Klotho locus, metabolic traits, and serum hemoglobin in hospitalized older patients: a genetic association analysis

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Abstract Klotho (KL) gene has been involved in severe alterations of physiological biochemical parameters leading to premature aging-like phenotypes and strikingly shortening lifespan. KL participates to the regulation of a number of intracellular biochemical pathways, including lipid profile and glucose metabolism. Aim of this study was to investigate the possible association between KL locus

and biological parameters commonly accepted as indicators of the clinical status in hospitalized older patients. We genotyped the single-nucleotide polymorphisms (SNPs) rs9536314, rs1207568, and rs564481 at the KL locus in 594 hospitalized older patients (65–99 years), consecutively attending a geriatric ward, and tested the association of these KL variants with biological quantitative traits using analyses of covariance and genetic risk score models. Significant associations of rs9536314 with serum levels of hemoglobin, albumin, and high-density lipoprotein cholesterol (HDL-C) as well as significant associations of rs564481 with serum levels of hemoglobin, fasting insulin, and fasting glucose were observed. Gender-segregated analyses confirmed these associations, and suggested that the associations of KL genotypes with HDL-C, fasting glucose and fasting insulin levels may be driven by the female gender, while the association with serum levels of hemoglobin may be driven by the male gender. The association of KL genotypes with creatinine levels was found only in females, while the association with insulin-like growth factor-1 (IGF-1) and lymphocytes count (LC) was found only in males. The genetic risk score (GRS) models further confirmed significant associations among KL SNPs and hemoglobin, total cholesterol, and HDL-C. Gender-segregated analyses with the GRS-tagged approach confirmed the associations with HDL-C, fasting glucose, and fasting insulin levels in females, and with hemoglobin and LC in males. Our findings suggested that KL locus

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may influence quantitative traits such as serum levels of lipid, fasting glucose, albumin and hemoglobin in hospitalized older patients, with some gender differences suggested for creatinine, IGF-1 levels, and LC, thus being one of the genetic factors possibly contributing to age-related diseases and longevity.

Keywords Klotho · Chromosome 13 · High-density lipoprotein cholesterol · Total cholesterol · Fasting glucose · Fasting insulin · Insulin-like growth factor-1 · Hemoglobin · Creatinine · Albumin · Lymphocytes

Introduction

In the past century, most of Western countries have experienced a significant increase in life expectancy, mainly due to a marked reduction in early-life mortality as well as in mortality at ages above 70 years (Vaupel et al. 1998). These changes are rapid on an evolutionary timescale, suggesting that important factors causing variation in aging process are unlikely to be of genetic origin. However, within a given birth cohort in a given country, there is still a large variation in lifespan (Christensen et al. 2006). The clarification at what extent this variation is related to genetic differences among individuals, and to understand the roles of specific genetic factors in this variation, is pivotal to understand the aging process.

The list of genetic factors influencing biological processes associated with aging, and possibly influencing life expectancy, is extremely long (Christensen et al. 2006). Recently, Klotho (KL) (AB005142) gene at locus 13q12 has been suggested to have a role in the aging process and the development of aging-like phenotypes (Kuro-o et al. 1997; Kurosu et al. 2005), throughout its role in important cellular pathways (Bartke 2006; Unger 2006). This gene encodes a protein very different from those involved in previously described premature-aging syndromes and cell senescence (Kuro-o et al. 1997). All these proteins function essentially in the nucleus. Conversely, KL gene, because of an alternative RNA splicing, produced two protein isoforms acting out of the nucleus, a membrane-linked form and a secreted form (Matsumura et al. 1998; Imura et al. 2004), the latter acting as an hormone in several cellular pathways (Russell and Kahn 2007; Kuro-o 2008; Razzaque

2009). In particular, the secreted KL isoform acts as an endocrine regulator of several cell surface glycoproteins, including ion channels and growth factor receptors, such as the insulin-like growth factor 1 receptor (IGF1R), that have been involved in aging control and stress resistance (Wang and Sun, 2009).

KL appeared to be an aging suppressor gene. In fact, mice homozygous for hypomorphic KL alleles, exhibit multiple aging phenotypes, including short lifespan and arteriosclerosis (Kuro-o et al. 1997). These mice have reduced blood glucose and insulin levels, as well as increased insulin sensitivity (Kuro-o et al. 1997; Utsugi et al. 2000). Conversely, mice over-expressing KL have an extended lifespan by about 20–30% and are insulin resistant (Kurosu et al. 2005). The membrane-linked KL isoform acts as an obligate co-receptor for fibroblast growth factor-23 (FGF23) (Kurosu et al. 2006; Urakawa et al. 2006) by interacting with FGF receptors in the kidney. FGF23 is a bone-derived hormone that inhibits both phosphate reabsorption as well as vitamin D biosynthesis. Interestingly, mice lacking either KL or FGF23 exhibit a premature-aging syndrome (Razzaque et al. 2006), which reveals an unexpected link between phosphate metabolism and aging. This link was also confirmed by a recent study showing that a missense mutation in KL gene results in decreased expression of KL protein and concomitant decreased FGF23 signaling, leading to a severe form of hyperphosphatemic familial tumoral calcinosis characterized by widespread cutaneous and visceral calcifications, diffuse osteopenia, and sclerodactyly (Ichikawa et al. 2007).

The secreted KL isoform is predominantly expressed in distal convoluted tubules of the kidneys and in the choroid plexus of the brain (Kurosu et al. 2005; Zhang and Zheng 2008). KL expression in the kidneys has been shown to be reduced in patients with chronic renal failure (Koh et al. 2001) and with acute renal failure in ischemia reperfusion injury murine models (Sugiura et al. 2005). These findings would imply that the reduction of KL protein may be relevant to the pathophysiology of renal failure. Interestingly, aged rats demonstrated a significant decrease in KL protein expression in renal cortex and medulla, and the aging-related kidney damage was exacerbated in aged rats with cognitive impairment (Zuo et al. 2010).

In humans, KL polymorphisms have been extensively associated with glucose metabolism (Rhee et al. 2006a; Shimoyama et al. 2009a; Lorenzi et al.

2010), lipid metabolism, serum levels of high-density lipoprotein cholesterol (HDL-C) (Arking et al. 2005; Walter 2009; Shimoyama et al. 2009a, b) and uric acid (Shimoyama et al. 2009b). KL polymorphisms have been also variously associated with some features of the aging process such as osteopenia/osteoporosis (Kawano et al. 2002; Shimoyama et al. 2009a; Mullin et al. 2005; Yamada et al. 2005), atherosclerosis (Saito et al. 2000), coronary artery disease (Arking et al. 2003; Low et al. 2005; Imamura et al. 2006; Rhee et al. 2006a, b; Walter 2009), stroke (Arking et al. 2005; Kim et al. 2006), blood pressure (Arking et al. 2005; Rhee et al. 2006a; Shimoyama et al. 2009a), type 2 diabetes mellitus (Freathy et al. 2006) and more generally with human lifespan, although with contrasting results (Utsugi et al. 2000; Arking et al. 2002, 2005; Novelli et al. 2008; Invidia et al. 2010). In particular, a common haplotype, termed “KL-VS”, composed of six single-nucleotide polymorphisms (SNPs) in perfect linkage disequilibrium (including rs9536314) is present in ~15% of Caucasians (Arking et al. 2002). It has been suggested that this KL variant influences the trafficking and the catalytic activity of KL protein (Arking et al. 2002), giving a survival advantage of KL-VS/wt elderly heterozygotes but not of KL-VS/KL-VS elderly homozygotes (Arking et al. 2002, 2005). Furthermore, some recent reports also proposed possible associations of the SNPs rs1207568 and rs564481 with glucose metabolism, lipid metabolism, and cardiovascular risk factors (Rhee et al. 2006a; Shimoyama et al. 2009a; Shimoyama et al. 2009b). Up to date, no data are available regarding the role of the KL polymorphisms in influencing biological parameters in hospitalized elderly patients. The aim of the present study was to investigate the possible association among the SNPs rs9536314, rs1207568, and rs564481 at the KL locus and biological parameters, mainly lipid and glucose metabolism-linked, commonly accepted as indicators of the clinical status in hospitalized older patients attending a geriatric ward.

Methods

Subjects

This was a prospective cross-sectional study fulfilling the Declaration of Helsinki, the guidelines for Good Clinical Practice and the Strengthening the Reporting of

Observational Studies in Epidemiology guidelines (available at URL <http://www.strobe-statement.org/>). The approval of the study for experiments using human subjects was obtained from the local ethic committee on human experimentation. Written informed consent for research was obtained from each patient or from relatives/legal guardian in the case of critically disabled demented patients. From January to December 2007, all patients consecutively admitted to the Geriatrics Unit of the Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) “Casa Sollievo della Sofferenza” were screened for the enrolment in the study. All screened subjects were Caucasians, and did not include people of Jewish, Eastern Europe, or Northern Africa descent, with most individuals having Central and Southern Italy ancestry.

Inclusion/exclusion criteria

Inclusion criteria were: (1) age ≥ 65 years; (2) ability to provide an informed consent and willingness to participate in the study; (3) availability of a comprehensive geriatric assessment (CGA) scored during hospitalization. Patients were excluded from the study if they refused to enter in the study or to sign the informed consent. Patients with a diagnosis of neoplasia or diseases of the blood and blood-forming organs were also excluded from the study.

