Association between *CST3* rs2424577 Polymorphism and Corpulence Related Phenotypes during Lifetime in Populations of European Ancestry

Henri Hooton^a Beatrice Dubern^a Corneliu Henegar^{a,b} Lavinia Paternoster^g Ellen A. Nohr^h
Rohia Alili^{a,b} Francis Rousseau^c Veronique Pelloux^{a,b} Pilar Galan^d Serge Hercberg^d
Peter Arner^e Thorkild I.A. Sørensen^f Karine Clément^{a,b}

^a INSERM U872, Paris, Nutriomique, France; Université Pierre et Marie Curie – Paris 6, Centre de Recherche de Cordeliers, UMS S 872, Paris, France; Université Paris Descartes, UMS S 872, Paris, France

^b Département de Nutrition, Assistance Publique-Hôpitaux de Paris, Hôpital Pitié-Salpêtrière,

^c INTEGRAGEN,

^d INSERM U556, INRA U1125, CNAM EA3200, University Paris 13, Nutritional Epidemiology Research Unit, Paris, France

^e Department of Medicine, Lipid Laboratory at Karolinska University Hospital, Karolinska Institute, Stockholm, Sweden

^f Institute of Preventive Medicine, Copenhagen University Hospital, Centre for Health and Society, Copenhagen, Denmark

^g MRC CAITE Centre, School of Social and Community Medicine, University of Bristol, UK

^h Department of Epidemiology, University of Aarhus, Denmark

Keywords

Genetics · Linkage disequilibrium · Flip-flop · Cystatin C · BMI evolution

Summary

Objective: Cystatin C, a protein coded by *CST3* gene, is implicated in adipose tissue biology. Our hypothesis is that common variants in *CST3* gene could play a role in the development of corpulence during lifetime. **Methods:** Two tag SNPs were selected to capture all SNPs in the *CST3* region. We first investigated the association of the two tag SNPs individually and combined into haplotypes with corpulence related phenotypes in 4,288 French subjects (BMI = 24.31 ± 3.74 kg/m²). Significant findings were replicated in five independent populations – 790 Danish lean men (BMI = 24.63 ± 2.30 kg/m²), 672 Danish obese men (BMI = 33.23 ± 2.34 kg/m²), 763 Swedish women (BMI = 21.73 ± 2.87 kg/m²), 1,848 Danish lean women (BMI = 22.66 ± 2.85 kg/m²) and 2,061 Danish obese women (BMI = 37.01 ± 3.59 kg/m²). **Results:** Rs2424577 was associated with BMI in three independent populations – G/G carriers were less corpulent than A/A carriers in the French individuals ($p = 0.045$) and in the Danish lean men ($p = 0.021$), and they were more corpulent in the group of Swedish women ($p = 0.004$). This phenomenon has been described as a flip-flop phe-

nomenon, probably caused by a multi-locus effect. **Conclusion:** *CST3* rs2424577 is associated with BMI in a complex fashion. This association is probably caused by the interaction between several functional variants.

Introduction

Obesity is due to a large number of causes that can be summarized as an interaction between a ‘deleterious’ environment and a predisposing genetic background. Depending on individuals, genetic background and environmental factors are forces driving energy imbalance, and participate in energy storage in adipose tissue. There is a wide spectrum ranging from genetically determined obesity to environmentally determined obesity with most individuals containing a complex mix of these factors (i.e. many individual effects of genes, environmental influences and the complex interaction between these). While epidemiological approaches, including twin studies, showed that genetic factors could cause 57–86% of BMI variations [1], there have been more than 400 genes referenced in the ‘NCBI gene’ database (www.ncbi.nlm.nih.gov/sites/entrez) as being associated with obesity, but each of these genes individually has a small effect on BMI variance. This is very well illustrated by the results of genome wide associa-

