Chapter 5: C-reactive protein and glucose regulation in familial longevity

Maarten P. Rozing[1,4] Simon P. Mooijaart[1,4], Marian Beekman[2,4], Carolien A. Wijsman[1,4], Andrea B. Maier[1,4], A. Bartke[3], Rudi G.J. Westendorp[1,4], P. Eline Slagboom[2,4], Diana van Heemst[1,4] , on behalf of the Leiden Longevity Study (LLS) Group

From the [1]Department of Gerontology and Geriatrics, Leiden University Medical Center, P.O. Box 9600, 2300 RC, Leiden, the Netherlands; [2]Department of Molecular Epidemiology, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands, [3]Department of Internal Medicine, Southern Illinois University School of Medicine, P.O. Box 19636, 62794-9628, Springfield, Illinois, United States of America, [4]Netherlands Consortium of Healthy Aging (NCHA).

Age (Dordr). 2011 Jan 19 Age Epub ahead of print

Abstract

Earlier we showed that the offspring from exceptionally long-lived families have a more favorable glucose metabolism when compared to controls. As chronic low-grade inflammation has been regarded as a strong risk factor for insulin resistance, we evaluated if and to what extent the favorable glucose metabolism in offspring from long-lived families could be explained by differences in subclinical inflammation, as estimated from circulating levels of C-reactive protein. We found no difference between the two groups in C-reactive protein levels or in the distribution of C-reactive protein haplotypes. However, among controls higher levels of C-reactive protein were related to higher glucose levels, whereas among offspring levels of C-reactive protein were unrelated to glucose levels. The current study suggests that the favorable glucose metabolism in offspring from long-lived families allows tight regulation of glucose levels despite elevated markers of low-grade inflammation. This finding indicates a robust insulin sensitivity which may render subjects from long-lived families resilient against the effects of low-grade inflammation and concomitant morbidity.

Introduction

The association between chronic subclinical inflammation and insulin resistance has been well established [1]. Insulin resistance in turn is regarded a strong risk factor for type 2 diabetes, hypertension and cardiovascular disease [2;3]. Levels of C-reactive protein (CRP), a marker of systemic inflammation, are elevated in subjects with impaired glucose tolerance as well as in overt diabetes [4;5] and increased levels of CRP are predictive for development of diabetes [6;7] Observational studies have shown a relation between elevated levels of CRP and elevated glucose levels in subjects without diabetes as well [8-10].

In the Leiden Longevity Study we have recruited exceptionally long-lived families based on proband siblings that both exhibit exceptional longevity. We also included the middle-aged offspring of the long-lived siblings and their partners as population based controls. Earlier we have shown that the offspring from these long-lived families have a lower prevalence of type 2 diabetes when compared to controls [11]. Moreover, we demonstrated that after exclusion of diabetic patients the offspring from long-lived families had relatively lower fasted and non-fasted glucose levels as well as a higher glucose tolerance [12;13]. The differences in glucose metabolism between offspring from long-lived families and controls could not be explained by differences in body composition or life style, such as smoking or physical activity.

In the current study, we sought to evaluate whether and to what extent the observed differences in glucose metabolism between the offspring from long-lived families and controls can be explained by differences in subclinical inflammation, as estimated from circulating levels of CRP. Therefore we examined high-sensitivity C-reactive protein (hsCRP) serum levels as well as CRP genotypes and their influence on glucose regulation in the offspring from exceptionally long-lived families and controls.

Materials and methods

The Leiden Longevity Study

The recruitment of 421 families in the Leiden Longevity Study has been described before.[14] A short outline is provided here. Families were recruited if at least two long lived siblings were alive and fulfilled the age-criterion of 89 years or older for males and 91 year or older for females. There were no selection criteria on health or demographic characteristics. For 2415 offspring of long-lived siblings and their partners as population controls, non-fasting serum samples were taken at baseline for the determination of endocrine and metabolic parameters. Additional information was collected from the generation of offspring and controls, including self-reported information on life style, information on medical history from the participants'

treating physicians and information on medication use from the participants' pharmacists. Subjects with hsCRP levels higher than 10.0 mg/L were excluded from the study (68 offspring (4.6%) and 31 controls (4.9%)) as were subjects with diabetes (65 offspring (4.4%) and 53 controls (8.3%)). Subjects were regarded as having diabetes if they had non-fasted glucose levels >11.0 mmol/L, a previous medical history of diabetes and/or used glucose lowering agents. In 84 subjects (61 offspring and 23 controls) data on hsCRP and/or glucose were not available.