Clinical assessment

At baseline, by means of a structured interview, a clinical evaluation, and a review of records from the patients' general practitioners, the following parameters were collected: date of birth, gender, clinical history, current pathologies, and medication history. To evaluate clinical, cognitive, and functional status, the CGA was carried out at admission in all patients (Pilotto et al., 2008). Briefly, functional status was evaluated by the activities of daily living (ADL) index (Katz et al. 1963) and the instrumental activities of daily living (IADL) scale (Lawton and Brody 1969). ADL defines the level of dependence/independence in six daily care activities including bathing, toileting, feeding, dressing, continence and transferring. IADL assess the independence in eight activities that are more cognitively and physically demanding than ADL, including managing finances, taking medications, using telephone, shopping, using transportation,

preparing meals, doing housework, and washing. Cognitive status was screened by the Short Portable Mental Status Questionnaire (SPMSQ) (Pfeiffer 1975), a ten-item questionnaire that assesses orientation, memory, attention, calculation, and language. Comorbidity was examined using the Cumulative Illness Rating Scale (CIRS) (Parmelee et al. 1995). The CIRS uses a five-point ordinal scale (score: 1–5) to estimate the severity of pathology in each of 13 systems, including cardiac, vascular, respiratory, eye–ear–nose–throat, gastrointestinal, hepatic, renal, genitourinary, musculoskeletal, skin, nervous system, endocrine-metabolic, and psychiatric behavioral disorders. Based on these ratings, the comorbidity index (CIRS-CI) score, which reflects the number of concomitant diseases, was derived from the total number of categories in which moderate or severe levels (grade 3–5) of disease were identified (range 0–13). Nutritional status was explored with the mini nutritional assessment (MNA) (Guigoz and Vellas, 1999), which includes information on (a) anthropometric measures; (b) lifestyle, medication, and mobility; (c) number of meals, food and fluid intake, and autonomy of feeding; and (d) self-perception of health and nutrition. A 30-point scoring system was proposed for the MNA, with an MNA score ≥ 24 points identifying a normal nutrition status, an MNA score of 17–23.5 points that suggested a borderline status/at risk for malnutrition, and an MNA score < 17 points identifying malnutrition (Guigoz and Vellas 1999). The Exton–Smith Scale (ESS) (Bliss et al. 1966) was used to evaluate the risk of developing pressure sores. This five-item questionnaire determines physical and mental condition, activity, mobility, and incontinence. For each item, a score from 1 to 4 is assigned. The main and secondary diagnoses at discharge from the hospital, coded according to the Italian Translation of the International Classification of Diseases, ninth revision, Clinical Modification (ICD-9-CM; available at URL <http://icd9cm.chrisendres.com/icd9cm/>) were also recorded in all patients. In particular, cardiovascular diseases included the following diseases classified as ICD-9-CM code: 401–405, 410–414, 415–429; neoplasia included diseases classified as ICD-9-CM code: 140–239; neurodegenerative diseases included diseases classified as ICD-9-CM code: 330–337; diseases of the blood and blood-forming organs included diseases classified as ICD-9-CM code: 280–289; endocrine, nutritional and metabolic diseases, and immunity

disorders included diseases classified as ICD-9-CM code: 249–259; cerebrovascular disease included diseases classified as ICD-9-CM code: 430–438.

Laboratory measurements

Serum white blood cell (WBC) and lymphocyte count (LC), erythrocyte sedimentation rate (ESR) and serum levels of hemoglobin, total cholesterol (TC), triglycerides (TG), iron, albumin, creatinine, fasting glucose and fasting insulin were determined in fresh blood samples using routine clinical chemistry methods. Serum levels of HDL-C and low-density lipoprotein cholesterol (LDL-C) were assayed enzymatically using the Roche HDL-C plus third generation and LDL-C plus second generation assays on Roche-automated clinical chemistry analyzer Modular P (Roche/Hitachi Diagnostics, GmbH, Mannheim, Germany). Quantitative measurement of IGF-1 in serum was made using solid-phase, chemiluminescent immunoassays (IMMULITE® 2000, Siemens Helthcare Diagnostics, Milano, Italy) on IMMULITE Analyzer. Serum free triiodothyronine (FT3) was measured using a commercial electrochemiluminescence immunoassay (Roche Diagnostics GmbH, Mannheim, Germany). Serum levels of C-reactive protein (CRP) were assessed by immunonephelometry.

SNP genotyping

Genomic DNA was purified from fresh/frozen blood samples following salting-out method (Miller et al. 1998). In this study, we analyzed the three single-nucleotide polymorphism (SNPs) rs1207568, rs9536314, and rs564481 spanning a 45 kb block at the KL locus (13p12) (33,590,184–33,634,983 bases from pter). Genotypes of the SNP rs1207568 (33,590,184 bases from pter), i.e., the C^{4,614}→T near the 5'UTR of the KL gene, were determined with the allele discrimination assay using TaqMan technology. Briefly, genomic DNA was analyzed with the ABI PRISM 7700 Sequence Detector system (Life Technologies Corporation, Carlsbad, CA, USA) using the assay c_7604792_10 according to manufacturer instructions. Genotypes of the SNP rs9536314 (33,628,138 bases from pter), i.e., the G^{42,568}→T in exon 2 of the KL gene causing the aminoacid change Phe³⁵²→Val, were determined as follows. Briefly, genomic DNA was amplified by PCR with a hot-start at 94°C for 7', followed by

30 cycles at 94°C for 30", 50°C for 30", and 72°C for 30". The PCR product (279 bp) was digested with Mae III for 6 h at 55°C. The analysis on a 3% agarose gel electrophoresis revealed the three genotypes T/T (232+47 bps), T/G (232+182+50+47 bps), and G/G (182+50+47 bps). Genotypes of the SNP rs564481 (33,634,983 bases from pter), i.e., the C^{49,413}→T causing the sense aminoacid change His⁵⁸⁹→His in exon 4 of the KL gene, were determined with the allele discrimination assay using TaqMan technology. Briefly, genomic DNA was analyzed with the ABI PRISM 7700 Sequence Detector system (Life Technologies Corporation, Carlsbad, CA, USA) using the assay c_592739_10 according to manufacturer instructions.

Statistical analysis

Patients' baseline characteristics were reported as mean±standard deviation (SD) or frequencies and percentages for continuous and categorical variables, respectively. For continuous variables, normal distribution was verified by the Shapiro–Wilk normality test and the 1-sample Kolmogorov–Smirnov test. Baseline comparisons between groups were made, for categorical variables, using the Fisher's exact test and, for continuous variables, using the *t* test or the analysis of variance and the analysis of covariance, using age, gender, diabetes mellitus, neurodegenerative diseases, cerebrovascular disease, and cardiovascular disease, as well as the use of statins, thyroid hormone, anti-inflammatory, and hypoglycemic drugs as covariates. For non-normally distributed variables, differences between groups were tested by the Wilcoxon rank-sum test with continuity correction or the Kruskal–Wallis rank sum test. In the genetic analysis the Hardy–Weinberg equilibrium was verified for each SNP, and the gene counting method was used to estimate the relative allele frequencies (Gerdes et al. 1992). Associations between SNPs and dichotomous or continuous outcomes were tested using multivariate logistic or linear regression models, respectively. Recessive, dominant, additive, and free genetic models of inheritance were tested in all hospitalized older patients and in gender-segregated analyses. The effect of multiple genetic risk loci on the outcomes was studied by constructing the genetic risk score (GRS) for each study participant. GRS was computed summing the number of risk alleles in all patients and in gender-segregated analyses. A *p* value<0.05 was

considered for statistical significance. All analyses were performed using SAS Release 9.1 (SAS Institute, Cary, NC, USA) and R Version 2.11.1 software package (The R Project for Statistical Computing. Available at URL <http://www.r-project.org/>).

Results

Demographic, clinical, and laboratory data

Demographic and clinical characteristics of patients according to gender were summarized in Table 1. No significant differences were observed between men and women in mean age, functional status assessed by means of ADL and IADL, comorbidity, and number of drugs taken, whereas significant differences were observed in cognitive status (SPMSQ, *p*=0.015), nutritional status (MNA, *p*=0.009), and ESS score (*p*<0.01).

Biochemical characteristics of patients according to gender were summarized in Table 2. No significant differences between men and women were observed in WBC count, LC, ESR, CRP, and the serum levels of iron, albumin, fasting glucose, TG, fasting insulin, and IGF-1. Conversely, females showed significant lower serum levels of hemoglobin (12.86±2.27 g/dl vs. 12.05±1.83 g/dl; *p*<0.001) and FT3 (2.81±0.68 pg/ml vs. 2.68±0.65 pg/ml; *p*=0.048), and higher serum levels of TC (153.39±42.98 mg/dl vs. 171.71±46.46 mg/dl; *p*<0.001), HDL-C (37.24±13.42 mg/dl vs. 42.58±16.97 mg/dl; *p*<0.001), and LDL-C (91.63±3.37 mg/dl vs. 102.42±37.62 mg/dl; *p*=0.001) than males.

Genetic analyses

Allele and genotype distribution of KL rs1207568, rs9536314, and rs564481 according to gender were summarized in Table 3. No significant differences were observed in the genotype and estimated allele frequency distribution between males and females.

