

Familial longevity is marked by enhanced insulin sensitivity

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Summary

Insulin resistance is a risk factor for various age-related diseases. In the Leiden Longevity study, we recruited long-lived siblings and their offspring. Previously, we showed that, compared to controls, the offspring of long-lived siblings had a better glucose tolerance. Here, we compared groups of offspring from long-lived siblings and controls for the relation between insulin and glucose in nonfasted serum ($n = 1848$ subjects) and for quantitation of insulin action using a two-step hyperinsulinemic-euglycemic clamp ($n = 24$ subjects). Groups of offspring and controls were similar with regard to sex distribution, age, and body mass index. We observed a positive bi-phasic linear relationship between \ln (insulin) levels and nonfasted glucose with a steeper slope from 10.7 mU L^{-1} insulin onwards in controls compared to offspring ($P = 0.02$). During the clamp study, higher glucose infusion rate was required to maintain euglycemia during high-dose insulin infusion ($P = 0.036$) in offspring, reflecting higher whole-body insulin sensitivity. After adjustment for sex, age, and fat mass, the insulin-mediated glucose disposal rate (GDR) was higher in offspring than controls (42.5 ± 2.7 vs. $33.2 \pm 2.7 \text{ } \mu\text{mol kg}^{-1} \text{ min}^{-1}$, mean \pm SE, $P = 0.025$). The

insulin-mediated suppression of endogenous glucose production and lipolysis did not differ between groups (all $P > 0.05$). Furthermore, GDR was significantly correlated with the mean age of death of the parents. In conclusion, offspring from long-lived siblings are marked by enhanced peripheral glucose disposal. Future research will focus on identifying the underlying biomolecular mechanisms, with the aim to promote health in old age.

Key words: aging; human; hyperinsulinemic-euglycemic clamp; insulin sensitivity; longevity.

Introduction

The degree of insulin resistance varies widely in the population at large (Ferrannini, 1992). The increase in insulin resistance with age may significantly contribute to the increased incidence of a variety of (age-related) diseases (Davidson, 1979; Chen *et al.*, 1985; Facchini *et al.*, 2001). The best known of these is type 2 diabetes, which occurs when insulin-resistant individuals are unable to secrete sufficient amounts of insulin to compensate for the defects in insulin action (Stumvoll *et al.*, 2008). Insulin resistance and compensatory hyperinsulinemia also are risk factors for hypertension (Skarfors *et al.*, 1991) and cardiovascular disease (Sjoholm & Nystrom, 2005) and have been associated with other adverse clinical outcomes, including Alzheimer's disease (Gustafson *et al.*, 2003; Craft, 2005) and cancer (Calle & Kaaks, 2004). It remains debatable whether the increase in insulin resistance with age results from chronological age *per se* or from lifestyle-related factors such as obesity and physical activity (Shimokata *et al.*, 1991; Ferrannini *et al.*, 1996). Insulin resistance shows familial clustering (Lillioja *et al.*, 1987; Martin *et al.*, 1992) and is more prominent in nondiabetic offspring of patients with diabetes type 2 (Haffner *et al.*, 1988). Remarkably, centenarians (Evert *et al.*, 2003) and their offspring (Atzmon *et al.*, 2004) as well as the offspring of nonagenarian siblings were found to have a reduced risk of cardiovascular disease and diabetes (Westendorp *et al.*, 2009).

Previous studies have suggested preserved insulin sensitivity in the oldest old. Cross-sectional data from the Italian population, covering an age range from 28 to 110 years, showed highest insulin resistance (as determined by homeostasis model assessment) at 80–90 years. In age categories beyond 80–90 years, insulin resistance was lower (Paolisso *et al.*, 2001). Centenarians were found to exhibit preserved glucose tolerance and preserved insulin sensitivity compared to elderly with a mean age of 78 years (Paolisso *et al.*, 1996). However, as the mean body mass index (BMI) of centenarians was much lower, it is not clear to what extent the preserved insulin sensitivity in centenarians reflects selective survival of subjects that have familial

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enrichment for enhanced insulin sensitivity or whether insulin sensitivity has been enhanced by lifestyle factors or because of processes that occur upon aging.