tions on large populations which have investigated the implication of several hundreds of thousands SNPs in BMI variance [2–5]. New variants influencing BMI were discovered, nevertheless, these variants even if combined only explain a very small part of the observed BMI variations. Therefore, it seems that most of the causal variants are still to be discovered. It is most likely that most of the variance of BMI attributable to genetic factors is due to a large number of variants, each of which has a very small effect [6–8]. Nevertheless most of these studies have been limited to data that was gathered at only a single time point and therefore only few studies have investigated the potential contribution of genetic factors to the development of corpulence during lifetime [9–11]. Understanding the mechanisms that underlie development of adipose tissue would certainly provide help to identify novel genes involved in BMI and fat mass variations during life. Using a large scale transcriptomic approach in human adipose tissue, we identified Cathepsin S as a novel biomarker of adiposity [12] with a correlation between Cathepsin S gene (*CTSS*) expression in adipose tissue and BMI in obese subjects. Cathepsin K or L knockout mice are protected against diet induced obesity, which highlights the potential role of Cathepsin S and K in obesity [13]. Cathepsins' enzymatic activity is regulated by endogenous inhibitors, the most powerful being cystatin C, which is coded by the *CST3* gene [14]. Emerging results from mice and cellular models suggest that cathepsin family members and cystatin C are involved in adipose tissue physiology, obesity and related complications. Several epidemiological studies have reported associations between obesity or increased adiposity and cystatin C circulating levels. Positive associations of serum cystatin C with BMI were found in an elderly population [15] and in the general population [16] and with body weight in a large population of European origin [17]. In apparently healthy subjects, higher waist circumference, an index of visceral adiposity, and increased % body fat were also associated with higher serum cystatin C [18, 19]. Recently, a graded association between higher BMI and elevated serum cystatin C was reported in American adults [20]. In addition, cystatin C appears to be a biomarker associated with vascular diseases known to be strongly associated with obesity [21]. Our team confirmed the associations between circulating cystatin C and corpulence in two populations of severely and moderately obese persons. Importantly, we showed that adipose tissue contributes to this

significant elevation. Obese patients had both higher expression rates in adipose tissue and higher circulating levels of cystatin C compared to lean subjects [22]. In addition we observed that cystatin C is expressed and released by human preadipocytes differentiated in vitro [23]. Based on these findings, our main aim was to evaluate if *CST3* (the gene that codes for cystatin C) is one of the numerous genetic factors that causes BMI variation in individuals. In this study, we thus searched for association between common variants (SNPs) located in the *CST3* gene and BMI development during time in several populations from different European countries.

Participants and Methods

Study Design

We carried out a two stage study, the first stage was a discovery stage and the second one was a replication stage – only associations that were nominally significant in the discovery stage were thus tested in the replication stage.

In the first stage we investigated the association of two selected *CST3* tag SNPs individually and then combined into haplotypes with corpulence related phenotypes – BMI, waist circumference, hip circumference and waist to hip ratio in a group of 4,288 French individuals from the SUVIMAX study. In the second stage, we only tested the association of the variants that were found to be associated with corpulence related phenotypes at a 0.05 significance threshold in the first stage. These variants were tested in five independent populations of European ancestry – 763 Swedish women from the Stockholm Pregnancy And Women's Nutrition study (SPAWN), a group of 790 Danish lean men, a group of 672 Danish obese men and two groups of Danish women from the Danish National Birth Cohort (DNBC) (1,848 lean and 2,061 obese women). In the Danish men and the Swedish women from the SPAWN cohort, BMI, waist circumference, hip circumference and waist to hip ratio were available. In the two groups of Danish women from the DNBC cohort, only BMI and waist circumference were available.

Populations

We selected these populations because BMI measures were available at several time points for each of them. BMI at each exam was calculated as weight (kg) divided by height (m) squared. The discovery population was a group of 4,288 French individuals from the SUVIMAX study [24, 25], a group representative of the French general population in many aspects. Only 4.4% of investigated participants were obese at inclusion in 1994–1995. Height of all participants was measured at inclusion. This study lasted 13 years. Participants were invited to a follow-up exam at year 2, 4, 6 and 13 of the study. At each exam, participants were weighed. Their waist and hip circumference were measured at each exam with an inelastic tape measure using standardized methods. This population is described in table 1.

Table 1. Evolution of corpulence related phenotypes of French individuals (SUVIMAX, N = 4,300)

Exam ID	Exam type	Exam description	Age, years	BMI, kg/m ²	Waist circumference, cm	Hip circumference, cm	Waist to hip ratio
1	follow-up	2 years	51.58 ± 6.24	24.31 ± 3.74	82.29 ± 12.13	98.84 ± 7.27	0.83 ± 0.089
2		4 years	53.58 ± 6.24	24.55 ± 3.80	81.79 ± 12.26	98.49 ± 8.02	0.83 ± 0.094
3		6 years	55.58 ± 6.24	24.92 ± 3.99	83.88 ± 12.56	98.17 ± 8.55	0.85 ± 0.091
4		13 years	62.10 ± 6.18	25.47 ± 4.16	87.33 ± 12.50	98.83 ± 8.53	0.88 ± 0.091