The associations of KL genotypes with biological parameters of patients, assuming different genetic models, were summarized in Tables 4, 5, and 6. Overall, the analysis of the SNP rs1207568 (Table 4, all patients), did not reveal important significances. Only an association with IGF-1 in males in the recessive model was observed (*p*=0.038; Table 4,

Table 1 Demographic and clinical characteristics of hospitalized older patients according to gender

	Men (<i>n</i> =231)	Women (<i>n</i> =363)	<i>p</i> value	All (<i>n</i> =594)
Age	78.18±7.04	79.04±7.14	0.151	78.71±7.11
ADL	3.73±2.52	3.47±2.47	0.227	3.57±2.49
IADL	3.27±3.06	3.34±3.18	0.797	3.31±3.13
SPMSQ	2.94±3.37	3.65±3.24	0.015	3.37±3.31
MNA	21.36±5.86	20.08±5.66	0.009	20.57±5.76
CIRS	3.29 ±1.82	3.22±1.83	0.656	3.25±1.82
ESS	15.55±3.84	14.77±3.77	0.016	15.08±3.81
Number of drugs	4.70±3.06	4.53±2.75	0.494	4.60±2.87

Data are mean±standard deviation

ADL Activities of Daily Living, IADL Instrumental Activities of Daily Living, SPMSQ Short Portable Mental Status Questionnaire, MNA Mini Nutritional Assessment, CIRS-CI Cumulative Illness Rating Scale-Comorbidity Index, ESS Exton-Smith Scale

males). The analysis of rs9536314 (Table 5, all patients), free from any genetic model, revealed an association with serum levels of HDL-C ($p=0.016$). This association was confirmed in the recessive model ($p=0.005$). We also observed an association with serum concentration of hemoglobin ($p=0.038$) in the

dominant model, and with albumin ($p=0.035$) in the recessive model (Table 5, all patients). In gender-segregated analyses, the association of rs9536314 with hemoglobin in the dominant model was further confirmed, but only in males ($p=0.042$; Table 5, males), while the association with HDL-C was

Table 2 Biological parameters of hospitalized older patients according to gender

	Males (<i>n</i> =231)	Females (<i>n</i> =363)	<i>p</i> value	All (<i>n</i> =594)
WBC ($10^3/\mu\text{l}$)	8.37±3.62	7.91±3.36	0.088	8.09±3.47
Lymphocytes ($10^3/\mu\text{l}$)	1.81±0.88	1.92±0.79	0.052	1.88±0.82
ESR (mm)	39.25±31.69	43.11±29.19	0.119	41.62±30.21
CRP (pg/ml)	3.95±6.34	3.14±5.39	0.081	3.45±5.79
Hemoglobin (g/dl)	12.87±2.27	12.05±1.83	<0.001	12.37±2.05
Iron ($\mu\text{g/dl}$)	61.24±39.46	58.73±32.48	0.519	59.72±35.38
Albumin (g/dl)	3.97±0.57	3.95±0.52	0.752	3.95±0.54
Fasting glucose (mg/dl)	112.52±52.37	110.74±45.04	0.819	111.43±45.99
TG (mg/dl)	121.93±84.19	121.56±64.67	0.821	121.70±72.85
TC (mg/dl)	153.39±42.98	171.71±46.46	<0.001	164.41±45.95
HDL-C (mg/dl)	37.24±13.42	42.58±16.97	<0.001	40.52±15.90
LDL-C (mg/dl)	91.63±37.37	102.42±37.96	0.001	98.25±38.06
FT3 (pg/ml)	2.81±0.68	2.68±0.65	0.048	2.73±0.67
Fasting insulin (IU/ml)	11.41±33.40	9.32±7.14	0.230	10.13±21.54
IGF-1 (ng/ml)	79.26±40.57	75.56±40.71	0.384	77.00±40.66

Data are mean±standard deviation

The analyses were adjusted for age and gender and the presence of diabetes mellitus, neurodegenerative diseases, cerebrovascular disease, and cardiovascular diseases, as well as for the use of statins, thyroid hormone, anti-inflammatory, and hypoglycemic drugs

WBC white blood cells, ESR erythrocyte sedimentation rate, CRP C-reactive protein, TG triglycerides, TC total cholesterol, HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, FT3 free triiodothyronine 3, IGF-1 insulin-like growth factor 1

Table 3 Allele and genotype distribution of Klotho rs1207568, rs9536314, and rs564481 according to gender in hospitalized older patients

		Males		Females		<i>p</i> value	(95% CI)		All	
		<i>n</i>	%	<i>n</i>	%				<i>n</i>	%
rs1207568 ^a	C/C	156	72.22	238	60.41	Reference			394	70.61
	C/T	52	24.07	97	28.36	0.325	0.818	0.553–1.210	149	26.70
	T/T	8	3.70	7	2.05	0.297	1.744	0.643–4.723	15	2.69
	C	364	0.843	573	0.838	Reference			937	0.840
	T	68	0.157	111	0.162	0.867	0.964	0.695–1.339	179	0.160
rs9536314 ^b	T/T	159	73.61	240	72.95	Reference			399	73.21
	T/G	54	25.00	82	24.92	0.999	0.994	0.669–1.478	136	24.95
	G/G	3	1.39	7	2.13	0.746	0.647	0.180–2.335	10	1.83
	T	372	0.861	562	0.854	Reference			934	0.857
	G	60	0.139	96	0.146	0.791	0.944	0.667–1.336	156	0.143
rs564481 ^c	C/C	83	38.07	116	33.92	Reference			199	35.54
	C/T	96	44.04	164	47.95	0.334	0.818	0.561–1.193	260	46.43
	T/T	39	17.89	62	18.13	0.621	0.879	0.540–1.432	101	18.04
	C	262	0.601	396	0.579	Reference			658	0.588
	T	174	0.399	288	0.421	0.494	0.913	0.715–1.166	462	0.413

Hardy–Weinberg equilibrium (rs1207568: $p=0.875$; rs9536314: $p=0.861$; rs564481: $p=0.337$)

^a $N=558$ (93.94% of the total sample); 216 male (38.71%) and 342 female (61.29%)

^b $N=545$ (91.75% of the total sample); 216 male (39.63%) and 329 female (60.37%)

^c $N=560$ (94.28% of the total sample); 218 male (38.93%) and 342 female (61.07%)

confirmed free from any genetic model ($p=0.01$) and in the recessive model ($p=0.005$), but only for females (Table 5, females). We also observed an association with LC in the additive ($p=0.035$) and in the dominant ($p=0.023$) models, but only in males (Table 5, males), while there was an association with creatinine free from any genetic model ($p=0.036$) and in the recessive model ($p=0.002$), but only in females (Table 5, females). The analysis of rs564481 (Table 6, all patients), free from any genetic model, revealed an association with serum concentration of hemoglobin ($p=0.048$), serum fasting glucose ($p=0.028$), and serum fasting insulin ($p=0.035$). These associations were confirmed in both additive ($p=0.041$, $p=0.018$, and $p=0.027$, respectively) and recessive ($p=0.014$, $p=0.009$, and $p=0.010$, respectively) models (Table 6, all patients). In gender-segregated analyses, the association of rs564481 with hemoglobin in the recessive model was also confirmed, but only in males ($p=0.05$) (Table 6, males), while for females the association with serum fasting glucose was confirmed in all genetic

models, and the association with serum fasting insulin was confirmed in all genetic models except for the dominant one (Table 6, females). Finally, the gender-segregated analyses, free from any genetic model, revealed only for males an association with lymphocytes ($p=0.027$), also confirmed in the additive ($p=0.023$) and dominant ($p=0.007$) models (Table 6, males).

The association of KL genetic variations with biological parameters was also assessed using the GRS (Table 7 and Fig. 1). Considering together rs1207568, rs9536314, and rs564481, a progressive increasing number of at risk alleles was associated with raising levels of HDL-C ($p=0.023$; $\beta=1.707$; Fig. 1a). In order to understand which of these alleles more influenced this association, we calculated the GRS also considering paired SNPs at a time. Considering only rs1207568 and rs9536314, the association with HDL-C levels resulted still significant ($p=0.022$), with higher serum HDL-C levels for each added risk allele ($\beta=2.369$; Fig. 1b). Associa-

