Lymphocytes of Type 2 Diabetic Women Carry a High Load of Stable Chromosomal Aberrations
A Novel Risk Factor for Disease-Related Early Death

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OBJECTIVE—Diabetes is associated with an increased risk of death in women. Oxidative stress due to chronic hyperglycemia leads to the generation of reactive oxygen species and loss of chromosomal integrity. To clarify whether diabetes is a premature aging syndrome, we determined telomere erosion dynamics and occurrence of structural chromosomal aberrations in women of the Ludwigshafen Risk and Cardiovascular Health (LURIC) Study.

RESEARCH DESIGN AND METHODS—Telomere lengths and karyotypes were examined in peripheral blood mononuclear cells. Regarding these parameters, surviving and deceased type 2 diabetic women of the LURIC study were compared with nondiabetic LURIC women with or without coronary heart disease and with healthy female control subjects.

RESULTS—Significantly enhanced telomere attrition was seen in all LURIC subjects compared with healthy control subjects. Although the average telomere-length loss is equivalent to well >10 years of healthy aging, telomere erosion was not associated with outcome within the LURIC cohort. However, strikingly high numbers of stable chromosomal aberrations were found in type 2 diabetic women but not in LURIC disease control subjects or in healthy individuals. Furthermore, within the younger age-groups, deceased type 2 diabetes patients had significantly more marker chromosomes than the surviving type 2 diabetic patients.

CONCLUSIONS—All women at high risk for cardiovascular death have accelerated telomere erosion, not caused by type 2 diabetes per se but likely linked to other risk factors, including dyslipidemia. By contrast, the occurrence of marker chromosomes is associated with type 2 diabetes and is a novel risk factor for type 2 diabetes–related early death. Diabetes 57:2950–2957, 2008

Type 2 diabetes is characterized by increased morbidity and all-cause mortality (1,2). The combination of excess caloric intake and reduced physical activity leading to obesity, dyslipidemia, and hypertension increases the risk for diabetes and coronary heart disease (CHD). Recent data show that among diabetic men, the mortality rate has decreased significantly, whereas in diabetic women, no such trend was found (3). The all-cause mortality rate difference between diabetic and nondiabetic women is considerable. Therefore, the combination of diabetes with multiple risk factors identifies women at particularly high risk (2,4).

The relative risk for morbidity and mortality in women with diabetes is increased compared with nondiabetic control subjects (2,5). Diabetes may therefore be regarded as a premature aging syndrome in which the overall metabolic shift leads to genotoxic stress that results in loss of chromosomal integrity (rev. in 6). Oxidative stress plays a crucial role in the pathogenesis of type 2 diabetes and in diabetes-associated complications. The generation of reactive oxygen species (ROS) is a common downstream mechanism whereby multiple by-products of glucose and (pro)inflammatory molecules exert adverse effects (7–11). DNA damage and telomere attrition can serve as markers of these processes and, consequently, mirror the pace of biological aging (rev. in 12–14).

Hypothesizing along these lines, we studied telomere erosion dynamics and/or the occurrence of structural chromosomal aberrations in women with type 2 diabetes who were participants of the Ludwigshafen Risk and Cardiovascular Health (LURIC) prospective cohort study (15). Life expectancy within the LURIC female cohort falls short by ~10 years compared with the general female population in Germany.

Telomeric erosion was much further advanced in all LURIC women, irrespective of type 2 diabetes, compared with age-matched control subjects, the difference amounting to >10 life-years. We further found a strikingly enhanced number of structural chromosomal aberrations in the peripheral lymphocytes of women with type 2 diabetes that was diabetes specific and, within the younger age-groups, associated with mortality.