CRP genotypes

Three single nucleotide polymorphisms (SNPs) were selected that associate with serum CRP levels: rs1205 (positioned in 3'UTR, alleles C/T), Rs1800947 (positioned in Codon 184, alleles G/C) and Rs1417938 (positioned in intron 1, alleles T/A). Genotyping of the SNPs was performed using Sequenom MassARRAY iPLEX®Gold. The high iPLEX primer design was performed by entering the sequences encompassing each polymorphism into SpectroDESIGNER provided by Sequenom®, Inc. (CA, USA). The high plex reaction protocol was used (www.sequenom.com/iplex). The average genotype call rate for genotyped SNPs was 96.3% and the average concordance rate was 99.7% among 4% duplicated control samples. All SNPs were in Hardy-Weinberg equilibrium ($P \geq 0.05$)

Plasma parameters

All serum measurements were performed with fully automated equipment. For insulin the Immulite 2500 from DPC (Los Angeles, CA, USA) was applied. For insulin the coefficient of variation was below 8%. For glucose and CRP, the Hitachi Modular P 800 from Roche, Almere, the Netherlands was applied. CV's of these measurements were below 5 %.

Statistical analysis

The program haploview was used to estimate allele frequencies, test the consistency of genotype frequencies at each SNP locus with Hardy-Weinberg equilibrium and estimate and plot pair wise LD between the examined SNPs. Haplotypes and haplotype frequencies were estimated using the SNPHAP software. The posterior probabilities of haplotypes <95% were excluded from the analyses (61 offspring and 24 controls).

Distributions of continuous variables were examined for normality and serum insulin levels and hsCRP levels were logarithmically transformed prior to analyses. Differences in age between the groups of offspring and controls were tested using a Mann-Whitney rank sum test. Differences in sex distribution between the groups of offspring and controls were calculated using a Chi-square test. Geometric means (with 95% confidence intervals (CI)) are reported for transformed

variables. Associations between serum levels as well as associations between *CRP* haplotypes and serum levels were tested using a linear mixed model with a random sibship effect to model correlation of sibling data. Age, sex, body mass index and use of lipid lowering agents were regarded as potential confounders in this association and were included in the analyses. The Statistical Package for the Social Sciences (SPSS) program for Windows, version 16.0 was used for data analysis.

Results

The principal features of the study groups, both without diabetes, are displayed in **table 1**. The proportion of males was slightly higher in the group of offspring than in the group of controls. Body mass index was similar between the two groups (P=0.57). Non-fasted glucose levels were lower in the group of offspring when compared to the group of controls (P=0.001), while non-fasted insulin levels did not differ (P=0.16). We did not observe a difference in serum levels of high sensitivity C-reactive protein (hsCRP) between the two groups. Also after adjustment for the potential confounders age, sex, body mass index and the use of lipid lowering agents, no difference in hsCRP levels was observed between the two groups (**table1**)

Potentially, genetic variation could mask true differences in CRP levels between the two groups. To distinguish between constitutional and acquired levels of hsCRP, we performed a genetic analysis of haplotypes constructed from the common *CRP* variants rs1205, rs1800947 and rs1417938, that have previously been associated with serum CRP levels. For the present analyses we report the results of the four most common haplotypes (frequency>5%) that cumulatively account for 99.9% of the haplotypes. The relation between the four selected haplotypes and serum hsCRP levels is depicted in **table 2**. All haplotypes correlated significantly with hsCRP levels. An increasing number of haplotype 1 and 2 gave rise to higher hsCRP levels, whereas an increasing number of haplotype 3 and 4 was related to a decrease in hsCRP serum levels. The change in hsCRP levels over the number of haplotypes was not different between offspring and controls.