Table 4 Association of Klotho rs1207568 genotypes with laboratory parameters under different genetic model of inheritance in all hospitalized older patients and gender-segregated analyses

rs1207568	C/C	C/T	T/T	<i>p</i> value			
				Free	Additive	Dominant	Recessive
All patients ^a							
WBC (10 ³ /μl)	8.21±3.57	7.56±2.89	8.94±3.43	0.087	0.341	0.133	0.226
Lymphocytes (10 ³ /μl)	1.88±0.88	1.84±0.69	1.80±0.78	0.594	0.309	0.317	0.672
ESR (mm)	41.78±29.78	41.60±30.52	46.92±40.51	0.687	0.797	0.994	0.398
CRP (pg/ml)	3.56±5.97	3.16±5.63	2.84±3.10	0.921	0.732	0.695	0.994
Hemoglobin (g/dl)	12.37±2.01	12.32±2.04	13.21±2.34	0.560	0.628	0.863	0.283
Iron (μg/dl)	60.27±34.57	58.55±39.15	61.67±30.77	0.872	0.891	0.776	0.722
Albumin (g/dl)	3.93±0.54	4.02±0.54	4.08±0.53	0.425	0.243	0.196	0.905
Creatinine (mg/dl)	1.14±0.84	1.10±0.64	1.04±0.46	0.629	0.387	0.487	0.414
Fasting glucose (mg/dl)	109.85±45.92	114.65±52.28	106.93±29.57	0.635	0.497	0.396	0.833
TG (mg/dl)	120.72±72.77	124.03±74.72	121.87±84.66	0.952	0.927	0.998	0.762
TC (mg/dl)	162.64±43.85	167.06±45.65	175.07±53.47	0.327	0.149	0.210	0.271
HDL-C (mg/dl)	39.79±14.41	41.89±18.99	44.20±12.54	0.255	0.099	0.123	0.338
LDL-C (mg/dl)	98.32±38.43	100.51±37.58	99.73±38.18	0.776	0.515	0.479	0.924
FT3 (pg/ml)	2.70±0.65	2.78±0.70	2.73±0.76	0.685	0.485	0.412	0.938
Fasting insulin IU/ml	9.12±6.89	9.16±6.96	10.78±7.40	0.715	0.840	0.962	0.434
IGF-1 (ng/ml)	76.22±41.36	76.72±34.05	74.67±62.97	0.681	0.494	0.626	0.409
Males ^b							
WBC (10 ³ /μl)	8.61±3.90	7.43±2.38	8.41±1.62	0.152	0.099	0.059	0.902
Lymphocytes (10 ³ /μl)	1.84±0.96	1.67±0.61	1.59±0.87	0.582	0.328	0.298	0.826
ESR (mm)	40.14±32.22	36.88±26.97	48.29±50.97	0.641	0.505	0.398	0.868
CRP (pg/ml)	4.23±7.00	2.88±4.13	3.18±2.95	0.246	0.105	0.095	0.532
Hemoglobin (g/dl)	12.85±2.22	12.65±2.24	14.29±2.45	0.118	0.540	0.942	0.051
Iron (μg/dl)	59.44±37.13	67.79±48.32	65.63±31.74	0.411	0.183	0.197	0.476
Albumin (g/dl)	3.94±0.58	4.10±0.52	4.01±0.63	0.180	0.100	0.066	0.752
Creatinine (mg/dl)	1.32±0.98	1.33±0.83	1.17±0.54	0.613	0.768	0.972	0.350
Fasting glucose (mg/dl)	112.79±52.05	110.88±49.45	92.13±18.34	0.876	0.793	0.920	0.609
TG (mg/dl)	118.42±81.68	124.79±90.76	130.88±114.67	0.889	0.733	0.837	0.630
TC (mg/dl)	150.99±41.10	155.98±42.28	172.13±61.60	0.248	0.124	0.218	0.149
HDL-C (mg/dl)	36.59±12.95	39.10±14.43	43.13±14.34	0.211	0.079	0.111	0.231
LDL-C (mg/dl)	92.08±38.88	90.98±34.10	99.88±39.24	0.816	0.594	0.699	0.549
FT3 (pg/ml)	2.77±0.70	2.88±0.65	3.00±0.74	0.311	0.130	0.172	0.298
Fasting insulin IU/ml	9.34±7.16	7.49±3.92	10.48±8.76	0.125	0.396	0.156	0.337
IGF-1 (ng/ml)	79.53±43.08	77.65±33.03	56.18±19.80	0.111	0.134	0.353	0.038
Females ^b							
WBC (10 ³ /μl)	7.94±3.31	7.62±3.14	9.48±4.71	0.268	0.942	0.605	0.164
Lymphocytes (10 ³ /μl)	1.91±0.82	1.93±0.71	1.98±0.73	0.932	0.817	0.888	0.713
ESR (mm)	42.85±28.09	44.14±32.12	45.33±28.56	0.875	0.779	0.874	0.607
CRP (pg/ml)	3.11±5.15	3.31±6.31	2.45±3.46	0.866	0.595	0.596	0.852
Hemoglobin (g/dl)	12.06±1.79	12.14±1.91	11.99±1.55	0.796	0.664	0.576	0.799
Iron (μg/dl)	60.82±32.8	53.39±32.08	57.14±31.45	0.334	0.179	0.143	0.927
Albumin (g/dl)	3.93±0.51	3.97±0.55	4.15±0.43	0.928	0.699	0.703	0.880

Table 4 (continued)

rs1207568	C/C	C/T	T/T	<i>p</i> value			
				Free	Additive	Dominant	Recessive
Creatinine (mg/dl)	.03±0.71	0.97±0.46	0.88±0.32	0.490	0.234	0.267	0.505
Fasting glucose (mg/dl)	107.92±41.41	116.67±53.88	123.86±31.96	0.397	0.175	0.200	0.476
TG (mg/dl)	122.24±66.36	123.61±64.93	111.57±33.91	0.690	0.679	0.848	0.389
TC (mg/dl)	170.62±43.98	173.26±46.52	178.43±47.12	0.651	0.359	0.358	0.743
HDL-C (mg/dl)	41.88±14.95	43.36±20.91	45.43±11.13	0.550	0.274	0.297	0.590
LDL-C (mg/dl)	102.38±37.67	105.53±38.51	99.57±40.07	0.608	0.413	0.346	0.903
FT3 (pg/ml)	2.66±0.61	2.72±0.73	2.45±0.73	0.577	0.953	0.729	0.372
Fasting insulin IU/ml	8.98±6.71	10.02±7.97	11.13±6.17	0.642	0.358	0.347	0.802
IGF-1 (ng/ml)	74.04±40.13	76.22±34.75	95.81±88.41	0.766	0.638	0.764	0.475

Data are mean±standard deviation

WBC white blood cells, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *TG* tryglycerides, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *FT3* free triiodothyronine, *IGF-1* insulin-like growth factor 1

^a The analysis was adjusted for age and gender and the presence of diabetes mellitus, neurodegenerative diseases, cerebrovascular disease, and cardiovascular diseases, as well as for the use of statins, thyroid hormone, anti-inflammatory, and hypoglycemic drugs

^b The analyses were adjusted for age and the presence of diabetes mellitus, neurodegenerative diseases, cerebrovascular disease, and cardiovascular diseases, as well as for the use of statins, thyroid hormone, anti-inflammatory, and hypoglycemic drugs

tions with increased serum TC ($p=0.006$; $\beta=7.804$) and hemoglobin ($p=0.035$; $\beta=0.267$) levels were also observed (Table 7, all patients and Fig. 1b). Considering the two SNPs rs9536314 and rs564481, a trend towards an association with decreased serum TG levels ($p=0.061$; $\beta=-8.465$) was observed (Table 7, all patients). In gender-segregated analyses, considering together rs1207568, rs9536314, and rs564481, a progressive increasing number of at risk alleles was associated with raising levels of HDL-C ($p=0.006$; $\beta=2.932$), but only in females (Table 7, females). In males, considering only rs1207568 and rs9536314, the association between the GRS and hemoglobin levels resulted still significant ($p=0.040$), while considering only rs1207568 and rs564481, the association with LC resulted significant ($p=0.031$) (Table 7, males). Finally, in females, considering only rs1207568 and rs9536314 or rs564481 and rs9536314, the association with increasing levels of HDL-C resulted still significant ($p=0.006$ and $p=0.022$), while considering only rs564481 and rs9536314, the association with fasting glucose ($p=0.025$) or fasting insulin levels resulted significant ($p=0.005$; Table 7, females).

Discussion

The present study demonstrated an influence of KL genotypes on serum levels of some biological parameters linked to lipid and glucose metabolisms, such as HDL-C, fasting glucose, fasting insulin, albumin, and hemoglobin under different genetic models. Gender-segregated analyses confirmed these associations, also suggesting that the influence of KL genotypes on HDL-C, fasting glucose or fasting insulin levels may be driven by the female gender, while the association with serum hemoglobin may be driven by the male gender. Notably, the association of KL genotypes with creatinine levels was found only in females, while the association with IGF-1 or LC was found only in males. The impact of KL SNPs on serum hemoglobin and HDL-C levels was further confirmed by a GRS-tagged approach that also suggested an association with serum TC. Gender-segregated analyses with the GRS-tagged approach confirmed the associations with HDL-C, fasting glucose, and fasting insulin levels in females, and with hemoglobin and LC in males.