In the Leiden Longevity Study (Schoenmaker *et al.*, 2006), we have recruited 421 long-lived families consisting of multiple nonagenarian siblings and their offspring (aged 33–81 years) from the Dutch population. The partners of the offspring (aged 30–80 years) were included as controls. Recently, we found that random and fasting glucose levels were lower, and we showed that glucose tolerance was better in the non-diabetic offspring when compared to controls. Offspring and controls did not differ with respect to age, sex distribution, BMI, and lifestyle indices such as the level of physical activity (Rozing *et al.*, 2010). Here, we further explored the relation between insulin and glucose in the two groups, after exclusion of diabetic subjects. To this end, we first compared the relationship between glucose and insulin levels as determined in random nonfasted serum samples ($n = 1838$), which include the physiological variation in insulin levels in response to everyday challenges, such as meals. Next, we performed a double tracer, two-step hyperinsulinemic-euglycemic clamp in two subgroups comprising 12 healthy offspring from long-lived siblings and 12 control subjects of which eight partners of eight of the healthy offspring. This gold standard technique allowed us to assess whole-body insulin sensitivity and distinguish between the effects of insulin on glucose disposal rate (GDR), endogenous glucose production, and lipolysis.

Results

The relationship between non-fasted glucose and insulin in the baseline cohort

Table 1 shows the characteristics of the baseline cohort ($n = 1838$). Baseline characteristics were similar between the offspring and controls. Figure 1 shows the nonfasted serum $\ln(\text{insulin})$ versus glucose levels for the two groups. For both groups, a biphasic positive association was observed between levels of glucose and $\ln(\text{insulin})$. Initially, slopes were similar between groups (0.38 in offspring vs. 0.32 in partners, $P = 0.73$); diverged from $\ln(\text{insulin}) = 2.37$ (corresponding to 10.7 mU L^{-1} insulin) onwards, after which slopes were significantly steeper in the partners (0.73 in offspring vs. 0.95 in partners, $P = 0.02$).

Table 1 Baseline characteristics of baseline cohort

	Offspring ($n = 1273$)	Controls ($n = 565$)
Female gender, n (%)	692 (54.4)	329 (58.2)
Age (year)	59.4 (6.4)	58.7 (7.4)
BMI (kg m^{-2})	25.3 (3.4)	25.5 (3.6)
Glucose (mmol L^{-1})	5.7 (1.1)	5.9 (1.2)
Insulin (mU L^{-1})	14.9 (2.2)	16.4 (2.2)

Continuous data are presented as means with SD. Insulin levels are presented as geometric means.

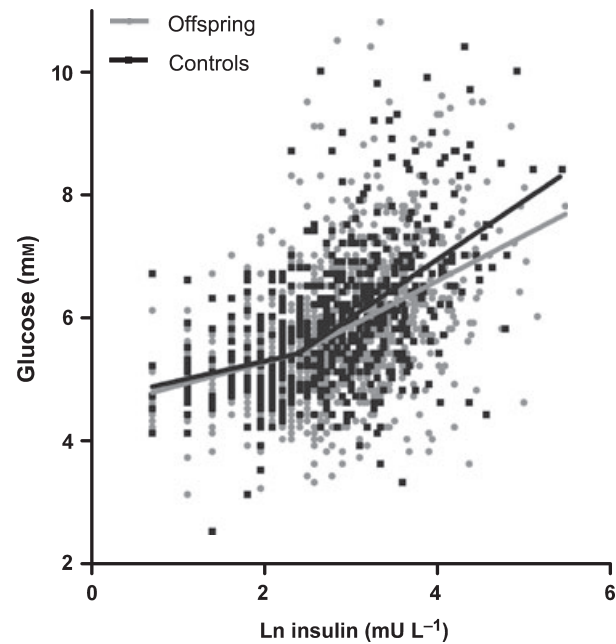


Fig. 1 Relation between nonfasted insulin and glucose values in the baseline cohort of the Leiden Longevity Study ($n = 1838$). Gray dots represent individuals from offspring group, and black squares represent individuals from control group. The two-phase relationship between serum levels of glucose and $\ln(\text{insulin})$, including the changeover point and the slopes of the lines before and after the changeover point, was modeled using a piecewise change-point model.

Baseline characteristics of the hyperinsulinemic-euglycemic clamp study groups

Data presented in Fig. 1 suggest that the significance of the different association between insulin and glucose is relevant to those with high insulin and that the effect could be most pronounced in tissues requiring high insulin levels. To test these hypotheses, we performed a hyperinsulinemic-euglycemic clamp in 24 subjects. Table 2 shows the baseline characteristics of the study groups. The group of offspring from long-lived siblings did not differ from the control group with respect to any of the baseline characteristics, although the offspring group showed a tendency towards a higher age and fat mass and higher age of parent(s).