Table 1. Baseline characteristics of the study population

	Offspring	Controls	p-value
Participants (N)	1479	63**5**	
Male sex (N, %)	691 (46.7)	264 (41.**6**)	***0.032***
Age in year (median (interquartile range)	59.1 (54.9 – 64.0)	58.**7** (53.8 – 63.6)	0.078
Body Mass Index in kg/m^2 (mean 95% CI)*	25.3 (25.0 – 25.5)	25.4 (25.1 – 25.7)	0.57
Lipid-lowering agent (N, %)	87 (5.9)	45 (7.1)	0.33
Currently smoking[§]	167 (13.2)	78 (14.0)	0.66
Insulin in µIU/L (mean 95% CI)	15.7 (14.9 – 16.4)	16.6 (15.5 – 17.7)	0.16
Glucose in mmol/L (mean 95% CI)	5.70 (5.64 – 5.77)	5.90 (5.81 – 5.99)	***0.001***
HsCRP in mg/dL (mean 95% CI)			
Adjusted for sex and age (model 1)	1.21 (1.15 – 1.28)	1.25 (1.16 – 1.34)	0.54
Model 1 and body mass index	1.20 (1.32 – 1.26)	1.23 (1.15 – 1.32)	0.56
Model 1 and lipid lowering agents	1.06 (0.97 – 1.16)	1.10 (1.00 – 1.21)	0.48
Model 1 and current smoking status[†]	1.30 (1.21 – 1.40)	1.34 (1.23 – 1.46)	0.53
Model 1 and body mass index and lipid lowering agents and current smoking status	1.11 (1.01 – 1.23)	1.14 (1.03 – 1.26)	0.54

Results for insulin and hsC-reactive protein (hsCRP) are presented as estimated geometric means with 95% confidence intervals. *Data on body mass was available for 1823 subjects (1266 offspring and 557 partners). Results for body mass index were adjusted for age and sex. [†]Data on [§]current smoking status were available for 1819 subjects (1262 offspring and 557 partners). Results for insulin and glucose were adjusted for age, sex and body mass index. 95% CI: 95% confidence interval.

Next we assessed if the distribution of *CRP* haplotypes was different between the two groups. In both the study groups haplotypes were in Hardy-Weinberg equilibrium. No difference in the frequencies of CRP haplotypes was observed between the group of offspring and the group of controls. (**table 3**)

Table 2. Association between CRP haplotypes and serum hsCRP levels

Haplotype	0-copies (mean (95% CI))	1-copy (mean (95% CI))	2-copies (mean (95% CI))	P for trend	P interaction
HsCRP (mg/dL)					
1	1.17 (1.09 – 1.24)	1.25 (1.18 – 1.34)	1.35 (1.20 – 1.51)	***0.015***	0.87
2	1.13 (1.06 – 1.20)	1.28 (1.20 – 1.36)	1.45 (1.28 – 1.65)	***<0.001***	0.63
3	1.32 (1.24 – 1.40)	1.14 (1.07 – 1.22)	1.04 (0.89 – 1.22)	***<0.001***	0.18
4	1.27 (1.21 – 1.34)	0.97 (0.87 – 1.09)	0.52 (0.29 – 0.93)	***<0.001***	0.23

Results for serum high-sensitivity C-reactive protein (hsCRP) are given as estimated geometric means and 95% confidence intervals for number of haplotypes. Results were adjusted for sex, age and study group (controls and offspring). P for interaction was calculated for the difference between controls and offspring in the trend of hsCRP over increasing number of haplotypes.

Table 3. CRP haplotype structure and frequencies

	SNP allele			Frequency		
Haplotype	rs1205	rs1800947	rs1417938	Offspring	Controls	p-value
1	T	G	T	0.335	0.344	0.57
2	T	G	**A**	0.336	0.315	0.17
3	**C**	G	T	0.260	0.273	0.38
4	**C**	**C**	T	0.069	0.068	0.94