Table 5 Association of Klotho rs9536314 genotypes with laboratory parameters under different genetic model of inheritance in all hospitalized older patients and gender-segregated analyses

rs9536314	T/T	T/G	G/G	<i>p</i> value			
				Free	Additive	Dominant	Recessive
All patients ^a							
WBC (10 ³ /μl)	8.16±3.68	8.16±3.38	7.55±2.08	0.885	0.929	0.829	0.706
Lymphocytes (10 ³ /μl)	1.86±0.75	1.99±1.07	2.01±0.59	0.455	0.230	0.210	0.822
ESR (mm)	41.52±29.89	39.91±30.25	42.11±33.15	0.994	0.924	0.935	0.932
CRP (pg/ml)	3.51±6.08	3.37±5.49	2.75±5.59	0.960	0.933	0.991	0.778
Hemoglobin (g/dl)	12.23±2.03	12.73±2.09	12.39±2.20	0.107	0.057	0.038	0.900
Iron (μg/dl)	60.75±36.57	60.39±33.33	68.11±50.37	0.795	0.834	0.713	0.639
Albumin (g/dl)	3.96±0.55	3.97±0.8	3.64±0.74	0.093	0.133	0.297	0.035
Creatinine (mg/dl)	1.15±0.83	1.07±0.49	1.43±1.41	0.268	0.940	0.625	0.163
Fasting glucose (mg/dl)	109.45±43.70	116.5±59.56	103.30±29.99	0.317	0.338	0.217	0.574
TG (mg/dl)	121.79±77.17	125.96±70.77	128.10±55.05	0.932	0.762	0.726	0.962
TC (mg/dl)	162.88±46.03	170.15±44.87	190.00±55.37	0.136	0.060	0.105	0.128
HDL-C (mg/dl)	40.80±15.31	39.94±13.96	54.30±46.82	0.016	0.494	0.939	0.005
LDL-C (mg/dl)	96.91±38.39	101.72±36.65	109.90±31.32	0.332	0.149	0.193	0.317
FT3 (pg/ml)	2.74±0.70	2.77±0.58	2.81±0.62	0.964	0.837	0.878	0.801
Fasting insulin IU/ml	9.24±7.35	8.92±5.50	7.88±3.39	0.734	0.472	0.540	0.536
IGF-1 (ng/ml)	78.59±41.08	74.70±37.93	77.43±41.98	0.399	0.188	0.175	0.725
Males ^b							
WBC (10 ³ /μl)	8.30±3.82	8.80±3.55	7.01±1.99	0.408	0.584	0.408	0.390
Lymphocytes (10 ³ /μl)	1.73±0.65	2.06±1.38	1.85±0.36	0.071	0.035	0.023	0.877
ESR (mm)	40.12±31.78	35.39±31.44	46.00±41.87	0.795	0.605	0.542	0.865
CRP (pg/ml)	3.81±6.65	4.19±5.73	6.06±9.56	0.756	0.461	0.493	0.668
Hemoglobin (g/dl)	12.67±2.20	13.41±2.53	12.50±1.73	0.107	0.066	0.042	0.880
Iron (μg/dl)	61.05±40.09	63.33±37.66	75.67±84.88	0.903	0.691	0.739	0.713
Albumin (g/dl)	3.99±0.56	3.94±0.57	3.43±1.25	0.298	0.210	0.323	0.168
Creatinine (mg/dl)	1.34±1.00	1.20±0.51	0.98±0.02	0.499	0.254	0.302	0.441
Fasting glucose (mg/dl)	108.91±41.44	127.5±77.40	93.00±12.17	0.169	0.084	0.063	0.957
TG (mg/dl)	121.03±90.30	130.34±77.11	91.67±6.66	0.446	0.986	0.774	0.248
TC (mg/dl)	151.86±43.04	156.67±43.84	153.00±46.51	0.734	0.431	0.446	0.728
HDL-C (mg/dl)	37.76±13.74	35.33±12.48	35.67±10.02	0.366	0.264	0.196	0.755
LDL-C (mg/dl)	89.71±37.20	95.67±37.34	101.00±45.90	0.209	0.113	0.177	0.165
FT3 (pg/ml)	2.80±0.71	2.77±0.63	3.07±0.43	0.566	0.813	0.982	0.298
Fasting insulin IU/ml	9.03±7.01	8.87±5.54	8.43±1.59	0.982	0.967	0.996	0.852
IGF-1 (ng/ml)	79.96±40.58	78.67±44.93	71.40±28.67	0.996	0.973	0.958	0.952
Females ^b							
WBC (10 ³ /μl)	8.07±3.60	7.72±3.22	7.78±2.23	0.963	0.907	0.856	0.879
Lymphocytes (10 ³ /μl)	1.94±0.80	1.94±0.79	2.13±0.76	0.950	0.810	0.775	0.932
ESR (mm)	42.44±28.62	42.87±29.27	40.17±32.29	0.776	0.612	0.534	0.848
CRP (pg/ml)	3.31±5.68	2.83±5.30	1.09±1.88	0.781	0.610	0.715	0.504
Hemoglobin (g/dl)	11.93±1.86	12.29±1.62	12.34±2.50	0.770	0.485	0.469	0.851
Iron (μg/dl)	60.54±34.09	58.44±30.21	64.33±33.55	0.597	0.381	0.326	0.979
Albumin (g/dl)	3.94±0.55	3.99±0.41	3.75±0.4	0.429	0.393	0.574	0.203

Table 5 (continued)

rs9536314	T/T	T/G	G/G	<i>p</i> value			
				Free	Additive	Dominant	Recessive
Creatinine (mg/dl)	1.03±0.66	0.99±0.46	1.66±1.73	0.036	0.230	0.579	0.010
Fasting glucose (mg/dl)	109.80±45.22	109.3±43.25	107.71±34.99	0.935	0.890	0.969	0.717
TG (mg/dl)	122.30±67.12	123.09±66.64	143.71±59.86	0.813	0.749	0.879	0.519
TC (mg/dl)	170.52±46.57	179.32±43.49	205.86±53.85	0.248	0.133	0.217	0.160
HDL-C (mg/dl)	42.79±15.97	42.98±14.11	62.29±54.84	0.010	0.183	0.597	0.002
LDL-C (mg/dl)	101.62±38.50	105.71±35.86	113.71±26.69	0.900	0.659	0.696	0.743
FT3 (pg/ml)	2.69±0.69	2.76±0.55	2.68±0.70	0.923	0.715	0.692	0.977
Fasting insulin IU/ml	9.37±7.58	8.96±5.51	7.60±4.14	0.667	0.379	0.425	0.559
IGF-1 (ng/ml)	77.67±41.47	72.09±32.56	80.01±48.41	0.314	0.183	0.138	0.976

Data are mean±standard deviation

WBC white blood cells, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *TG* tryglicerides, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *FT3* free triiodothyronine, *IGF-1* insulin-like growth factor 1

^a The analysis was adjusted for age and gender and the presence of diabetes mellitus, neurodegenerative diseases, cerebrovascular disease, and cardiovascular diseases, as well as for the use of statins, thyroid hormone, anti-inflammatory, and hypoglycemic drugs

^b The analyses were adjusted for age and the presence of diabetes mellitus, neurodegenerative diseases, cerebrovascular disease, and cardiovascular diseases, as well as for the use of statins, thyroid hormone, anti-inflammatory, and hypoglycemic drugs

In the last 10 years, there was an increasing body of evidence suggesting a possible role of KL locus in cardiovascular and cerebrovascular diseases. In fact, several studies reported an association of different KL polymorphisms with atherosclerosis (Saito et al. 2000), coronary artery disease (Arking et al. 2003; Low et al. 2005; Imamura et al. 2006; Rhee et al. 2006a, b; Walter 2009), and stroke (Arking et al. 2005; Kim et al. 2006). These suggested associations could be partly explained by a regulatory role of the KL locus on lipid metabolism (Shimoyama et al. 2009a). In the present study, we observed a significant association of the SNP rs9536314 with serum HDL-C levels, that was confirmed also when it was considered together with the other two SNPs, or when it was paired with rs1207568 in the GRS model. Probably, rs9536314 may play a major role in respect to the KL locus as a whole. This association has been extensively discussed (Arking et al. 2005; Walter 2009; Freathy et al. 2006). In particular, subjects V/V homozygotes for the KL-VS haplotype had lower HDL-C levels than subjects with V/F or F/F haplotypes (Arking et al. 2005). We confirmed these findings in both free and recessive genetic models, while in another recent study there was weak evidence

that V/V males had lower HDL-C levels than F/F or F/V males (recessive model), also after additional correction for age and body mass index (Freathy et al. 2006). The GRS analysis showed that an increased number of at risk alleles in the three SNPs was associated with increasing variations of HDL-C levels, in particular for the SNPs rs9536314 and rs1207568, showing an higher β regression coefficient. These findings from the GRS model, also involving the other two KL SNPs investigated in the association with the HDL-C, confirmed recent studies showing that other KL polymorphism were variously associated with HDL-C levels in Japanese healthy subjects (Shimoyama et al. 2009a) and patients underwent renal dialysis (Shimoyama et al. 2009a). Finally, a further association was observed in the GRS analysis using only SNPs rs9536314 and rs1207568. We observed that the presence of an increased number of at risk alleles in these two SNPs was associated with raising TC levels. Notably, despite no single association was observed for the SNP rs1207568, to the best of our knowledge, this is the first study in which, this SNP was always present in all the association observed by means of the GRS analysis among lipid quantitative traits and KL variants. Finally, GRS-tagged gender-