Table 2. Evolution of corpulence related phenotypes in replication populations

Exam ID	Exam type	Exam description	Age, years	BMI, kg/m ²	Waist circumference, cm	Hip circumference, cm	Waist to hip ratio
Danish men (lean) n = 790							
1	school health record	birth	0	–	–	–	–
2		6 years	6	15.36 ± 1.05	–	–	–
3		7 years	7	15.62 ± 1.10	–	–	–
4		8 years	8	16.06 ± 1.18	–	–	–
5		9 years	9	16.47 ± 1.47	–	–	–
6		10 years	10	16.91 ± 1.65	–	–	–
7		11 years	11	17.32 ± 1.85	–	–	–
8		12 years	12	17.86 ± 2.02	–	–	–
9		13 years	13	18.45 ± 2.15	–	–	–
10		14 years	14	19.09 ± 1.90	–	–	–
11	follow-up	1943–1977	19.98 ± 1.89	24.63 ± 2.30	–	–	–
12		1981–1983	37.16 ± 8.51	24.65 ± 3.08	–	–	–
13		1992–1994	47.79 ± 8.55	26.13 ± 3.60	93.49 ± 10.53	100.26 ± 7.30	0.93 ± 0.07
14		1998–2000	49.75 ± 6.13	26.18 ± 3.81	93.94 ± 10.83	98.08 ± 6.94	0.95 ± 0.06
Danish men (obese) n = 672							
1	school health record	birth	0	–	–	–	–
2		6 years	6	17.76 ± 1.97	–	–	–
3		7 years	7	18.54 ± 2.39	–	–	–
4		8 years	8	19.69 ± 2.51	–	–	–
5		9 years	9	20.63 ± 2.65	–	–	–
6		10 years	10	21.59 ± 2.79	–	–	–
7		11 years	11	22.68 ± 2.93	–	–	–
8		12 years	12	24.02 ± 3.08	–	–	–
9		13 years	13	25.09 ± 3.12	–	–	–
10		14 years	14	26.25 ± 3.27	–	–	–
11	follow-up	1943–1977	19.84 ± 1.81	33.23 ± 2.34	–	–	–
12		1981–1983	32.52 ± 6.2	33.60 ± 5.06	–	–	–
13		1992–1994	43.00 ± 6.23	35.61 ± 5.64	116.89 ± 14.08	114.75 ± 11.18	1.02 ± 0.09
14		1998–2000	47.46 ± 5.21	36.01 ± 5.88	118.12 ± 14.34	114.14 ± 11.67	1.03 ± 0.07
Swedish women (SPAWN) n = 750							
1	before pregnancy	1984	29.16 ± 4.54	21.73 ± 2.87	–	–	–
2	follow-up	1 year later	30.16 ± 4.54	22.41 ± 3.28	–	–	–
3		2000–2001	46.12 ± 4.62	24.64 ± 4.10	–	–	–
4		2001–2002	45.96 ± 5.20	24.30 ± 4.14	81.96 ± 10.94	99.53 ± 9.17	0.82 ± 0.07
Danish women (DNBC lean) n = 1,848							
1	before pregnancy	before pregnancy	29.64 ± 4.21	22.66 ± 2.85	–	–	–
2	during pregnancy	12th gestational week	29.87 ± 4.17	23.68 ± 2.71	–	–	–
3		30th gestational week	30.24 ± 4.22	25.35 ± 2.49	–	–	–
4	after pregnancy	6 months after birth	31.02 ± 4.13	23.30 ± 3.02	–	–	–
5		18 months after birth	32.16 ± 4.20	23.05 ± 3.09	79.14 ± 8.70	–	–
6		7 years after birth	36.72 ± 4.12	23.44 ± 2.90	82.42 ± 8354	–	–
Danish women (DNBC obese) n = 2,061							
1	before pregnancy	before pregnancy	29.31 ± 4.24	37.01 ± 3.59	–	–	–
2	during pregnancy	12th gestational week	29.55 ± 4.25	36.89 ± 3.69	–	–	–
3		30th gestational week	29.91 ± 4.23	37.83 ± 4.14	–	–	–
4	after pregnancy	6 months after birth	30.72 ± 4.24	36.07 ± 3.83	–	–	–
5		18 months after birth	31.81 ± 4.14	35.85 ± 3.91	104.88 ± 12.70	–	–
6		7 years after birth	36.53 ± 4.16	36.95 ± 4.79	110.04 ± 12.65	–	–

The first and second replication populations were 2 samples of Danish men – 790 lean and 672 obese subjects. Both of these groups were part of a study carried out in Denmark; participants were recruited between 1943 and 1977, they underwent 3 follow-up exams – one in 1981–1983, one in 1992–1994, and one in 1998–2000. At each exam, they were weighed and measured [10]. Data concerning their height and weight during childhood were also available (from 6 to 14 years old, measured once a year). These populations are described in table 2.

The third replication population included 763 Swedish women (1.8% of whom were obese at inclusion) from the SPAWN cohort [26] described in table 2. These women were part of a longitudinal study describing the development of corpulence after pregnancy; they underwent four exams – before pregnancy (1984), at least 1 year after pregnancy, in 2000–2001 and in 2001–2002. Height and weight were measured at inclusion and participants were weighed again at each exam.