Minor alleles are in bold. SNP=single nucleotide polymorphism

Since high levels of CRP have been associated with insulin resistance, we examined the association between serum levels of hsCRP and serum levels of non-fasted insulin as well as glucose for the group of offspring and the group of controls. Higher levels of hsCRP were consistently related to higher levels of insulin in both groups (**figure 1A**). In the group of offspring one standard deviation increase in ln hsCRP was associated with an increase in levels of ln serum insulin of 0.08 (95% confidence interval: 0.04 – 0.13) µIU/L (p<0.001). In the group of controls one standard deviation increase in ln hsCRP was associated with an increase in levels of ln serum insulin of 0.11 (0.04 – 0.19) (p=0.004). The relation between hsCRP and insulin was not different between the two groups (p for interaction=0.28). Next we examined the relation between hsCRP and glucose in the two groups. Among offspring no significant relation was observed between one standard deviation increase in ln hsCRP and glucose (mmol/L): 0.01 (-0.05 – 0.08)

(p=0.71). (**figure 1B**). In contrast, among controls one standard deviation increase in ln hsCRP was related with a 0.13 (0.02 – 0.24) mmol/L increase in serum glucose levels (p=0.017). The association between hsCRP and glucose was significantly different in the group of controls when compared to the group of offspring (p for interaction=0.020).

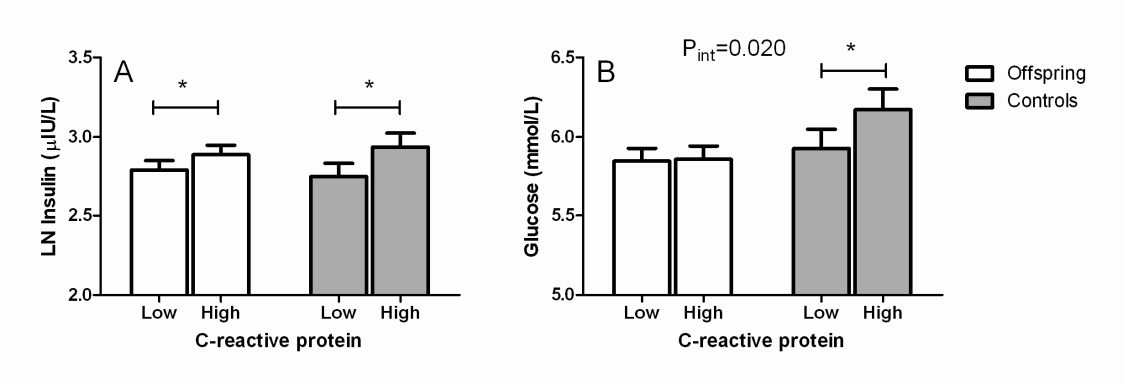

Figure 1. Association between hsCRP levels and non-fasted serum insulin levels (A) and serum glucose levels (B) for offspring and controls. *For the figure HsCRP levels were dichotomized into categories of low and high hsCRP levels based on the median value of CRP of the whole population (1.15 mg/dL). * denotes P-value lower than 0.05.*

If *CRP* haplotypes associate with CRP levels and CRP levels associate with markers of glucose metabolism, it could be expected that *CRP* haplotypes associate with markers of glucose metabolism. To tease out the causal relation between serum hsCRP and glucose metabolism we assessed the relationship between *CRP* haplotypes and serum levels of insulin as well as glucose. (**table 4**) In both groups, none of the haplotypes demonstrated an association with levels of serum insulin nor serum glucose.

Table 4. Association between CRP haplotypes and serum glucose parameters

Haplotype	0-copies (mean (95% CI))	1-copy (mean (95% CI))	2-copies (mean (95% CI))	P for trend	P interaction
Serum insulin (μIU/L)					
1	16.1 (15.2 – 17.1)	16.2 (15.3 – 17.2)	16.9 (15.2 – 18.7)	0.54	0.68
2	16.4 (15.5 – 17.4)	16.0 (15.1 – 17.0)	16.6 (14.8 – 18.7)	0.84	0.15
3	16.4 (15.5 – 17.2)	16.5 (15.5 – 17.5)	14.4 (12.4 – 16.6)	0.09	0.23
4	16.1 (15.5 – 16.9)	17.3 (15.6 – 19.2)	11.9 (6.57 – 21.6)	0.41	0.83
Serum glucose (mmol/L)					
1	5.80 (5.72 – 5.87)	5.82 (5.74 – 5.89)	5.83 (5.68 – 5.97)	0.66	0.49
2	5.80 (5.73 – 5.88)	5.79 (5.71 – 5.87)	5.92 (5.76 – 6.07)	0.42	0.39
3	5.84 (5.77 – 5.91)	5.79 (5.71 – 5.87)	5.65 (5.46 – 5.85)	0.09	0.40
4	5.80 (5.74 – 5.86)	5.88 (5.74 – 6.02)	5.39 (4.67 – 6.11)	0.57	0.18