RESEARCH DESIGN AND METHODS
The LURIC study is an ongoing prospective cohort study investigating risk factors for cardiovascular death in Caucasian individuals (15). Between June 1997 and January 2000, 3,266 individuals (2,266 men and 1,002 women) who had undergone coronary angiography were included. All LURIC participants...
had elective coronary angiography and left ventriculography. CHD was assessed by angiography using the maximum luminal narrowing estimated by visual analysis. Clinically relevant CHD was defined as the occurrence of at least one stenosis $\geq 20\%$ in at least 1 of 15 coronary segments. Individuals with stenosis $<20\%$ were considered as control subjects (16). The institutional review board at the Arztekammer Rheinland-Pfalz approved the study. Informed written consent was obtained from each participant. All participants were profiled in detail with regard to established risk factors for cardiovascular disease. The cohort is followed for morbidity and mortality. Information on vital status was obtained from local registries. No patient was lost to follow-up. Death certificates were obtained in 97% of dead participants. Two modes of death were classified: cardiovascular death and others. Cardiovascular death included the following categories: sudden death, fatal myocardial infarction, death due to congestive heart failure, death immediately after intervention to treat CHD, fatal stroke, and other causes of death due to CHD. Other modes of death covered all kinds of noncardiovascular deaths; those succumbing to this mode of death were censored.

Because the chance to find a novel risk factor in diabetes mortality appeared more promising looking into a high-risk cohort, the present analysis is restricted to women (Fig. 1). Among the 1,002 women who entered the LURIC study, 204 had type 2 diabetes, and 798 had no signs of diabetes at the time of enrollment. Median time of follow-up was 5.45 years. Up to the fixed day (20 January 2000), of the 204 type 2 diabetes subjects, 72 had died (referred to as L-T2Dmdec) and 132 had survived (referred to as L-T2Dmsur). From the nondiabetic subgroup 129 died, and 669 were still alive. Clinical data of these patients are described in detail in Table 1.

The study combines a cross-sectional analysis and a follow-up approach. From the L-T2Dmdec and L-T2Dmsur, 63 matching pairs were found on the basis of CHD status, presence versus absence of dyslipidemia and/or hypertension, and age. To test the impact of type 2 diabetes against this high-risk profile, two LURIC control groups were drawn from the surviving nondiabetic patients. Of those, 23 and 20 matched with individuals of the type 2 diabetes profile, two LURIC control groups were drawn from the surviving nondiabetic population. Clinical chemistry. Standard laboratory procedures were used as described previously (15). Specifically, diabetes was diagnosed if 2 h after the oral glucose load, plasma glucose was $>1.25$ g/l in the fasting state or $>2$ g/l, respectively, or if individuals were receiving oral antidiabetics or insulin.

Cell culture and metaphase preparation. Peripheral blood mononuclear cells were isolated on density gradients (Lymphoprep, Nynas Pharma, Oslo, Norway) and washed in RPMI-1640 (Life Technologies, Paisley, U.K.) twice before use. Peripheral lymphocytes were cultured and phytohemagglutinin stimulated as described previously to obtain metaphase cells (17). Fifteen metaphases from each individual were karyotyped according to standard criteria (19) and analyzed under an Axioscope microscope (Zeiss, Jena, Germany) equipped with a charged-coupled device camera, linked to the Isis and telomere software (MetaSystems, Altlussheim, Germany). Abnormal chromosomes were detected and further characterized by inverted gray scales using the software.

Pepptide nucleic acid probes and fluorescence in situ hybridization. The peptide nucleic acid (PNA) probe for telomeric sequences is a ready-to-use probe included in the Telomere PNA FISH Kit/Cy3 (Dako, Glostrup, Denmark). The PNA centromeric probe for chromosome 2 was generated by Dako and is available on request. The hybridization was performed according to the manufacturer's instructions and as detailed by Perner et al. (17). Reliability and reproducibility of the telomere/centromere fluorescence in situ hybridization (T/C-FISH) method had been tested by Perner et al. (17). The examiner of the T/C-FISH and cytogenetic analyses (see below) was blinded to the clinical status of the subjects analyzed.