Familial longevity associates with higher whole-body glucose metabolism

A two-step hyperinsulinemic clamp was performed using a low ($10 \text{ mU m}^{-2} \text{ min}^{-1}$) and a high ($40 \text{ mU m}^{-2} \text{ min}^{-1}$) insulin dose in the first and second clamp step, respectively. Mean insulin levels during the last 30 min of the clamp periods were similar between groups, both during low-dose insulin infusion ($11.0 \pm 1.0 \text{ mU L}^{-1}$ in offspring vs. $11.2 \pm 1.0 \text{ mU L}^{-1}$ in controls, $P = 0.89$) and during high-dose insulin infusion ($42.5 \pm 2.2 \text{ mU L}^{-1}$ in offspring vs. $38.9 \pm 2.2 \text{ mU L}^{-1}$ in controls $P = 0.25$). Throughout the entire clamp, glucose levels remained stable and were similar between groups (Fig. 2A). Figure 2B

Table 2 Baseline characteristics of clamp group

	Offspring (n = 12)	Controls (n = 12)
Female gender (%)	50.0	50.0
Age (year)	62.7 (2.4)	61.2 (5.5)
Systolic blood pressure (mmHg)	142.5 (20.6)	143.2 (25.0)
Diastolic blood pressure (mmHg)	86.8 (10.7)	86.2 (11.1)
Weight (kg)	79.3 (10.3)	80.1 (9.7)
BMI (kg m ⁻²)	26.0 (2.0)	26.1 (2.3)
Fat mass (%)	33.0 (7.3)	30.9 (9.7)
Lean mass (kg)	53.6 (11.5)	53.8 (12.2)
Waist circumference (cm)	93.0 (10.8)	93.6 (7.7)
Waist/Hip ratio	0.90 (0.1)	0.89 (0.1)
Total cholesterol (mmol L ⁻¹)	6.1 (1.0)	5.9 (0.8)
HDL-cholesterol (mmol L ⁻¹)	1.7 (0.4)	1.7 (0.4)
LDL-cholesterol (mmol L ⁻¹)	3.9 (0.9)	3.8 (0.7)
Mean age parents (year)	88.3 (4.0)	76.6 (8.2)
Age oldest parent (year)	97.0 (3.8)	82.3 (10.5)

Continuous data are presented as means with SD.

shows the glucose infusion rates during the clamp. During high-dose insulin infusion, offspring had significantly higher glucose infusion rates ($P = 0.036$) compared to controls, despite a slightly higher age and fat mass in the offspring.

Familial longevity is characterized by enhanced peripheral insulin sensitivity, but not hepatic insulin sensitivity

Next, we assessed whether the higher glucose infusion rate required to maintain euglycemia in offspring was accounted for by increased glucose disposal or by enhanced insulin-mediated

suppression of endogenous glucose production (Table 3, Fig. 3). At low-dose insulin infusion ($10 \text{ mU m}^{-2} \text{ min}^{-1}$), the groups did not differ with respect to endogenous glucose production. At high-dose insulin infusion ($40 \text{ mU m}^{-2} \text{ min}^{-1}$), the mean GDR, expressed as glucose rate of disappearance (Rd), was higher in offspring than in controls (42.5 ± 2.7 vs. $33.2 \pm 2.7 \text{ } \mu\text{mol kg}^{-1} \text{ min}^{-1}$, $P = 0.025$). When analysis was carried out stratified according to gender, a similar trend was observed in both sexes, (45.4 ± 3.7 vs. $33.5 \pm 3.7 \text{ } \mu\text{mol kg}^{-1} \text{ min}^{-1}$, $P = 0.057$ for women, 39.7 ± 4.2 vs. $32.8 \pm 4.2 \text{ } \mu\text{mol kg}^{-1} \text{ min}^{-1}$, $P = 0.29$ for men). To determine the insulin sensitivity of adipose tissue, we assessed the capacity of insulin to suppress the rate of glycerol appearance (Ra). At baseline and during both clamp conditions, the Ra of glycerol was similar in offspring and controls (Table 3, Fig. 3), (all $P > 0.05$).

Insulin sensitivity correlates positively with the age at death of the subjects' parents

The presented results suggest a relation between familial longevity and GDR. To explore whether this association was specific for offspring of long-lived siblings only or of a more general nature, we assessed the relationship between parental age (at death or censorship) and the GDR under high-dose insulin infusion in all subjects (Fig. 4, Table 4).