Results for serum insulin are given as estimated geometric means and 95% confidence intervals for number of haplotypes. Results for glucose are given as estimated means and 95% confidence intervals for number of haplotypes. Results were adjusted for sex, age and study group (controls and offspring). P for interaction was calculated for the difference between controls and offspring in the trend of insulin or glucose over increasing number of haplotypes.

Discussion

Earlier it has been demonstrated that the offspring from exceptionally long-lived families have a more favorable glucose metabolism when compared to population based controls.[12;13] In the present study we show that this difference in glucose metabolism could not be explained by current differences in subclinical inflammation, as estimated from serum levels of hsCRP. We did not find a difference in the levels of hsCRP between the group of offspring from long-lived families and the group of environmentally matched controls, nor in the frequencies of the common genetic CRP variants and haplotypes. All CRP haplotypes correlated significantly with hsCRP levels, however no association was found between CRP haplotypes and insulin or glucose levels. We observed a distinct association between levels of hsCRP and levels of glucose in the

two groups: in the group of controls increasing levels of hsCRP were associated with higher non-fasted glucose levels, whereas in the group of offspring this relation was absent.
The lack of difference between the group of offspring and controls in hsCRP levels as an approximate measure for low-grade inflammation is remarkable given the higher prevalence of metabolic syndrome in the group of controls [13]. The metabolic syndrome is a combination of cardio-vascular risk factors for which the dominant underlying factor appears to be insulin resistance [15]. A chronic subclinical inflammatory state is considered a crucial factor in the development of insulin resistance [5;5;16]. In accordance, insulin resistance has been shown to correspond closely with elevated levels of inflammatory markers as CRP [17;18]. The current study demonstrates that the offspring from long-lived families compared to controls are able to tightly regulate glucose levels despite elevated markers of low-grade inflammation. This suggests that the more favorable glucose metabolism reported earlier in offspring is sustained and even more manifest under challenging conditions as for example during low-grade inflammation. Subjects from long-lived families seem to be protected by their robust insulin sensitivity against the effects of low-grade inflammation and concomitant morbidity, as for example metabolic syndrome. Alternatively, CRP might be causal in the observed difference in insulin sensitivity in response to inflammation. However, the consistent lack in both groups of association between CRP haplotypes with glucose levels strongly argues against this explanation.

During inflammation, acute phase reactants and pro-inflammatory cytokines are thought to induce a hypermetabolic state. This hypermetabolic state, which is aimed at mobilizing energy to support immune function and tissue repair, is characterized by an accelerated gluconeogenesis [19] as well as induced insulin resistance resulting in elevated glucose levels [20] (**figure 2A**). Preserved insulin action in the offspring presumably allows for disposal of the excess glucose and/or reduced hepatic gluconeogenesis, thereby abolishing the inflammatory induced hyperglycemia (**figure 2B**). Hypothetically, a favorable balance between insulin-sensitizing and diabetogenic components underlies this phenomenon. Multiple endogenous diabetogenic components and insulin-sensitizing factors have been identified, as for example IL-6, TNF-α, PAI-1 and leptin on the one hand, and adiponectin on the other hand.[21] Our future research will focus on unraveling the specific mechanisms of the robust glucose metabolism in offspring from long-lived families.

In conclusion, we found that the previously observed favorable glucose metabolism in middle-aged offspring from exceptionally long-lived families is unaffected by low-grade inflammation, as estimated by the inflammatory marker hsCRP, suggesting a resilience against the hyperglycemic effects of a chronic low-grade inflammatory state.