Cyto genetic analysis. Stable chromosomal aberrations (translocations, insertions, interstitial deletions, and inversions) are easily detectable while performing T/C-FISH and were further characterized by inverted gray scales using the software.
of the original DAPI counterstaining. The frequencies of marker chromosomes were quantified in all metaphases within each age-group (15 metaphases per person) and expressed in percent. The same procedure was done with healthy control subjects. Normalized data were derived by calculating the ratio of absolute telomere intensities and the centromeric reference signal intensities of each chromosome 2 (T/C value). For each individual, 15 ing the ratio of absolute telomere intensities and the centromeric reference

Data are means \( \pm \) SD or \( n \) (% of patients). *Diabetes was defined according to World Health Organization/American Diabetes Association criteria; in all probands without known history of diabetes, diabetes was ruled out by an oral glucose tolerance test. †Hypertension defined by the use of antihypertensive drugs and/or systolic blood pressure >140 and/or diastolic blood pressure >90 mmHg. ‡Dyslipidemia defined by use of lipid lowering drugs and/or HDL <35 mg/dl, triglycerides >170 mg/dl, and total cholesterol >240 mg/dl. CAD, angiographically proven coronary artery disease; MI, myocardial infarction; ND, not done; OAD, oral antidiabetic drug.

of telomeric signals in a single metaphase, Fig. 2B shows the same metaphase in inverted gray scales for karyotyping, and Fig. 2C gives the resulting karyogram. Telomere length was significantly shorter in the entire type 2 diabetes cohort (\( P = 0.0001 \)) compared with healthy control subjects (see telomere lengths of healthy control subjects in Fig. 3A and telomere lengths of all LURIC subgroups in Fig. 3B). Focusing on outcome, a difference in telomere length between the L-T2DMsur and the L-T2DMdec subjects was not demonstrated using conditional logistic regression (Fig. 3B, black and white dots) (\( P = 0.104 \) without adjustment and 0.070 after adjustment for C-reactive protein and the number of marker chromosomes). Overall, the loss in telomere length in type 2 diabetic patients corresponded to a remarkable telomere attrition of \(~1\) kb corresponding to \(~10\) years compared with normal age-matched control subjects (Fig. 3B). This number is compatible with the reduced life expectancy of all female LURIC patients. Figure 4 shows the mortality curves of LURIC type 2 diabetic patients, LURIC patients without type 2 diabetes, and for comparison, the mortality curve of the general female population in Germany as issued by the Statistische Bundesamt (20). To verify these results, two further nondiabetic LURIC control groups were defined for comparison with the type 2 diabetes cohort, L-ctr.surCHD+ and L-ctr.surCHD− (Fig. 1). The sample sizes were estimated based on the observed average telomere lengths and SDs of the type 2 diabetes cohort

### RESULTS

**Telomere-length analysis.** Metaphase chromosomes from mononuclear cells taken at the baseline examination of the type 2 diabetes cohort were subjected to T/C-FISH telomere-length assessment and compared with age-matched healthy control subjects. Figure 2A gives an example of telomeric signals in a single metaphase, Fig. 2B shows the same metaphase in inverted gray scales for karyotyping, and Fig. 2C gives the resulting karyogram. Telomere length was significantly shorter in the entire type 2 diabetes cohort (\( P = 0.0001 \)) compared with healthy control subjects (see telomere lengths of healthy control subjects in Fig. 3A and telomere lengths of all LURIC subgroups in Fig. 3B). Focusing on outcome, a difference in telomere length between the L-T2DMsur and the L-T2DMdec subjects was not demonstrated using conditional logistic regression (Fig. 3B, black and white dots) (\( P = 0.104 \) without adjustment and 0.070 after adjustment for C-reactive protein and the number of marker chromosomes). Overall, the loss in telomere length in type 2 diabetic patients corresponded to a remarkable telomere attrition of \(~1\) kb corresponding to \(~10\) years compared with normal age-matched control subjects (Fig. 3B). This number is compatible with the reduced life expectancy of all female LURIC patients. Figure 4 shows the mortality curves of LURIC type 2 diabetic patients, LURIC patients without type 2 diabetes, and for comparison, the mortality curve of the general female population in Germany as issued by the Statistische Bundesamt (20). To verify these results, two further nondiabetic LURIC control groups were defined for comparison with the type 2 diabetes cohort, L-ctr.surCHD+ and L-ctr.surCHD− (Fig. 1). The sample sizes were estimated based on the observed average telomere lengths and SDs of the type 2 diabetes cohort
and of the healthy control subjects (26.3 ± 7.5). Under conservative assumption of a difference between means of 7 and a common SD of 10, the power of the comparison is >80% for a control group of a size \( n = 20 \). Although L-ctr.surCHD\(^{+}\) (\( n = 20 \)) and L-ctr.surCHD\(^{-}\) (\( n = 23 \)) showed mean T/C values of 16.3 ± 6 and 17.4, respectively, which are lower than the mean value in type 2 diabetes (18.4 ± 9.1), the differences were not statistically significant (\( P = 0.57 \) and 0.94, respectively) (Fig. 3B, red and blue dots). Thus, all LURIC women had accelerated telomere erosion and reduced life expectancy irrespective of type 2 diabetes and/or CHD.