The two last replication populations were two samples of Danish pregnant women from the DNBC [27] – a group of 1,848 lean women and a group of 2,061 obese women (table 2). These women were involved in a

longitudinal study addressing the impact of intrauterine exposures on the health of the offspring. Data was available at 6 time points – before pregnancy, at the 16th gestational week, at the 30th gestational week as well as 6 months, 18 months and 7 years after birth. Participants provided their height and weight during telephone interviews except for the 7-year follow-up, which was a web-based questionnaire. They also provided their waist circumference at the 2 last exams (18 months and 7 years after birth).

Phenotypes of Interest

Four phenotypes were studied in the French SUVIMAX group – BMI, waist circumference, hip circumference and waist to hip ratio. In the groups of Danish lean and obese men and in the group of Swedish women from the SPAWN cohort, BMI was available at each follow-up time point. Waist circumference, hip circumference and waist to hip ratio were also available in these populations but not at each time point. BMI was also available in the two groups of Danish women from the DNBC cohort at each time point. Waist circumference was also available in these two groups but not at each time point.

Table 3. Description of *CST3* variability in study populations.

	French individuals (SUVIMAX)	Danish men (lean)	Danish men (obese)	Swedish women (SPAWN)	Danish women (DNBC lean)	Danish women (DNBC obese)
<i>rs2424577</i>						
A						
frequency	0.61	0.61	0.61	0.62	0.59	0.60
G						
frequency	0.39	0.39	0.39	0.38	0.41	0.40
A/A						
frequency	0.38	0.36	0.35	0.38	0.35	0.36
n	1,600	289	236	288	641	744
A/G						
frequency	0.46	0.49	0.51	0.49	0.49	0.48
n	1,916	379	345	372	899	983
G/G						
frequency	0.16	0.15	0.14	0.13	0.17	0.16
n	653	122	91	101	308	334
<i>rs3827143</i>						
A						
frequency	0.80	–	–	–	–	–
G						
frequency	0.20	–	–	–	–	–
A/A						
frequency	0.62	–	–	–	–	–
n	2,588	–	–	–	–	–
A/G						
frequency	0.34	–	–	–	–	–
n	1,438	–	–	–	–	–
G/G						
frequency	0.04	–	–	–	–	–
n	147	–	–	–	–	–
<i>LD</i>						
r ²	0.41	–	–	–	–	–
<i>Haplotype</i>						
Estimated frequencies						
AA	0.6117	–	–	–	–	–
AG	0.0007	–	–	–	–	–
GA	0.1806	–	–	–	–	–
GG	0.2069	–	–	–	–	–

Table 4. Genotype phenotype and haplotype phenotype association results in the discovery stage (significant results are shown in italics)

<i>CST3</i> variant	BMI, kg/m ²	Waist circumference, cm	Hip circumference, cm	Waist to hip ratio
<i>rs2424577</i>				
A/A vs. A/G				
p	0.535	0.484	0.324	0.853
estimate	−0.096	−0.225	−0.262	0.000
95% CI lower	−0.347	−0.968	−0.767	−0.004
95% CI upper	0.155	0.518	0.243	0.003
A/A vs. G/G				
p	<i>0.045</i>	<i>0.009</i>	0.099	<i>0.009</i>
estimate	0.359	1.139	0.609	0.007
95% CI lower	0.015	0.257	−0.086	0.002
95% CI upper	0.703	2.021	1.303	0.012
A/G vs. G/G				
p	0.111	<i>0.032</i>	0.339	<i>0.011</i>
estimate	0.263	0.912	0.347	0.006
95% CI lower	−0.073	0.051	−0.331	0.001
95% CI upper	0.599	1.772	1.024	0.011
A/A and A/G vs. G/G (dominant)				
p	0.055	<i>0.011</i>	0.170	<i>0.005</i>
estimate	0.307	1.015	0.466	0.006
95% CI lower	−0.009	0.205	−0.172	0.002
95% CI upper	0.623	1.825	1.103	0.011
A/A vs. A/G and G/G (recessive)				
p	0.217	0.13	0.164	0.266
estimate	0.163	0.458	0.350	0.002
95% CI lower	−0.073	−0.146	−0.126	−0.002
95% CI upper	0.399	1.062	0.825	0.005
<i>rs3827143</i>				
A/A vs. A/G				
p	0.780	0.955	0.959	0.981
estimate	0.035	−0.057	−0.016	0.000
95% CI lower	−0.210	−0.685	−0.510	−0.004
95% CI upper	0.280	0.570	0.477	0.003
A/A vs. G/G				
p	0.574	0.378	0.973	0.099
estimate	−0.180	−0.766	−0.015	−0.008
95% CI lower	−0.807	−2.376	−1.281	−0.017
95% CI upper	0.447	0.843	1.251	0.001
A/G vs. G/G				
p	0.511	0.400	0.958	0.108
estimate	0.215	0.709	−0.001	0.008
95% CI lower	−0.426	−0.936	−1.295	−0.002
95% CI upper	0.856	2.354	1.292	0.017
<i>Haplotypes</i>				
AA				
p	0.552	0.859	0.681	0.857
estimate	−0.231	0.175	0.329	−0.001
95% CI lower	−0.993	−1.755	−1.239	−0.012
95% CI upper	0.530	2.105	1.897	0.010
AG				
p	–	–	–	–
estimate	–	–	–	–
95% CI lower	–	–	–	–
95% CI upper	–	–	–	–