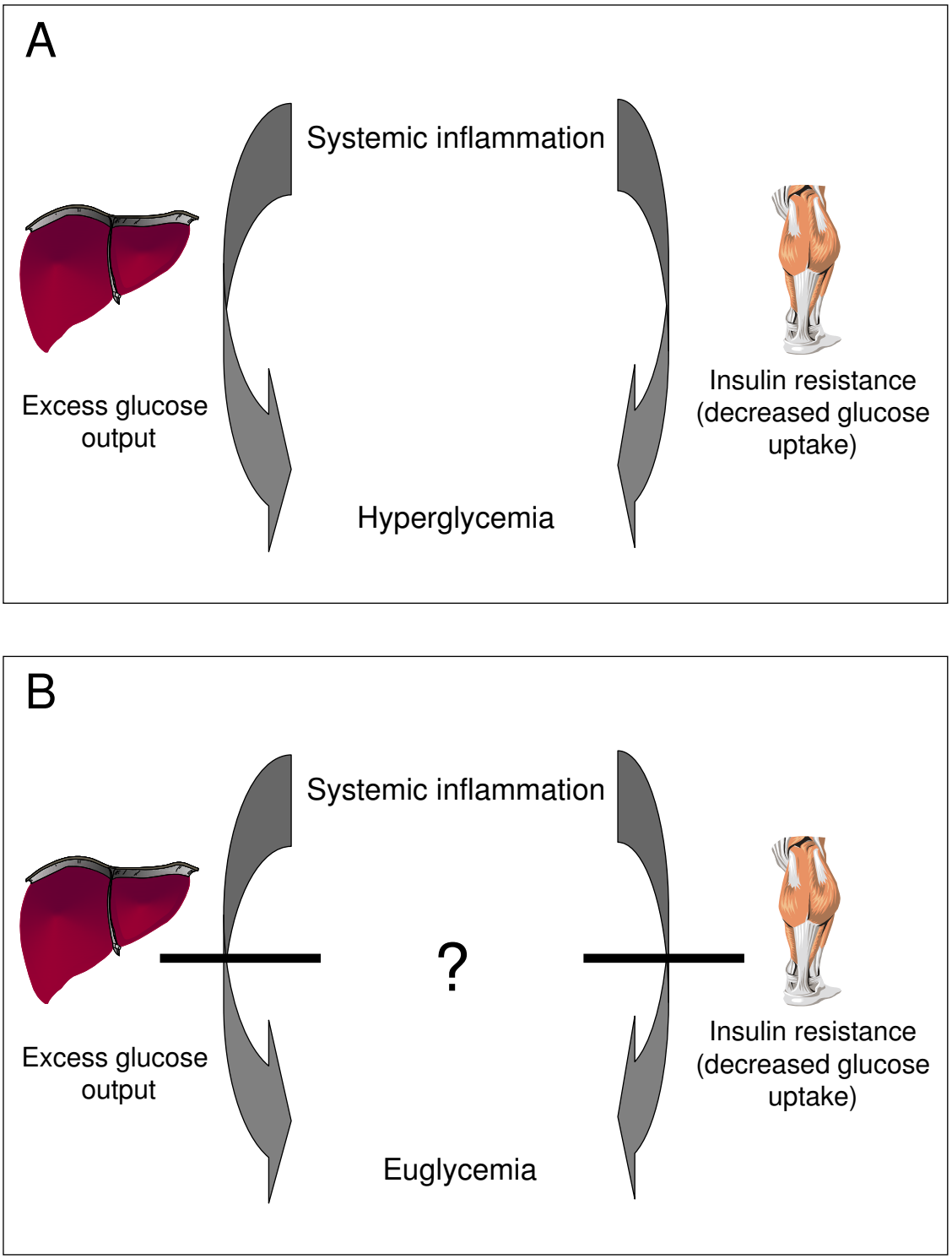

Figure 2. Mechanism of inflammatory induced hyperglycemia. *The state is characterized by an accelerated hepatic gluconeogenesis as well as induced peripheral insulin resistance leading to hyperglycemia (A). Preserved insulin action in the offspring from long-lived families presumably allows for disposal of excess glucose and/or reduced hepatic gluconeogenesis, thereby abolishing the inflammatory induced hyperglycemia (B).*

Acknowledgements

We thank Stella Trompet for her valuable assistance with the statistical analyses of the data. This study was supported by the Innovation Oriented research Program on Genomics (SenterNovem; IGE01014 and IGE5007), the Centre for Medical Systems Biology (CMSB), the Netherlands Genomics Initiative/Netherlands Organization for scientific research (NGI/NWO; 05040202) and the EU funded Network of Excellence Lifespan (FP6 036894).