After adjustment for sex, age, and fat mass, we found a positive correlation between the mean age of the parents and GDR ($P = 0.007$), and between the age of the oldest parent and the GDR ($P = 0.034$). To exclude the possibility that these results were driven by the high age of parental death in the offspring group, we repeated the analyses for the control group only, and

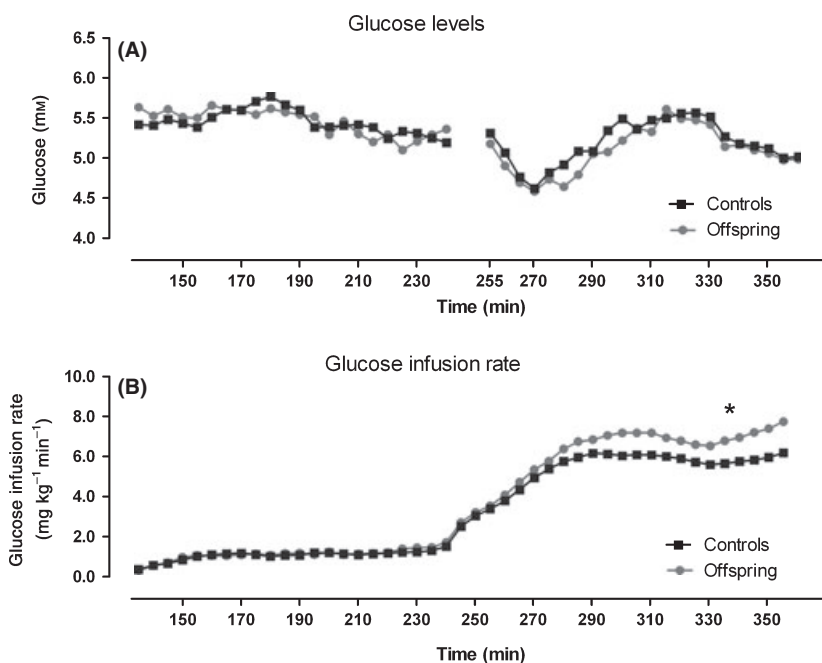


Fig. 2 (A) Mean glucose levels and (B) mean glucose infusion rates during hyperinsulinemic conditions for offspring ($n = 12$) and controls ($n = 12$). Time = 120 to time = 240: $10 \text{ mU m}^{-2} \text{ min}^{-1}$ insulin. Time = 240 to time = 360: $40 \text{ mU m}^{-2} \text{ min}^{-1}$. Asterisk (*) represents $P < 0.05$ for the difference in M -value (whole-body glucose metabolism) between groups during the last 30 min of high-dose insulin infusion (time = 330 to time = 360) after adjusting for sex, age, and fat mass (%).

Table 3 Glucose and fat metabolism in offspring enriched for longevity and controls under different clamp conditions

	Basal steady state			Insulin (10 mU m ⁻² min ⁻¹)			Insulin (40 mU m ⁻² min ⁻¹)		
	Offspring	Controls	P-value	Offspring	Controls	P-value	Offspring	Controls	P-value
Plasma glucose (mmol L ⁻¹)	6.1 (0.1)	5.9 (0.1)	0.27	5.6 (0.1)	5.6 (0.1)	0.69	5.5 (0.1)	5.4 (0.1)	0.64
Plasma insulin (mU L ⁻¹)	5.4 (1.1)	4.7 (1.1)	0.68	10.8 (1.1)	11.5 (1.1)	0.66	41.9 (2.2)	39.5 (2.2)	0.45
Glucose Rd (μmol kg ⁻¹ min ⁻¹)	12.9 (0.3)	12.6 (0.3)	0.43	15.5 (1.0)	14.3 (1.0)	0.57	42.5 (2.7)	33.2 (2.7)	0.025
Clamp EGP (μmol kg ⁻¹ min ⁻¹)	12.7 (0.3)	12.4 (0.3)	0.57	7.8 (0.3)	7.9 (0.3)	0.85	1.8 (0.3)	2.1 (0.3)	0.36
Glycerol Ra (μmol kg ⁻¹ min ⁻¹)	2.1 (0.2)	2.4 (0.2)	0.34	0.9 (0.1)	1.0 (0.1)	0.36	0.6 (0.1)	0.7 (0.1)	0.52
FFA (mmol L ⁻¹)	0.60 (0.1)	0.76 (0.1)	0.07	0.14 (0.03)	0.21 (0.03)	0.08	< 0.05 (0.005)	< 0.05 (0.005)	0.94
Triglycerides (mmol L ⁻¹)	1.17 (0.2)	1.0 (0.2)	0.49	1.0 (0.2)	0.9 (1.2)	0.52	0.91 (0.2)	0.74 (0.2)	0.49

Glucose Rd, rate of disappearance of glucose; EGP, endogenous glucose production; glycerol Ra, rate of appearance of glycerol; FFA, free fatty acids.

P-value obtained after linear analysis with adjustment for sex, age, and fat mass (%). Bold value: $P < 0.05$.

During basal steady state, glucose Rd is composed of endogenous glucose production and tracer infusion.