Table 6 Association of Klotho rs564481 genotypes with laboratory parameters under different genetic model of inheritance in all hospitalized older patients and gender-segregated analyses

rs564481	C/C	C/T	T/T	<i>p</i> value			
				Free	Additive	Dominant	Recessive
All patients ^a							
WBC (10 ³ /μl)	8.13±3.28	8.18±3.51	7.56±3.29	0.438	0.306	0.626	0.199
Lymphocytes (10 ³ /μl)	1.93±1.01	1.85±0.72	1.82±0.67	0.626	0.366	0.339	0.636
ESR (mm)	42.53±31.04	42.28±29.95	38.99±29.18	0.660	0.453	0.697	0.363
CRP (pg/ml)	3.48±5.09	3.65±6.48	2.79±5.37	0.510	0.458	0.850	0.255
Hemoglobin (g/dl)	12.50±1.96	12.45±2.02	12.01±2.14	0.048	0.041	0.286	0.014
Iron (μg/dl)	61.67±37.78	58.80±35.98	59.95±31.31	0.803	0.689	0.540	0.983
Albumin (g/dl)	3.95±0.50	3.95±0.59	3.99±0.51	0.901	0.686	0.810	0.655
Creatinine (mg/dl)	1.18±0.92	1.11±0.74	1.08±0.55	0.537	0.296	0.272	0.569
Fasting glucose (mg/dl)	115.78±49.96	111.37±50.55	101.40±30.65	0.028	0.018	0.158	0.009
TG (mg/dl)	128.26±85.95	121.48±71.20	110.43±48.51	0.175	0.072	0.194	0.087
TC (mg/dl)	162.71±45.85	166.36±45.30	161.36±40.59	0.459	0.525	0.999	0.242
HDL-C (mg/dl)	39.92±15.90	40.30±16.40	41.99±13.97	0.642	0.461	0.734	0.346
LDL-C (mg/dl)	95.03±38.82	102.78±39.47	96.37±32.75	0.102	0.953	0.259	0.196
FT3 (pg/ml)	2.71±0.67	2.75±0.68	2.68±0.63	0.615	0.925	0.676	0.489
Fasting insulin IU/ml	9.67±7.55	9.34±6.82	7.71±5.52	0.035	0.027	0.225	0.010
IGF-1 (ng/ml)	77.70±39.90	75.86±40.46	75.78±41.18	0.901	0.735	0.892	0.648
Males ^b							
WBC (10 ³ /μl)	7.98±2.80	8.71±4.10	8.09±3.46	0.404	0.817	0.390	0.520
Lymphocytes (10 ³ /μl)	2.00±1.18	1.66±0.58	1.76±0.72	0.027	0.023	0.007	0.394
ESR (mm)	39.50±31.81	39.23±33.02	40.58±28.67	0.889	0.629	0.653	0.735
CRP (pg/ml)	3.58±4.87	4.45±7.63	3.18±5.50	0.514	0.921	0.511	0.519
Hemoglobin (g/dl)	12.97±2.10	13.01±2.40	12.26±2.03	0.142	0.093	0.349	0.050
Iron (μg/dl)	64.21±41.00	61.91±43.11	57.18±29.81	0.680	0.379	0.439	0.502
Albumin (g/dl)	3.96±0.53	3.97±0.63	4.02±0.52	0.954	0.891	0.983	0.780
Creatinine (mg/dl)	1.29±0.99	1.33±0.97	1.32±0.68	0.971	0.949	0.863	0.923
Fasting glucose (mg/dl)	112.87±49.46	112.11±56.10	105.77±37.01	0.172	0.601	0.661	0.124
TG (mg/dl)	124.43±99.10	123.14±84.23	105.00±41.21	0.332	0.250	0.596	0.137
TC (mg/dl)	152.84±39.86	154.06±45.91	150.24±40.05	0.801	0.557	0.723	0.513
HDL-C (mg/dl)	37.81±13.87	37.17±14.44	37.26±11.33	0.732	0.721	0.509	0.871
LDL-C (mg/dl)	89.20±33.38	95.97±43.17	87.74±32.77	0.431	0.790	0.691	0.316
FT3 (pg/ml)	2.83±0.71	2.81±0.70	2.72±0.64	0.565	0.321	0.514	0.307
Fasting insulin IU/ml	8.86±6.52	9.12±6.30	8.41±7.70	0.520	0.782	0.761	0.357
IGF-1 (ng/ml)	82.50±46.18	74.64±34.32	79.17±42.68	0.281	0.226	0.117	0.770
Females ^b							
WBC (10 ³ /μl)	8.23±3.59	7.87±3.08	7.21±3.16	0.271	0.107	0.149	0.239
Lymphocytes (10 ³ /μl)	1.88±0.87	1.96±0.77	1.86±0.63	0.588	0.726	0.422	0.717
ESR (mm)	44.71±30.44	44.02±28.02	37.98±29.69	0.509	0.266	0.431	0.281
CRP (pg/ml)	3.41±5.26	3.18±5.68	2.54±5.33	0.646	0.354	0.390	0.521
Hemoglobin (g/dl)	12.16±1.78	12.12±1.69	11.85±2.20	0.246	0.180	0.521	0.094
Iron (μg/dl)	59.84±35.36	56.95±30.98	61.81±32.40	0.775	0.841	0.860	0.555
Albumin (g/dl)	3.95±0.47	3.94±0.56	3.96±0.51	0.941	0.771	0.728	0.919

Table 6 (continued)

rs564481	C/C	C/T	T/T	<i>p</i> value			
				Free	Additive	Dominant	Recessive
Creatinine (mg/dl)	1.10±0.85	0.98±0.54	0.92±0.38	0.167	0.083	0.060	0.391
Fasting glucose (mg/dl)	117.88±50.43	110.94±47.20	98.65±25.83	0.050	0.015	0.043	0.046
TG (mg/dl)	131.06±75.19	120.52±62.63	113.90±52.68	0.330	0.142	0.165	0.327
TC (mg/dl)	170.30±48.80	173.69±43.44	168.77±39.57	0.429	0.684	0.773	0.272
HDL-C (mg/dl)	41.44±17.10	42.09±17.21	44.97±14.72	0.577	0.301	0.426	0.359
LDL-C (mg/dl)	99.20±41.93	106.68±36.75	101.79±31.80	0.171	0.741	0.219	0.363
FT3 (pg/ml)	2.64±0.64	2.71±0.66	2.66±0.62	0.470	0.517	0.275	0.870
Fasting insulin IU/ml	10.26±8.20	9.46±7.12	7.31±3.75	0.042	0.019	0.120	0.017
IGF-1 (ng/ml)	74.26±34.52	76.57±43.74	73.65±40.41	0.459	0.750	0.373	0.608

Data are mean±standard deviation

WBC white blood cells, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *TG* tryglicerides, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *FT3* free triiodothyronine, *IGF-1* insulin-like growth factor 1

^a The analysis was adjusted for age and gender and the presence of diabetes mellitus, neurodegenerative diseases, cerebrovascular disease, and cardiovascular diseases, as well as for the use of statins, thyroid hormone, anti-inflammatory, and hypoglycemic drugs

^b The analyses were adjusted for age and the presence of diabetes mellitus, neurodegenerative diseases, cerebrovascular disease, and cardiovascular diseases, as well as for the use of statins, thyroid hormone, anti-inflammatory, and hypoglycemic drugs

segregated analyses also confirmed these associations, suggesting that the association between KL genotypes and HDL-C levels may be driven by the female gender.

At present, the mechanisms by which KL locus is associated with lipid metabolism are unclear. In particular, HDL-C plasma concentrations decline with age in prospective studies (Wilson et al. 1994; Ferrara et al. 1997) may be also due to secondary KL-linked pathways such as hormonal changes, inflammatory processes, and diabetes mellitus (Wilson et al. 1994; Rohrer et al. 2004; van der Westhuyzen et al. 2007). In cross-sectional studies, by contrast, HDL levels did not change much, or even slightly increase with age, suggesting that only people with still high HDL concentrations survive (Barzilai et al. 2003; Barter 2004; Atzmon et al. 2006). A selection bias by HDL lowering genetic variation may explain why HDL deficiency is extremely rare among centenarians. Conversely, HDL may modulate the aging process, not only by its well-known antiatherogenic effects, but possibly also by direct interfering with aging signaling or survival factor such as KL. In fact, HDL-C levels are inversely associated with the detrimental effect of a dysfunctional KL protein in humans (Walter 2009). HDL-C levels in the high normal

range seem to be completely protective against KL dysfunction (Arking et al. 2003, 2005). The underlying mechanism of this relationship is unknown. It is obvious, however, that HDL and KL modulate similar signaling pathways. Both molecules induce nitric oxide (NO) synthesis, counteract apoptosis, induce angiogenesis, and counteract insulin signaling in cell culture models (McCallum and Epanand 1995; Nofer et al. 2002). HDL may compensate for KL dysfunction by its ability to remove cellular lipids. One recently proposed model is that the main anti-aging effect of KL is its ability to prevent cellular lipid overload (and lipooapoptosis) by reducing insulin-stimulated availability of the lipogenic substrate glucose (Unger 2006). The effects of KL, however, rather induce a moderate insulin-resistance state (Unger 2006). The HDL-binding protein encoded by the gene sphingosine-1-phosphate receptor 3 (S1PR3) at locus 9q22.1-q22.2 belongs to a family of closely related “survival receptors” that interact with various heterotrimeric G proteins and growth factor receptors to trigger multiple functional responses involved in differentiation, proliferation, and angiogenesis. Therefore, these proteins may be candidates as regulatory factors in various KL/HDL pathways (Walter 2009).