Table 4 continued on next page

Table 4. *Continued*

<i>CST3</i> variant	BMI, kg/m ²	Waist circumference, cm	Hip circumference, cm	Waist to hip ratio
GA				
p	0.260	0.351	0.208	0.741
estimate	−0.151	−0.321	−0.342	−0.001
95% CI lower	−0.414	−0.995	−0.875	−0.005
95% CI upper	0.112	0.354	0.190	0.003
GG				
p	0.192	0.393	0.271	0.697
estimate	0.172	0.288	0.294	0.001
95% CI lower	−0.086	−0.373	−0.229	−0.003
95% CI upper	0.431	0.950	0.817	0.004

Table 5. Genotype phenotype association results in the replication stage (significant results are shown in italics)

Population and phenotype	<i>CST3</i> rs2424577				
	A/A vs. A/G	A/A vs. G/G	A/G vs. G/G	A/A and A/G vs. G/G (dominant)	A/A vs. A/G and G/G (recessive)
Danish men (lean) BMI					
p	<i>0.010</i>	<i>0.022</i>	0.670	0.181	<i>0.004</i>
estimate	−0.510	−0.621	0.111	−0.332	−0.537
95% CI lower	−0.895	−1.153	−0.402	−0.819	−0.901
95% CI upper	−0.125	−0.089	0.624	0.155	−0.173
Danish men (lean) waist circumference					
p	<i>0.027</i>	<i>0.040</i>	0.634	0.211	<i>0.012</i>
estimate	−1.784	−2.298	0.515	−1.283	−1.907
95% CI lower	−0.206	−0.100	2.633	0.729	−0.413
95% CI upper	−3.361	−4.497	−1.604	−3.294	−3.400
Danish men (lean) waist to hip ratio					
p	<i>0.025</i>	<i>0.027</i>	0.535	0.158	<i>0.010</i>
estimate	−0.011	−0.015	0.004	−0.009	−0.012
95% CI lower	−0.001	−0.002	0.017	0.003	−0.003
95% CI upper	−0.021	−0.028	−0.009	−0.021	−0.021
Danish men (obese) BMI					
p	0.355	0.655	0.260	0.370	0.534
estimate	0.263	−0.186	0.449	−0.341	0.169
95% CI lower	0.822	0.631	1.231	0.406	0.703
95% CI upper	−0.295	−1.003	−0.333	−1.089	−0.365
Danish men (obese) waist circumference					
p	0.635	0.605	0.377	0.435	0.820
estimate	0.561	−0.895	1.456	−1.230	0.258
95% CI lower	2.886	2.497	4.691	1.864	2.482
95% CI upper	−1.763	−4.286	−1.779	−4.323	−1.966
Danish men (obese) waist/hip ratio					
p	0.946	0.461	0.411	0.401	0.860
estimate	0.000	−0.007	0.007	−0.007	−0.001
95% CI lower	0.013	0.012	0.025	0.010	0.011
95% CI upper	−0.012	−0.026	−0.010	−0.024	−0.013
Swedish women BMI					
p	0.297	<i>0.019</i>	0.090	<i>0.027</i>	0.165
estimate	0.143	0.846	−0.704	0.766	0.293
95% CI lower	−0.350	0.119	−1.409	0.094	−0.178
95% CI upper	0.636	1.574	0.002	1.437	0.763

