)</td>
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<td>Self-reported diabetes or HbA1c ≥6.5% (%)</td>
<td>44 (14)</td>
<td>25 (8)</td>
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Table 2. Comparison of geometric mean LTL values at different levels of baseline variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>All (n=662)</th>
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<th>Controls (n = 331)</th>
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<td>LTL p-value&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>55 - 59</td>
<td>0.55</td>
<td>0.57</td>
<td>154</td>
</tr>
<tr>
<td>60 - 64</td>
<td>0.56</td>
<td>0.56</td>
<td>163</td>
</tr>
<tr>
<td>65 - 69</td>
<td>0.50</td>
<td>0.49</td>
<td>75</td>
</tr>
<tr>
<td>70+</td>
<td>0.49</td>
<td>0.49</td>
<td>39</td>
</tr>
<tr>
<td><strong>Alcohol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5 g/d</td>
<td>0.56</td>
<td>0.393</td>
<td>312</td>
</tr>
<tr>
<td>5-14 g/d</td>
<td>0.53</td>
<td>0.54</td>
<td>161</td>
</tr>
<tr>
<td>15-29 g/d</td>
<td>0.56</td>
<td>0.54</td>
<td>104</td>
</tr>
<tr>
<td>30+ g/d</td>
<td>0.53</td>
<td>0.52</td>
<td>77</td>
</tr>
<tr>
<td><strong>Diabetes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0.55</td>
<td>0.290</td>
<td>588</td>
</tr>
<tr>
<td>Yes</td>
<td>0.52</td>
<td>0.54</td>
<td>40</td>
</tr>
<tr>
<td><strong>Smoking status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Life-long non-smoker</td>
<td>0.56</td>
<td>0.494</td>
<td>277</td>
</tr>
<tr>
<td>Current smoker</td>
<td>0.55</td>
<td>0.54</td>
<td>141</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total may not add up due to missing values

<sup>b</sup> <em>P</em><em>value</em> = <em>P</em><em>value</em> for trend test
Table 3. Pearson correlation between HbA1c and C-peptide and LTL

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases and controls (N)</th>
<th>Correlation coefficient</th>
<th>P-value</th>
<th>Cases (N)</th>
<th>Correlation coefficient</th>
<th>P-value</th>
<th>Controls (N)</th>
<th>Correlation coefficient</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c</td>
<td>653</td>
<td>-0.077</td>
<td>0.049</td>
<td>326</td>
<td>-0.080</td>
<td>0.152</td>
<td>327</td>
<td>-0.101</td>
<td>0.067</td>
</tr>
<tr>
<td>C-peptide</td>
<td>617</td>
<td>-0.131</td>
<td>0.001</td>
<td>309</td>
<td>-0.116</td>
<td>0.042</td>
<td>308</td>
<td>-0.150</td>
<td>0.008</td>
</tr>
</tbody>
</table>
Table 4. Associations between LTL and pancreatic cancer risk

<table>
<thead>
<tr>
<th>Relative LTL</th>
<th>Quartile boundaries</th>
<th>Conditional analysis(^a)</th>
<th>Conditional analysis adj.by C-peptide and HbA1c(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>P(_{\text{value}})</td>
</tr>
<tr>
<td>Quartile 1</td>
<td>(0.18 - 0.45)</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Quartile 2</td>
<td>(0.46 - 0.54)</td>
<td>0.86 (0.54-1.37)</td>
<td>0.32 (0.51-1.76)</td>
</tr>
<tr>
<td>Quartile 3</td>
<td>(0.55 - 0.67)</td>
<td>0.94 (0.59-1.51)</td>
<td>0.92 (0.55-1.56)</td>
</tr>
<tr>
<td>Quartile 4</td>
<td>(0.68 - 1.55)</td>
<td>1.18 (0.71-1.97)</td>
<td>1.38 (0.80-2.41)</td>
</tr>
<tr>
<td>Continuous</td>
<td>1.09 (0.99-1.21)</td>
<td>0.07</td>
<td>1.13 (1.01-1.27)</td>
</tr>
</tbody>
</table>

\(P_{\text{trend}} = 0.3081; \ OR = \text{Odds Ratio}; 95\% \text{ Confidence interval.}\)

\(^a\) Conditional analysis with matching variables (age, gender, country of origin, fasting status)

\(^b\) Conditional analysis with matching variables (age, gender, country of origin) and adjustment by C-peptide and HbA1c levels
Leukocyte telomere length in relation to pancreatic cancer risk: a prospective study.
Daniele Campa, Björn Mergarten, Immaculata De Vivo, et al.

Cancer Epidemiol Biomarkers Prev  Published OnlineFirst August 7, 2014.

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