Table 7 Association of Klotho genotypes with some laboratory parameters according to different Genetic Risk Scores in all hospitalized older patients and gender-segregated analyses

	rs1207568			rs1207568			rs1207568			–		
	rs9536314			rs9536314			–			rs9536314		
	rs564481			–			rs564481			rs564481		
	<i>P</i> value	β	SE	<i>P</i> value	β	SE	<i>P</i> value	β	SE	<i>P</i> value	β	SE
All patients^a												
WBC ($10^3/\mu\text{l}$)	0.160	−0.237	0.169	0.408	−0.193	0.232	0.217	−0.190	0.153	0.226	−0.257	0.212
Lymphocytes ($10^3/\mu\text{l}$)	0.694	−0.017	0.044	0.704	0.023	0.060	0.229	−0.049	0.040	0.991	0.001	0.056
ESR (mm)	0.387	−1.235	1.425	0.880	−0.300	1.982	0.650	−0.612	1.350	0.262	−2.022	1.800
CRP (pg/ml)	0.253	−0.325	0.284	0.529	−0.246	0.390	0.439	−0.201	0.259	0.246	−0.416	0.359
Hemoglobin (g/dl)	0.964	0.004	0.092	0.035	0.267	0.127	0.220	−0.106	0.086	0.679	−0.048	0.117
Iron ($\mu\text{g/dl}$)	0.775	0.498	1.744	0.648	1.106	2.419	0.765	−0.478	1.599	0.913	−0.242	2.214
Albumin (g/dl)	0.885	0.004	0.025	0.962	−0.002	0.035	0.333	0.023	0.024	0.570	−0.019	0.033
Creatinine (mg/dl)	0.088	−0.061	0.036	0.220	−0.061	0.050	0.228	−0.04	0.03	0.069	−0.082	0.045
Fasting glucose (mg/dl)	0.430	−1.560	1.973	0.312	2.743	2.711	0.177	−2.482	1.835	0.105	−4.058	2.496
TG (mg/dl)	0.148	−5.158	3.560	0.958	0.257	4.896	0.158	−4.557	3.222	0.061	−8.465	4.515
TC (mg/dl)	0.233	2.467	2.066	0.006	7.804	2.832	0.737	0.654	1.947	0.771	0.767	2.632
HDL-C (mg/dl)	0.023	1.707	0.747	0.022	2.369	1.029	0.135	1.035	0.692	0.178	1.286	0.954
LDL-C (mg/dl)	0.214	2.125	1.709	0.077	4.164	2.349	0.676	0.673	1.607	0.293	2.284	2.167
FT3 (pg/ml)	0.427	0.026	0.032	0.255	0.051	0.045	0.717	0.011	0.030	0.731	0.014	0.041
Fasting insulin IU/ml	0.717	0.413	1.139	0.384	−1.360	1.560	0.549	0.610	1.016	0.414	1.172	1.434
IGF-1 (ng/ml)	0.209	−2.334	1.855	0.154	−3.642	2.549	0.577	−0.974	1.744	0.248	−2.716	2.350
Males^b												
WBC ($10^3/\mu\text{l}$)	0.339	−0.267	0.279	0.167	−0.530	0.382	0.467	−0.192	0.264	0.931	0.031	0.353
Lymphocytes ($10^3/\mu\text{l}$)	0.326	−0.073	0.074	0.457	0.078	0.105	0.031	−0.148	0.068	0.667	−0.041	0.094
ESR (mm)	0.238	−2.718	2.297	0.203	−4.044	3.166	0.939	−0.174	2.256	0.508	−1.962	2.956
CRP (pg/ml)	0.205	−0.606	0.477	0.138	−0.973	0.653	0.379	−0.403	0.457	0.971	−0.022	0.611
Hemoglobin (g/dl)	0.583	0.091	0.165	0.040	0.463	0.224	0.382	−0.140	0.160	0.849	0.040	0.211
Iron ($\mu\text{g dl}$)	0.359	2.805	3.051	0.129	6.298	4.135	0.877	0.454	2.924	0.996	−0.019	3.927
Albumin (g/dl)	0.355	0.039	0.042	0.426	0.046	0.058	0.287	0.043	0.040	0.740	−0.018	0.055
Creatinine (mg/dl)	0.390	−0.058	0.068	0.257	−0.106	0.093	0.915	−0.007	0.067	0.389	−0.074	0.086
Fasting glucose (mg/dl)	0.809	0.792	3.268	0.372	4.008	4.479	0.595	−1.674	3.142	0.738	1.397	4.161
TG (mg/dl)	0.442	−4.833	6.269	0.801	2.176	8.623	0.447	−4.504	5.911	0.213	−10.009	8.009
TC (mg/dl)	0.393	2.700	3.153	0.061	8.097	4.296	0.617	1.548	3.088	0.914	−0.443	4.074
HDL-C (mg/dl)	0.835	−0.204	0.979	0.976	0.041	1.346	0.383	0.832	0.951	0.158	−1.817	1.281
LDL-C (mg/dl)	0.339	2.583	2.694	0.110	5.918	3.686	0.847	0.513	2.653	0.515	2.266	3.476
FT3 (pg/ml)	0.589	0.029	0.053	0.110	0.119	0.074	0.897	0.007	0.051	0.575	−0.038	0.068
Fasting insulin IU/ml	0.754	−0.162	0.515	0.749	−0.226	0.705	0.491	−0.340	0.493	0.919	−0.067	0.657
IGF-1 (ng/ml)	0.192	−3.880	2.966	0.447	−3.113	4.084	0.101	−4.632	2.811	0.366	−3.459	3.821
Females^b												
WBC ($10^3/\mu\text{l}$)	0.325	−0.213	0.216	0.819	0.068	0.299	0.241	−0.223	0.19	0.108	−0.434	0.269
Lymphocytes ($10^3/\mu\text{l}$)	0.843	0.011	0.056	0.948	0.005	0.076	0.892	0.007	0.051	0.972	0.002	0.070
ESR (mm)	0.946	0.124	1.823	0.366	2.332	2.576	0.505	−1.113	1.669	0.655	−1.023	2.289
CRP (pg/ml)	0.835	−0.074	0.353	0.584	0.268	0.489	0.680	−0.128	0.311	0.251	−0.511	0.444

Table 7 (continued)

	rs1207568			rs1207568			rs1207568			–		
	rs9536314			rs9536314			–			rs9536314		
	rs564481			–			rs564481			rs564481		
	<i>P</i> value	β	SE	<i>P</i> value	β	SE	<i>P</i> value	β	SE	<i>P</i> value	β	SE
Hemoglobin (g/dl)	0.520	−0.071	0.110	0.365	0.138	0.152	0.449	−0.076	0.100	0.265	−0.154	0.138
Iron (μ g/dl)	0.466	−1.538	2.108	0.268	−3.329	2.999	0.591	−1.009	1.877	0.673	−1.123	2.657
Albumin (g/dl)	0.548	−0.019	0.032	0.570	−0.025	0.045	0.670	0.012	0.029	0.538	−0.025	0.041
Creatinine (mg/dl)	0.097	−0.064	0.038	0.602	−0.028	0.054	0.064	−0.069	0.037	0.094	−0.081	0.048
Fasting glucose (mg/dl)	0.338	−2.399	2.500	0.431	2.739	3.476	0.307	−2.320	2.269	0.025	−7.107	3.153
TG (mg/dl)	0.163	−5.913	4.230	0.764	−1.769	5.888	0.205	−4.730	3.724	0.130	−8.154	5.369
TC (mg/dl)	0.546	1.689	2.792	0.065	7.206	3.885	0.886	0.364	2.538	0.897	0.461	3.537
HDL-C (mg/dl)	0.006	2.932	1.064	0.006	4.092	1.480	0.194	1.240	0.952	0.022	3.112	1.346
LDL-C (mg/dl)	0.422	1.802	2.241	0.329	3.051	3.117	0.519	1.316	2.038	0.524	1.801	2.823
FT3 (pg/ml)	0.412	0.034	0.041	0.593	0.031	0.058	0.553	0.022	0.037	0.347	0.049	0.052
Fasting insulin IU/ml	0.101	−0.730	0.444	0.757	0.192	0.620	0.195	−0.527	0.405	0.005	−1.576	0.555
IGF-1 (ng/ml)	0.702	−0.917	2.399	0.415	−2.717	3.327	0.614	1.121	2.219	0.450	−2.284	3.020

SE standard error, β beta regression coefficient, *WBC* white blood cells, *ESR* erythrocyte sedimentation rate, *CRP* C-reactive protein, *TG* triglycerides, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *FT3* free triiodothyronine, *IGF-1* insulin-like growth factor 1

^a The analysis was adjusted for age and gender and the presence of diabetes mellitus, neurodegenerative diseases, cerebrovascular disease, and cardiovascular diseases, as well as for the use of statins, thyroid hormone, anti-inflammatory, and hypoglycemic drugs

^b The analyses were adjusted for age and the presence of diabetes mellitus, neurodegenerative diseases, cerebrovascular disease, and cardiovascular diseases, as well as for the use of statins, thyroid hormone, anti-inflammatory, and hypoglycemic drugs