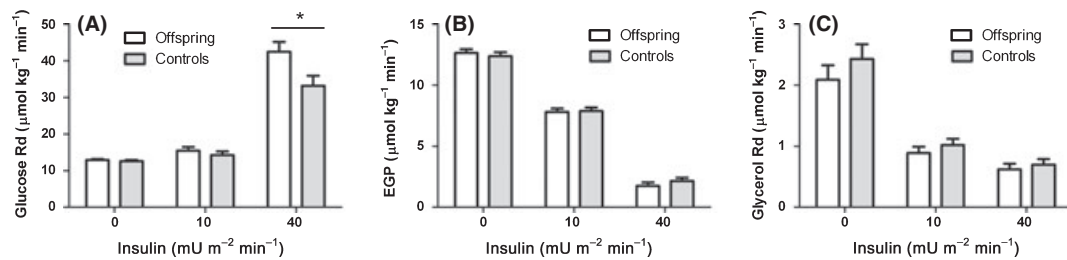


Fig. 3 Glucose and fat metabolism in offspring ($n = 12$) and controls ($n = 12$) under different clamp conditions; (A) glucose disposal rate (Rd), (B) endogenous glucose production (EGP), (C) rate of disappearance of glycerol (Rd). Results are means with standard error, after adjustment for age, sex, and fat mass (%). Asterisk (*) represents significant difference ($P < 0.05$) between groups using linear regression with correction for age, sex, and fat mass (%).

results did not change materially (Table 4). Also, excluding subjects with parents who were still alive at date of censorship did not change results (data not shown).

Discussion

Here, we show that familial longevity in humans is characterized by enhanced peripheral insulin sensitivity, i.e. compared to a control group with similar distribution of age, sex, and body composition, healthy offspring of long-lived siblings had a higher insulin-mediated GDR. In contrast, the capacity of insulin to suppress endogenous glucose production or lipolysis did not

differ between the groups. Interestingly, the GDR during hyperinsulinemia was positively correlated with the age at death of the parents of the entire group, suggesting that longevity genes are involved in the control of insulin action in the general population.

This is the first study to show that subjects with familial predisposition for healthy longevity have higher whole-body insulin sensitivity when compared to a control group similar in age, sex, and body composition. A previous study showed preserved whole-body insulin sensitivity in healthy centenarians, but different from our study, these data could not be compared to a control group of similar age and BMI (Paolisso *et al.*, 1996).

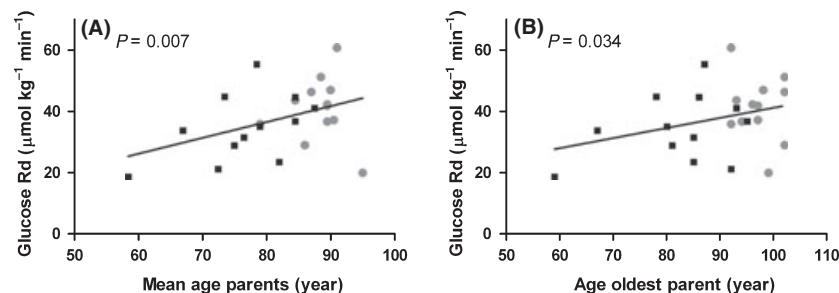


Fig. 4 (A) Association between mean age of both parents (at death or censorship) and glucose disposal rate (GDR) under 40 mU m⁻² min⁻¹ insulin infusion; (B) Association between age of oldest parent only (at death or censorship) and GDR under 40 mU m⁻² min⁻¹ insulin infusion. Gray circles represent the offspring ($n = 12$), and black squares represent controls ($n = 12$). P-values for association between age of parent and GDR, using linear regression with adjustment for age, sex, and fat mass (%) of participants.

Table 4 Relation between parental age and glucose disposal rate

	All subjects (<i>n</i> = 24)			Controls only (<i>n</i> = 12)		
	β	SE	<i>P</i> -value	β	SE	<i>P</i> -value
Mean age of both parents						
Crude	0.52	0.2	0.045	0.65	0.4	0.11
Multivariate	0.65	0.2	0.007	0.69	0.2	0.022
Age of oldest parent only						
Crude	0.33	0.2	0.12	0.33	0.3	0.30
Multivariate	0.43	0.2	0.034	0.37	0.3	0.19

SE, standard error. When parents were still alive, current age was used. β represents increase in glucose disposal rate ($\mu\text{mol kg}^{-1} \text{min}^{-1}$) per year increase in age of death of the parent(s). Multivariate: adjusted for age, sex, and fat mass. *P*-value obtained using linear regression analysis. Bold values: *P* < 0.05.

Moreover, here we document that it is insulin action on glucose metabolism, and glucose disposal in particular that distinguishes offspring of long-lived siblings from controls. Insulin action on lipolysis did not differ between the groups.