### Chromosomal aberrations

The T/C-FISH technique further allows structural chromosomal aberrations to be identified. The results are based on the number of marker chromosomes (Fig. 2, asterisk) per metaphase, where the proband is the statistical unit. Although the prevalence of marker chromosomes was negligible in newborns and low in the age-matched healthy control subjects, the number of marker chromosomes found in L-T2DMdec and L-T2DMsur was significantly increased (\( P < 0.0001 \)) and even exceeded that in the advanced-age healthy control subcohort (Fig. 5A).

Specifically, we verified the statistically significant difference between the type 2 diabetes cohort and healthy control subjects by comparing type 2 diabetes with L-ctr.surCHD\(^{+}\) and L-ctr.surCHD\(^{-}\). Although in type 2 diabetes, a mean frequency of 12.45% per metaphase was observed, the corresponding values in the 50–80 years subgroup of healthy control subjects were 1.75% (\( n = 20 \)).

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**FIG. 2.** A representative metaphase and the corresponding karyogram. *A:* Metaphase hybridized with PNA probes and counterstained with DAPI. Blue, DAPI; red, FISH with telomere- and chromosome 2-specific probes. *B:* The corresponding gray scale image (black and white) reveals the chromosome bands that allowed marker chromosomes to be identified (*). *C:* Corresponding karyogram with the following karyotype: 49, XX,~2,+4mar. (Please see http://dx.doi.org/10.2337/db08-0274 for a high-quality digital representation of this image.)
1.65% in L-ctr.surCHD (n = 20), and 2.5% in L-ctr.surCHD (n = 23). No differences could be shown between the three control groups (P > 0.3 in each pairwise comparison); however, each of the control groups differed from type 2 diabetes (P < 0.0001 in each comparison). Therefore, the high frequency of marker chromosomes can be attributed to type 2 diabetes.

Regarding survival, the number of stable chromosomal aberrations was higher in L-T2DMdec when compared with L-T2DMsur (P = 0.09) (Figs. 5 and 6). Regression analysis (Fig. 5B) yielded an age-dependent increase in the number of marker chromosomes in L-T2DMsur (y = 2.7543x - 0.1629; r² = 0.8326). However, in L-T2DMdec, only a weak association with age was found (y = 1.17x + 9.7086; r² = 0.4208). In Fig. 6, the corresponding regression curves fitting the proportion of marker chromosomes per person are given together with 95% CIs for the curves (Fig. 6). They were determined using a generalized linear model with log link function and negative binomial distribution. For L-T2DMsur but not for L-T2DMdec, an association with age was found (P = 0.042 and 0.65, respectively) (Fig. 5B). Furthermore, Fig. 5B suggests a difference in the proportion of marker chromosomes between L-T2DMdec and L-T2DMsur and a rising risk of dying with increase in marker chromosomes for the younger women. Therefore, the 63 case-control pairs (which were all matched for age in yearly intervals) were split into two groups: those younger than the median age (being 69 years) and those older. In the younger group (32 pairs) L-T2DMdec had, in median, one marker chromosome more than the matched L-T2DMsur (P = 0.034, Wilcoxon’s signed-rank test), whereas in the older group (31 pairs), no such difference was found (P = 0.86).