Table 5 continued on next page

Table 5. *Continued*

Population and phenotype	CST3 rs2424577				
	A/A vs. A/G	A/A vs. G/G	A/G vs. G/G	A/A and A/G vs. G/G (dominant)	A/A vs. A/G and G/G (recessive)
Swedish women waist circumference					
p	0.514	0.131	0.046	0.058	0.945
estimate	−0.623	2.186	−2.808	2.540	−0.063
95% CI lower	−2.495	−0.654	−5.562	−0.091	−1.857
95% CI upper	1.250	5.025	−0.055	5.171	1.732
Swedish women waist to hip ratio					
p	0.164	0.363	0.993	0.676	0.146
estimate	−0.008	−0.008	0.000	−0.003	−0.008
95% CI lower	−0.020	−0.025	−0.024	−0.020	−0.019
95% CI upper	0.003	0.009	−0.017	0.013	0.003
Danish women (DNBC lean) BMI					
p	0.425	0.989	0.537	0.711	0.532
estimate	0.117	0.003	0.114	−0.064	0.086
95% CI lower	−0.171	−0.376	−0.248	−0.405	−0.184
95% CI upper	0.404	0.382	0.476	0.276	0.357
Danish women (DNBC lean) waist circumference					
p	0.957	0.900	0.863	0.870	0.995
estimate	−0.032	0.097	−0.129	0.115	0.003
95% CI lower	−1.190	−1.425	−1.595	−1.260	−1.083
95% CI upper	1.126	1.619	1.337	1.490	1.089
Danish women (DNBC obese) BMI					
p	0.666	0.413	0.236	0.270	0.961
estimate	0.078	−0.202	0.280	−0.247	0.008
95% CI lower	−0.277	−0.686	−0.184	−0.686	−0.328
95% CI upper	0.434	0.282	0.744	0.192	0.345
Danish women (DNBC obese) waist circumference					
p	0.994	0.140	0.025	0.040	0.908
estimate	0.893	−1.914	2.807	−2.411	0.107
95% CI lower	−1.058	−4.455	0.353	−4.708	−1.723
95% CI upper	2.845	0.628	5.262	−0.114	1.937

SNP Selection

Tag SNPs were selected using data from 60 unrelated individuals from the CEU HapMap population (HapMap Data Rel 27 Phase II + III, February 2009 on NCBI B36 assembly, dbSNP B126) using the tagger pairwise method in order to capture all SNPs in the *CST3* gene sequence and nearby flanking sequences (chromosome 20; 20p11.2; position 23559434 to 23569433) that are referenced in HapMap and that have a minor allele frequency equal or above 5%. We required the minimum estimated r^2 between the tagged and tag SNPs sets to be > 0.90 , implying only a slight loss of power in typing only tags.

Genotyping

Genotypes were determined for all individuals either by Taqman semi quantitative PCR assays (Applied Biosystem) according to the manufacturer's instructions, by SNPlexTM at INTEGRAGEN facility (Evry, France) or using the Illumina 610-QUAD chip.

LD Measure and Haplotype Reconstruction

The amount of linkage disequilibrium (LD) between tag SNPs was measured by computing the r^2 correlation coefficient [28] with FAMHAP 18

[29]. Associations between haplotypes and phenotypes were tested with HAPSTAT 3.0 [30].

Statistical Analysis

All statistical analysis was performed with R 2.10.1 [31]. Hardy-Weinberg (HW) equilibrium was tested for each SNP in each population by chi-square test. This test provided exact p values (without Yates' correction for continuity). Deviation from HW equilibrium was considered as significant for $p < 0.025$ in the discovery sample and for $p < 0.05$ in the replication samples.

Normality of the distribution of each phenotype was tested by Cramer von Misses test. Phenotypes were transformed either logarithmically or by box cox method, using R car package [32], in order to obtain a normal distribution.

Associations between phenotypes and genotypes were tested by linear mixed models assuming an additive model using R nlme package [33] as recommended in the case of longitudinal observational studies [34].

The model that was used for the linear mixed models procedure was phenotype = genotype + exam ID indicator + age + population specific covariates, random effects = Individual ID; this model only contains main effects.

Fig. 1. Evolution of BMI according to *CST3* rs2424577 genotypes.

Evolution (means $\pm$ SE) of BMI according to rs2424577 genotype (A/A: red circle, A/G: blue triangle and G/G: green diamond) during study period in **a** French individuals from the SUVIMAX group (2 to 13 years follow-up), **b** the group of Danish lean men, and **c** Swedish women from the SPAWN group (1984–2000) (exams are described in table 1 and 2). G/G and A/G carriers had a lower BMI than A/A carriers at each exam in the group of French individuals and in the group of Danish lean men. On the contrary, G/G carriers had a higher BMI at each exam than A/G and A/A carriers in the group of Swedish women.

*Significant p values.

All models were adjusted for age. Models were also adjusted for gender in the French SUVIMAX group, for number of children in the Swedish women group (SPAWN cohort) and for number of pregnancies and number of children in the two groups of Danish women from the DNBC cohort.