Reference List

(1) Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. *J Clin Invest* 2006;116:1793-1801.

(2) McFarlane SI, Banerji M, Sowers JR. Insulin resistance and cardiovascular disease. *J Clin Endocrinol Metab* 2001;86:713-718.

(3) Shen DC, Shieh SM, Fuh MM, Wu DA, Chen YD, Reaven GM. Resistance to insulin-stimulated-glucose uptake in patients with hypertension. *J Clin Endocrinol Metab* 1988;66:580-583.

(4) Ford ES. Body mass index, diabetes, and C-reactive protein among U.S. adults. *Diabetes Care* 1999;22:1971-1977.

(5) Temelkova-Kurktschiev T, Siegert G, Bergmann S et al. Subclinical inflammation is strongly related to insulin resistance but not to impaired insulin secretion in a high risk population for diabetes. *Metabolism* 2002;51:743-749.

(6) Barzilay JI, Abraham L, Heckbert SR et al. The relation of markers of inflammation to the development of glucose disorders in the elderly: the Cardiovascular Health Study. *Diabetes* 2001;50:2384-2389.

(7) Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA* 2001;286:327-334.

(8) Doi Y, Kiyohara Y, Kubo M et al. Relationship between C-reactive protein and glucose levels in community-dwelling subjects without diabetes: the Hisayama Study. *Diabetes Care* 2005;28:1211-1213.

(9) Festa A, D'Agostino R, Jr., Tracy RP, Haffner SM. C-reactive protein is more strongly related to post-glucose load glucose than to fasting glucose in non-diabetic subjects; the Insulin Resistance Atherosclerosis Study. *Diabet Med* 2002;19:939-943.

(10) Nakanishi N, Shiraishi T, Wada M. Association between fasting glucose and C-reactive protein in a Japanese population: the Minoh study. *Diabetes Res Clin Pract* 2005;69:88-98.

(11) Westendorp RG, van HD, Rozing MP et al. Nonagenarian siblings and their offspring display lower risk of mortality and morbidity than sporadic nonagenarians: The Leiden Longevity Study. *J Am Geriatr Soc* 2009;57:1634-1637.

(12) Rozing MP, Westendorp RG, Frolich M et al. Human insulin/IGF-1 and familial longevity at middle age. *Aging (Albany NY)* 2009;1:714-722.

(13) Rozing MP, Westendorp RG, de Craen AJ et al. Favorable glucose tolerance and lower prevalence of metabolic syndrome in offspring without diabetes mellitus of nonagenarian siblings: the Leiden longevity study. *J Am Geriatr Soc* 2010;58:564-569.

(14) Schoenmaker M, de Craen AJ, de Meijer PH et al. Evidence of genetic enrichment for exceptional survival using a family approach: the Leiden Longevity Study. *Eur J Hum Genet* 2006;14:79-84.

(15) Hanley AJ, Karter AJ, Festa A et al. Factor analysis of metabolic syndrome using directly measured insulin sensitivity: The Insulin Resistance Atherosclerosis Study. *Diabetes* 2002;51:2642-2647.

(16) Spranger J, Kroke A, Mohlig M et al. Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Diabetes* 2003;52:812-817.

(17) Festa A, D'Agostino R, Jr., Howard G, Mykkanen L, Tracy RP, Haffner SM. Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). *Circulation* 2000;102:42-47.

(18) Yudkin JS, Stehouwer CD, Emeis JJ, Coppack SW. C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *Arterioscler Thromb Vasc Biol* 1999;19:972-978.

(19) Mizock BA. Alterations in carbohydrate metabolism during stress: a review of the literature. *Am J Med* 1995;98:75-84.

(20) Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 2006;7:85-96.

(21) Arai Y, Kojima T, Takayama M, Hirose N. The metabolic syndrome, IGF-1, and insulin action. *Mol Cell Endocrinol* 2009;299:124-128.