Earlier, after exclusion of diabetic subjects, we found higher nonfasted glucose levels in the control group in a larger sample of the Leiden Longevity Study (Rozing *et al.*, 2009b), as well as enhanced glucose tolerance in the offspring in a smaller subgroup of the Leiden Longevity Study (Rozing *et al.*, 2010). Here, in the large group excluding the diabetics, we plotted the association between nonfasted $\ln(\text{insulin})$ and nonfasted glucose levels in offspring and controls, and we observed a steeper rise in glucose levels in the high nonfasted insulin range in the control group compared to the offspring group. This finding suggested that the difference in glucose metabolism between the groups might be most pronounced under high insulin conditions and in tissues where insulin acts in higher concentrations, such as the peripheral tissues. Our findings in the clamp conditions confirmed these hypotheses. It should be emphasized that the enhanced peripheral insulin sensitivity that we describe here is independent of fat mass and most likely also of exercise (Rozing *et al.*, 2010). In line with the earlier studies on the oral glucose tolerance (Rozing *et al.*, 2010), the difference in insulin sensitivity between groups was slightly more pronounced in women. Because of the small sample size in our current clamp studies, we cannot be sure whether a true gender effect exists. It is not clear that biological mechanisms contribute to the preservation of insulin sensitivity in familial longevity. Interestingly, centenarians and their offspring were shown to have higher serum levels of insulin-sensitizing hormones, most notably adiponectin (Atzmon *et al.*, 2008).

Interestingly, enhanced insulin sensitivity in the offspring of long-lived siblings co-occurs with other phenotypic features, including lower levels of active thyroid hormone (Rozing *et al.*, 2009a), a different spectrum of cellular responses to oxidative stress *in vitro* (Dekker *et al.*, 2009) and larger low-density lipoprotein (LDL) particle sizes (Heijmans *et al.*, 2006). The co-occurrence of multiple beneficial features is reminiscent of the phenotype seen in genetically modified long-lived mammals as

well as in calorie-restricted mammals (Colman *et al.*, 2009). Pathways implicated in mediating longevity phenotypes in genetically modified long-lived mammals as well as in calorie-restricted mammals include modulation of FOXO, AMPK, Sirtuins, and mTOR (Guarente, 2008). Interestingly, genetic variants of FOXO3A have been linked to human longevity in seven different cohorts, including Hawaiians of Japanese descent, Italians, Ashkenazi Jews, Californians, New Englanders, Germans, and Chinese [reviewed in (Kenyon, 2010)]. Given the complexity of pathways and the generally small but possibly additive effects observed for individual genetic variants (Kuningas *et al.*, 2008), stronger effects will possibly be observed when entire genetic pathways will be analyzed (Pawlikowska *et al.*, 2009). However, besides genetic factors, familial factors affecting lifestyle, especially early in life, may have affected the later phenotypes observed in the Leiden Longevity Study.

The strict selection criteria for the clamp study participants may have diminished the experimental contrast between the groups and masked even greater differences in insulin action. The groups were comparable for age, gender, environmental conditions, and lifestyle indices, and type 2 diabetes and/or any other chronic disease were reasons to exclude individuals from participation (whether it concerned offspring or control). Because the prevalence of age-related pathology associated with insulin resistance, including diabetes and cardiovascular disease, is higher in controls (Westendorp *et al.*, 2009), inclusion of all cohort members (irrespective of the presence of chronic disease) would probably have revealed an even more explicit difference in insulin action between offspring and controls but would have hampered causal inference.

The insulin levels during the hyperinsulinemic clamp study were comparable to insulin levels in the nonfasted, randomly obtained serum samples in the larger baseline cohort of the Leiden Longevity Study. Likewise, the different response to insulin in offspring under experimental high insulin clamp conditions was reflected by a comparable difference in the relationship between randomly taken nonfasted insulin and glucose levels in the higher range of insulin levels. This suggests that the differences in insulin sensitivity found between offspring and controls under controlled, experimental conditions may reflect everyday physiological conditions.

In conclusion, familial longevity in humans is marked by an increased capacity of insulin to stimulate glucose disposal. Moreover, the age at death of the parents predicts the GDR in response to insulin infusion in their offspring, suggesting that familial factors are involved in the control of insulin action in man. Our future research will focus on identifying the underlying biomolecular mechanisms and pathways.