Also, glucose metabolism has been associated with genetic KL variants (Rhee et al. 2006a; Shimoyama et al. 2009a; Lorenzi et al. 2010). Although KL is a type 2 diabetes mellitus candidate gene due to its importance in insulin signaling, as demonstrated by murine models, and its potential role in longevity (Kuro-o et al. 1997; Utsugi et al. 2000; Kurosu et al. 2005; Arking et al. 2002, 2005), results from several studies on the KL-VS variant gave no support for this (Freathy et al. 2006; Arking et al. 2002, 2003). Furthermore, a large study on glucose metabolism-linked quantitative traits in 1,177 healthy individuals showed no significant association of KL-VS with fasting insulin and glucose (Freathy et al. 2006). In the present study, the analysis of rs564481, free from any genetic model, revealed an association with serum fasting glucose and serum fasting insulin, also confirmed in both additive and recessive models. Furthermore, gender-segregated analyses suggested an association between KL SNP rs1207568 and serum IGF-1 in the recessive model but only for

males, and confirmed only for females the association between rs564481 and serum fasting glucose in all genetic models and with serum fasting insulin in all genetic models except for the dominant one. Gender-segregated analyses with the GRS-tagged approach confirmed the associations of KL genotypes with fasting glucose and fasting insulin levels in females. Therefore, we confirmed findings from studies in which fasting glucose was significantly higher in females carriers of the T allele of KL rs564481 compared with non-carriers in Japanese (Shimoyama et al. 2009a) and Korean women (Rhee et al. 2006a). However, we did not confirm a regulatory role of rs1207568 on fasting glucose suggested in Japanese women (Shimoyama et al. 2009a). Notwithstanding the present promising findings, between the two actions of KL, e.g., modulation of the insulin/IGF signaling pathway or regulation of calcium and phosphate homeostasis, the latter pathway appears to be more important in regulating aging. In fact, normalization of vitamin D activity in mice lacking

Fig. 1 Estimated mean increment (β) of laboratory parameters according to the presence of mutated Klotho alleles. **a** Estimated increment of high-density lipoprotein cholesterol (HDL-C) ($\beta=1.707$) according to the number of mutated allele of the three Klotho SNPs rs1207068, rs9536314, and rs564481. **b** Estimated increment of hemoglobin (Hb) ($\beta=0.267$), total cholesterol (TC) ($\beta=7.804$), and HDL-C ($\beta=2.369$) according to the number of mutated allele of the two Klotho SNPs rs1207068 and rs9536314

either KL or FGF23 either by feeding a vitamin D-deficient diet or knockout for 1 α -hydroxylase substantially rescues the progeroid phenotype (Nabeshima 2006). Similarly, mice lacking KL fed a vitamin D-deficient diet lived substantially longer (>6 months) than the insulin receptor substrate heterozygous rescue of KL^{-/-} mouse lifespan. Furthermore, the enhanced insulin sensitivity in KL^{-/-} mice normalized on a vitamin D-deficient diet suggests the insulin response is secondary to the altered mineral metabolism (Bellino 2006). Finally, recent findings suggested that decreased KL expression is not a general feature of rodent models of insulin resistance and that the soluble KL protein does not inhibit IGF-1 and/or insulin signaling in HEK293, L6, and HepG2 cultured cells, arguing against a direct

role of KL in insulin signaling (Lorenzi et al. 2010). However, the hypothesis that KL indirectly regulates insulin sensitivity via FGF23 activation remains to be investigated.

In the present study, we observed a significant association of the two KL SNPs rs9536314 and rs564481 with serum concentrations of hemoglobin that was confirmed also when rs9536314 was paired with rs1207568 in the GRS model. Gender-segregated analyses confirmed these associations, also suggesting that the relationship between KL genotypes and serum hemoglobin may be driven by the male gender. This association was also confirmed by the GRS-tagged approach indicating associations of KL variants with hemoglobin in males. To the best of our knowledge, this is the first study reporting an association of the KL locus with serum concentration of hemoglobin. In particular, this association was observed for the analysis free from any genetic model as well as under the additive and dominant models for rs9536314, whereas it was observed only in the recessive model for rs564481. The GRS analysis also demonstrated that an increased number of at risk alleles in the two SNPs rs1207568 and rs9536314 was associated to a slightly increased variations in hemoglobin serum concentration. These findings may suggest a role of the KL locus as a whole in influencing serum hemoglobin concentrations in this population. Although there was no evidence of association among KL and markers of hemolysis, polymorphisms in KL showed an association with some hemolysis-related subphenotypes of sickle cell disease (Steinberg and Adewoye 2006). In particular, it was reported an association of KL SNPs with priapism by genotype and haplotype analyses (Nolan et al. 2005), with sickle cell stroke (Sebastiani et al. 2005), with osteonecrosis (Baldwin et al. 2005), and with leg ulcers by genotype analysis (Nolan et al. 2006). KL directly or indirectly promotes endothelial NO production, and recent studies of the effects of plasma hemoglobin on NO bioavailability suggested that some subphenotypes of sickle cell disease were related to the hemolytic rate (Nolan et al. 2005). How KL might regulate markers of hemolysis such as serum hemoglobin concentration or the occurrence of these hemolysis-related subphenotypes of sickle cell disease is unknown, but disordered NO and oxidative biology are features of the pathobiology of these disorders (Jison et al. 2004). However, to explain the

possible links between KL variants and hemoglobin levels we proposed these suggestions of a regulatory role of KL genotypes on markers of hemolysis in a model like that of sickle cell disease that is rarely present in older age, although prolonged survival in homozygous sickle cell disease is more common than previously thought (Serjeant et al. 2007).

At the best of our knowledge, the present is the first study suggesting an association of the KL SNP rs9536314 with serum concentration of albumin in the recessive model, but this finding was not confirmed in gender-segregated analyses. Although there were no association findings among KL genotypes and serum albumin, this gene locus was associated in some animal models with another similar transport proteins, transthyretin (TTR), also called prealbumin (Park et al. 2009; Li et al. 2010). In fact, gene expression profiling of brain tissue from a mouse model of phenylketonuria, an autosomal recessive disorder caused by a deficiency of phenylalanine hydroxylase which catalyzes the conversion of phenylalanine to tyrosine, revealed overexpression of both TTR and KL (Park et al. 2009). Furthermore, recent animal studies identified KL and TTR proteins as physiological targets of amyloid precursor protein (APP) that are regulated by β -secretase cleaved soluble APP, an extracellular large soluble derivative APP fragment, independent of developmental APP functions (Li et al. 2010). This novel and unexpected APP-mediated signaling pathway may play an important role in maintaining TTR and KL levels and their respective functions in β -amyloid sequestration and aging.

Finally, gender-segregated analyses suggested an association of the KL SNPs rs9536314 and rs564481 with LC under different models, but only in males, while there was an association between rs9536314 and creatinine free from any genetic model and in the recessive model, but only in females. Gender-segregated analyses with the GRS-tagged approach also confirmed the association of KL variants with LC in males. The present findings confirmed recent studies that have shown that the expression of the KL gene is extremely reduced in the CD4+ lymphocytes of patients with rheumatoid arthritis (Witkowski et al. 2007), mostly in the cells of relatively younger patients (Soroczyńska-Cybula et al. 2011), making them similar to the lymphocytes of healthy elderly in this aspect. Moreover, an impairment of B lymphopoiesis was demonstrated in mice homozygous for hypomorphic

KL alleles and attributed to a defect in the “microenvironment” in the bone marrow (Okada et al. 2000). The association between the KL SNP rs9536314 and serum creatinine levels found in our female older patients confirmed a series of studies in which the KL production was severely reduced in human chronic renal failure (Koh et al. 2001), while KL deficiency causes kidney damage (Aizawa et al. 1998; Koh et al. 2001; Wang and Sun 2009), suggesting that KL is essential in maintaining normal kidney structure and function. Interestingly, also the aging-related kidney damage was associated with downregulation of KL (Zuo et al. 2010). Finally, acute kidney injury (AKI) diagnosis is based on an increase in serum creatinine or a decrease in urine output. To be effective, treatment of AKI should be started very early after the insult and well before the rise of serum creatinine (Aiello and Noris 2010). Very recent findings on rodents suggested that KL could be a novel biomarker and a renoprotective factor with therapeutic potential against ischemia-induced AKI (Hu et al. 2010).

Limitations of the present study should also be considered in interpreting our findings. Since KL has been recently reported as an important genetic factor in determining aging process and development of aging-like phenotypes (Kuro-o et al. 1997; Kurosu et al. 2005), throughout his pivotal role in important metabolic pathways (Bartke 2006; Unger 2006), we enrolled in this study only hospitalized older patients accurately selected and adjusted all genetic analyses for possible confounders, i.e., age-related diseases and possible pharmacological treatments. Such adjustments were necessary to try reduce the probability that any observed associations were not spurious and caused by confounders such as the possibility that our findings could be a type I (i.e., false positive) statistical error often common in genetic association studies (Deng 2001; Koller et al. 2004). Nonetheless, we have to acknowledge that our broad definition of cardiovascular diseases as an adjustment factor may also be masking genuine associations with sub-types of cardiovascular diseases which will inevitably have gene–gene and/or gene environment-related etiology (Kulminski et al. 2010). Limitations also include the lack of the lifestyle and dietary data for the hospitalized older patients, which may affect the laboratory data. Finally, investigating possible associations between genetic variants and clinical parameters in hospitalized older patients may introduce some confounding factors. Such studies

should be ideally carried in healthier populations where individual clinical parameters can be interpreted as such, and in which mortality and clinical outcomes can be studied with a long-term follow-up. In conclusion, whereas further studies on wider samples of selected ambulatory- and hospital-based patients are needed to confirm the present findings, these results suggested that KL locus may, at least in part, influence serum levels of laboratory markers, mainly linked to lipid and glucose metabolisms, and commonly accepted as indicators of the clinical status of hospitalized older patients.

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