When phenotype data was only available at one time point (waist circumference, waist to hip ratio in the group of Swedish women) associations between phenotypes and genotypes were tested by linear regression with the following model: phenotype = genotype + age + number of children.

P values < 0.05 were considered significant since we carried out a discovery analysis followed by a replication stage. The significant findings were then tested assuming a dominant and recessive model in the discovery population and tested in the five independent replication groups. Importantly, a meta-analysis was performed on the estimates from the mixed models carried out in each study using standard procedures for meta-analysis using R *rmeta* package [35].

Results

Two tag SNPs were selected in order to capture all SNPs referenced in HapMap that are located in *CST3* gene and nearby flanking sequences – rs2424577 (HapMap reference allele: G) and rs3827143 (HapMap reference allele: A). SNP rs2424577 captured no other SNP, whereas rs3827143 captured five other SNPs – rs3787499, rs3827142, rs3787498, rs35610040 and rs3827141 – all with $r^2 = 1$. Tag SNP frequencies, haplotype frequencies, and LD between both tag SNPs (r^2) are described in table 3. All genotyped SNPs were found to be in HW equilibrium except for rs3827143 which deviated moderately from HW equilibrium in the discovery sample. Association tests carried out in each population are summarized in tables 4 and 5.

Fig. 2. Evolution of waist circumference according to *CST3* rs2424577 genotypes. Waist circumference (means $\pm$ SE) according to rs2424577 genotype (A/A: red, A/G: blue and G/G: green) in **a** the group of French individuals (SUVIMAX) during study period (2–13 years follow-up), **b** the group of Danish obese women (DNBC obese), 18 months and 7 years after birth, **c** the group of Danish lean men in 1992–1994 and in 1998–2000, and in **d** the group of Swedish women (SPAWN) in 2001–2002 (all exams are described in table 1 and 2). G/G carriers have a lower waist circumference than A/G and A/A carriers in the group of French individuals, the group of Danish obese women and the group of Danish lean men. However, G/G carriers have higher waist circumference than A/A and A/G carriers in the group of Swedish women. *Significant p values.

Discovery Sample (French SUVIMAX Population)

All tests carried out in this population were adjusted for age and gender; all results are for genotype main effects.

Association between *CST3* rs2424577 and Corpulence Related Phenotypes

We found significant association between rs2424577 and BMI as well as waist circumference and waist to hip ratio in the SUVIMAX population. At each time point of the study, G/G carriers had significantly lower BMI (fig. 1a, table 4) than A/A carriers. G/G carriers also had lower waist circumference (fig. 2a, table 4) and waist to hip ratio (table 4) than

A/A and A/G carriers. When carrying out the analysis under a dominant model (A/A and A/G vs. G/G), the association with BMI was of borderline significance and the associations with waist circumference and waist to hip ratio remained significant (table 4). No association was found when carrying out the analysis in a recessive manner (A/A vs. A/G and G/G). BMI accounts for general corpulence, whereas waist circumference, hip circumference and waist to hip ratio account for abdominal adiposity which is a risk factor for obesity associated co-morbidities. In order to investigate the specific influence of rs2424577 on adiposity while taking global corpulence into account we tested for association between rs2424577 and waist circumference as well as hip circumfer-

ence and waist to hip ratio, and we included BMI as a cofactor in each model; no significant association was found; however, the association between rs2424577 and waist to hip ratio when including BMI as cofactor in the model was of borderline significance ($p = 0.0534$, data not shown) when carrying out the analysis in a dominant manner (G/G vs. A/G and A/A). This probably suggests that the associations found with markers of body fat distribution strongly depend on global corpulence (i.e. BMI) in this population. Although G/G carriers already had a lower BMI at baseline, this difference did not reach statistical significance. Nevertheless it appeared that the association between rs2424577 and corpulence was stable during lifetime.

Association between CST3 rs3827143 and Corpulence Related Phenotypes

No significant associations between *CST3* rs3827143 and any of the investigated phenotypes were found in this population.

Association between CST3 Haplotypes and Corpulence Related Phenotypes

Moderate LD was found between the two tag SNPs (table 2). Because of their ability to capture LD more informatively, testing associations with haplotypes may be more powerful than those with individual SNPs. We hence reconstructed *CST3* haplotypes in order to investigate their role in the phenotypes of interest. In order to look for specific haplotype effects beyond the already identified SNP effects, we added this variant as an extra cofactor in the haplotype analysis. No association was found. Thus only *CST3* rs2424577 was investigated in our five replication samples.

Replication Samples

We investigated the association between rs2424577 and three corpulence related phenotypes – BMI, waist circumference and waist to hip ratio (when available) – in the five replication populations.