Methods

Subjects

The Leiden Longevity Study comprises 421 families, as described more extensively elsewhere (Schoenmaker *et al.*, 2006). Families

were recruited if at least two long-lived siblings were alive and fulfilled the age-criterion of 89 years or older for men and 91 years or older for women. As no proper controls exist for this age group, for further studies, the offspring of these long-lived nonagenarians were included. This generation carries on average 50% of the genetic advantage of their long-lived parent and was shown to have a 35% lower mortality rate compared with their birth cohort (Schoenmaker et al., 2006). Their partners, with whom most have had a relationship for decades, were included as population-based controls.

Blood samples were taken at baseline for the extraction of DNA and RNA and the determination of nonfasted serum and plasma parameters. Blood samples were obtained throughout the day between 9:30 hours and 17:00 hours. Nonfasted serum samples and BMI were available for 1930 subjects. After exclusion of subjects with nonfasted glucose levels above 11 mmol L⁻¹ (indicative of possible diabetes), history of diabetes or use of glucose lowering medication, nonfasted serum samples of 1838 subjects were available for the current study.

For the hyperinsulinemic-euglycemic clamp study, we aimed to include 12 couples, each consisting of an offspring from long-lived siblings and his or her current partner as control subject. Subjects were selected from the database based on the following inclusion criteria: middle-age (50–75 years), residence in close proximity of the research center (< 45 min by car) and BMI that fell within the range of the mean \pm 1 SD of the BMI of the eligible subjects (22 kg m⁻² < BMI < 30 kg m⁻²). Eligible subjects were screened for the following exclusion criteria: fasting plasma glucose > 6.9 mmol L⁻¹ (American Diabetes Association, 2005), presence of endocrine, renal, hepatic or other significant chronic diseases, use of medication known to influence lipolysis, glucose metabolism or GH-secretion, recent weight changes or attempts to loose weight (> 3 kg weight change within last 3 months), smoking, extensive sporting activities (> 10 h per week), and inaccessible peripheral veins for intravenous catheter insertion, as assessed by clinical examination and routine laboratory tests. During the screening interview, information on age (of death) of the parents was obtained.

In total, 87 subjects were approached, of which 17 subjects did not fulfill the inclusion criteria (19%), 44 subjects refused participation (51%), and 26 subjects agreed to participate in the study (30%). Two subjects (one offspring, one control) did not complete the study because of medical technical reasons. One of the partners of an offspring also had a long-lived parent with a long-lived sibling and was therefore included in the offspring group. In total, the group consisted of 24 subjects, of which 16 participated as couple (eight couples), and eight did not participate as couple (eight singletons). The Medical Ethical Committee of the Leiden University Medical Center approved the study, and written informed consent was obtained from all subjects.

Clinical protocol

All clamp studies started at 8:00 hours after an overnight fast. Anthropometric measurements (height, weight, waist, and hip

circumference) and blood pressure measurements were taken according to standard methods. Body composition was measured using bioelectrical impedance analysis (BIA). In a larger sample of the Leiden Longevity Study, body composition as measured with BIA was highly correlated with dual energy X-ray absorptiometry (DEXA) measurements. (Ling, de Craen, Slagboom, Gunn, Stokkel, Westendorp, Maier, unpublished data) Metabolic studies were performed as described previously (Jazet et al., 2005). Subjects were requested to lie down on a bed in a semirecumbent position. A polyethylene catheter was inserted into an antecubital vein for infusion of test substances. Another catheter was inserted into a contralateral dorsal hand vein for blood sampling; this hand was kept in a heated box (60 °C) throughout the study day to obtain arterialized venous blood samples. Basal samples were taken for measurement of glucose, insulin, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, free fatty acids (FFA), glycerol, and background enrichment of [6,6-²H₂]-glucose and [²H₅]-glycerol. At 08:30 hours ($t = 0$ min), an adjusted primed (17.6 μ mol kg⁻¹) continuous (0.22 μ mol kg⁻¹ min⁻¹) infusion of [6,6-²H₂]-glucose (enrichment 99.9%; Cambridge Isotopes, Cambridge, MA, USA) was started and continued throughout the study. At 09:00 hours ($t = 30$ min), a primed (1.6 μ mol kg⁻¹), continuous (0.11 μ mol kg⁻¹ min⁻¹) infusion of [²H₅]-glycerol (Cambridge Isotopes) was started and continued throughout the study. At the end of the basal period ($t = 90$ min), three blood samples were taken at 10-min intervals for the determination of glucose, insulin, glycerol, triglycerides, FFA's, and enrichment of [6,6-²H₂]-glucose and [²H₅]-glycerol. Subsequently, a primed continuous infusion of human recombinant insulin (10 mU m⁻² min⁻¹; Actrapid, Novo Nordisk Pharma BV, Alphen aan de Rijn, The Netherlands) was started ($t = 120$ min) for 2 h. This low-dose insulin infusion was used to determine differences in insulin sensitivity of the liver and whole-body lipolysis. Exogenous glucose 20% enriched with 3% [6,6-²H₂]-glucose was infused at a variable rate to maintain the plasma glucose level at 5.0 mmol L⁻¹. From $t = 210$ to $t = 240$ min, blood samples were taken at 10-min intervals for the determination of [6,6-²H₂]-glucose and [²H₅]-glycerol-specific activities, glucose, insulin, glycerol, triglycerides, and FFA. Next, at $t = 240$, a primed continuous infusion of insulin was started at 40 mU m⁻² min⁻¹. This second high dose of insulin infusion was used to determine whole-body glucose disposal. From $t = 330$ to $t = 360$ min, blood samples were taken at 10-min intervals for the determination of [6,6-²H₂]-glucose and [²H₅]-glycerol-specific activities, glucose, insulin, glycerol, triglycerides, and FFA. Plasma samples were put on ice immediately after withdrawal, and all samples were centrifuged at 1610 g at 4 °C for 20 min and stored at -80 °C until assay.