**DISCUSSION**

In LURIC women, pan-genomic telomere length was significantly shorter compared with healthy control subjects. The accelerated telomere attrition amounts to well >10 years of healthy aging (6,12,18). Although telomere length was not associated with outcome within this cohort, the cohort itself, however, has an increased risk of death, reducing life expectancy by ~10 years compared with the general female population in Germany (Fig. 4). This study was designed to find chromosomal abnormalities associated with diabetes-related mortality. We intentionally restricted our analysis to women, who are known to carry the greater burden (21,22). Obviously, our core finding that stable marker chromosomes in peripheral lymphocytes are associated with an elevated risk of diabetes-related death in younger women will have to be reevaluated in a similar male setting to gain information on the overall power of this novel risk factor. Our data are in line with recent studies showing that enhanced telomere attrition is associated with atherosclerosis (23,24). Accelerated telomere erosion was found in diabetic patients (25–27). This is confirmed here; however, as evidenced by our disease control subjects, acceleration of telomere attrition is independent of type 2 diabetes. Alternatively, our data strongly suggest that accelerated erosion is caused by dyslipidemia (hypercholesterolemia and hyperglycemia), a state that is frequently associated with type 2 diabetes.

Dyslipidemia leads to an increased production of aldehydes, including methylglyoxal. A direct role for methylglyoxal in telomeric attrition has not been observed so far. However, methylglyoxal induces an increase of free radicals (ROS) (28), and ROS might, albeit indirectly, accelerate telomere attrition (29). Furthermore, methylglyoxal causes stable modification of DNA bases, which in turn induces chromosomal aberrations, sister chromatid exchanges, and micronuclei in human lymphocytes treated in vitro (30). Although there is an enzymatic defense against methylglyoxal-induced DNA mutation, e.g., the enzymes glyoxalase I and aldehyde reductase (31), both enzymes are highly dependent on the concentration of glutathione, which is severely reduced in diabetes (32). Hence, it is conceivable that methylglyoxal-induced chro-
mosomal breaks are incorrectly repaired in diabetes patients.

The rate of chromosome aberrations in human peripheral lymphocytes of healthy individuals has been known to increase with advancing age (rev. in 33), possibly caused by the permanent attack of ROS (14,34), diminished DNA repair (35), altered DNA repair mechanisms (36), and modified T-cell repertoire (37).

The most important finding of our study is the high load of stable chromosomal aberrations in type 2 diabetes. These stable chromosomal aberrations in type 2 diabetic women outnumbered by far the marker chromosomes in our control populations, i.e., healthy control subjects and both LURIC control subgroups, and can be regarded as dramatic also against the background of published data.

At present, a conceptional gap exists between the biomarker “load of marker chromosomes in lymphocytes” and the mechanism of death in type 2 diabetic patients. It is even unclear whether lymphocytes are in this aspect representative of somatic cells or of other tissues or cell systems with their well-known differences in cellular turnover rates. Theoretically, the key players in CHD, i.e., blood monocytes, lymphocytes, endothelial cells, vascular fibroblasts, and smooth muscle cells, while engaged in the injury and repair struggle eventually giving rise to fatal plaque formation and rupture (41), might acquire fatal dysfunctions caused by diabetes-induced DNA damage. There is a wealth of detailed data on diabetes-related malfunctioning of these cells and platelets and on oxidative stress on endothelial cells in atherosclerosis (42). In addition, chromosomal alterations have been found in atherosclerotic plaques (43). Further research aiming at a
potentially causal link between DNA damage and cellular malfunction is warranted in the light of our finding that the load of chromosomal markers in lymphocytes of diabetic women is a senescence marker prognosticating premature death.

In summary, the current study provides the largest dataset currently available to address the relationship between stable chromosomal aberrations and telomere length and an association with survival outcome in women affected by type 2 diabetes. We show that telomere short-
ening is associated with classical cardiovascular disease risk factors other than type 2 diabetes, whereas, in younger women, the occurrence of chromosomal aberrations is associated with type 2 diabetes and directly correlated with the risk of death. Our observation that most women with an increased load of marker chromosomes died during the observation period suggests the presence of a threshold value (6). This assumption might be supported by the fact that the state-of-the-art polyphony used to treat the LURIC cohort did not alter the survival outcome (15).

In conclusion, future prospective studies in high-risk populations should take into account telomere dynamics and stable chromosomal alterations as global biomarkers for outcome.

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