Assays

All serum measurements were taken with fully automated equipment. For glucose, cholesterol, HDL-cholesterol, triglyce-

rides, and FFA, the Modular P2 analyzer was used from Roche (Almere, the Netherlands). Insulin was measured using the Immulite 2500 from DPC (Los Angeles, CA, USA). CVs for these measurements were all below 9%.

[6,6-²H₂]-glucose and [²H₅]-glycerol were determined in a single analytical run using gas chromatography–mass spectrometry as described previously (Jazet *et al.*, 2005). LDL-cholesterol was calculated using the Friedewald formula (11). In case insulin and glycerol values were below threshold for correct estimation of concentration, we estimated the concentration to be half of the threshold value.

Calculations

An isotopic steady state was achieved during the steady state and during the last 30 min of the hyperinsulinemic clamp periods. Therefore, steady-state equations were used to calculate tracer infusion rates, according to the modified Steele's steady-state equations (Steele, 1959; Finegood *et al.*, 1987). The rates of appearance (Ra) and disappearance (Rd) for glucose and glycerol were calculated by dividing the tracer infusion rate by the tracer-to-tracee ratio. Glucose disposal rates were expressed in $\mu\text{mol kg}^{-1}$ body weight per min. Endogenous glucose production (EGP) during the basal steady state and during the hyperinsulinemic state was calculated as the difference between the rates of glucose appearance and glucose infusion.

Statistical analyses

Initially, a nonparametric curve (locally weighted scatterplot smoothing) was fitted to model the relation between glucose and $\ln(\text{insulin})$ for the two groups ($n = 1838$). This curve showed a clear bi-phasic relation which we subsequently modeled using a two-phase linear regression model (Seber, 1977). Within each group, the expected glucose level for a person at a certain level of $\ln(\text{insulin})$ was modeled using the formula: predicted glucose = $\alpha_1 + \beta_1 \times \ln(\text{insulin})$, for $\ln(\text{insulin}) < \gamma$ and $\alpha_2 + \beta_2 \times \ln(\text{insulin})$, for $\gamma < \ln(\text{insulin})$, with restrictions on α_1 and α_2 such that the function is continuous in the changeover point γ , i.e., $(\alpha_1 + \beta_1 \times \gamma) = (\alpha_2 + \beta_2 \times \gamma)$. Because changeover points were similar between groups, group differences in slopes before (difbeta1) and after (difbeta2) the changeover point (γ) were modeled using the formula: predicted glucose = $\alpha + \text{difalpha} \times \text{partner} + (\beta_1 + \text{difbeta1} \times \text{partner}) \times (\ln(\text{insulin}) - \gamma) \times ((\ln(\text{insulin}) - \gamma) < 0) + (\beta_2 + \text{difbeta2} \times \text{partner}) \times (\ln(\text{insulin}) - \gamma) \times ((\ln(\text{insulin}) - \gamma) > 0)$. The model was fitted using software for nonlinear regression models. Data are presented as mean with standard deviation (baseline characteristics) or mean with standard error (SE) to assess differences between groups. Differences in outcomes between groups as well as the associations of GDR with age of parents were calculated using a linear regression model with correction for age, sex, and fat mass. Statistical significance was set $P < 0.05$. All analyses were performed using SPSS version 17.0 (SPSS Inc, Chicago, IL, USA).

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Author contributions

CAW and MPR performed the clamp studies, analyzed the data, and drafted the manuscript. TCMS performed tracer measurements, SleC contributed to data analysis. SPM contributed to revision of the manuscript. RGJW, PES, HP and DvH designed the study and edited the manuscript.

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