Danish Men

Analysis in the group of Danish lean men revealed an association between rs2424577 and BMI (fig. 1b) in the same way as in the SUVIMAX group – G/G carriers were less corpulent than A/A carriers (table 5). In addition, we also found that A/G carriers were significantly less corpulent than A/A carriers (table 5). In this group, when carrying out the analysis under a dominant model (A/A and A/G vs. G/G), the association with BMI was no longer significant, but when the analysis was carried out under a recessive model (A/A vs. A/G and G/G), the association with BMI was significant (table 5). We also found an association between rs2424577 and both waist circumference and waist to hip ratio in the same way as in the SUVIMAX group. G/G carriers had lower waist circumfer-

ence (table 5, fig. 2c) and waist to hip ratio (table 5) than A/A carriers. A/G carriers also had lower waist circumference (table 5, fig. 2c) and waist to hip ratio (table 5) than A/A carriers. When carrying out the analysis under a dominant model (A/A and A/G vs. G/G), the associations with waist circumference and waist to hip ratio were no longer significant, but when the analysis was carried out under a recessive model (A/A vs. A/G and G/G), the association with waist circumference and waist to hip ratio were significant (table 5). In contrast to lean men, we found no association between rs2424577 and BMI, waist circumference and waist to hip ratio in the group of Danish obese men (table 5).

Swedish Women (SPAWN)

In the third replication group (763 women from the SPAWN cohort), *CST3* rs2424577 was significantly associated with BMI (table 5, fig. 1c) and with waist circumference (table 5, fig. 2d). Surprisingly, G/G carriers were more corpulent than A/A carriers (table 5, fig. 1c and 2d). When carrying out the analysis under a dominant model (A/A and A/G vs. G/G), only the association with BMI remained significant (table 5), but when the analysis was carried out under a recessive model (A/A vs. A/G and G/G), these associations were no longer significant.

Danish Women (DNBC)

In the two last replication cohorts, associations between rs2424577 and BMI as well as waist circumference were investigated. No significant result was found in the 1,848 lean women from the DNBC cohort (table 5). However, in the 2,091 obese women from the DNBC cohort, waist circumference was significantly associated with rs2424577 – as found in other groups, G/G carriers had a lower waist circumference than A/G carriers (table 5, fig. 2b). When carrying out the analysis under a dominant model (A/A and A/G vs. G/G), the association with waist circumference remained significant (table 5, fig. 2b).

Gene × Gender × Age Interactions

Since our results were heterogeneous, we decided to perform additional investigations. First we searched for a gender specific action of *CST3* rs2424577 on BMI that could explain the different findings. We investigated the role of *CST3* rs2424577 on BMI separately in men and women in our discovery sample (French SUVIMAX group). In men, G/G carriers had a lower BMI than A/A and A/G carriers (data not shown), but this difference did not reach statistical significance. In women, we found that G/G carriers had a significantly lower BMI than A/A and A/G carriers ($p = 0.045$ under a dominant model – A/A and A/G vs. G/G; data not shown).

Second, we searched for gene × gender, gene × age and gene × age × gender interactions in the French cohort, fo-

Fig. 3. Meta analysis of *CST3* rs2424577 A/A versus G/G association with BMI.

Estimates (black rectangles) $\pm$ 95% CI (grey lines) for *CST3* rs2424577 G/G versus A/A comparison in each group and global estimate (black diamond). The overall estimate of G/G versus A/A is -0.18 (95% CI -0.37 to 0.02) and the overall p value is 0.0312 . Globally, G/G carriers have a significantly lower BMI than A/A carriers.

cusing on the association between rs2424577 (data not shown) and BMI, waist circumference and waist to hip ratio. We thus added a 'gene $\times$ age' or a 'gene $\times$ gender' or 'gene $\times$ age $\times$ gender' interaction term to the model described in 'Participants and Methods' (see above). While the 'gene $\times$ gender' interaction term did not reach statistical significance, the 'gene $\times$ age' interaction term yielded several significant results – age of the individuals apparently influenced the difference in BMI between A/G and G/G carriers ($p = 0.0432$), the difference in waist circumference between A/A and G/G carriers ($p = 0.0358$), the difference in waist circumference between A/G and G/G carriers ($p = 0.0333$) and the difference in waist to hip ratio between A/A and G/G carriers ($p = 0.0493$). The 'gene $\times$ age $\times$ gender' interaction term also yielded several significant results – the interaction between age and gender influenced the difference in waist circumference between A/A and G/G carriers ($p = 0.0126$) and between A/G and G/G carriers ($p = 0.0045$). In this model, gender influenced the difference in waist circumference between A/A and G/G carriers and between A/G and G/G carriers ($p = 0.008$ and $p = 0.0039$, respectively). The interaction between age and gender influenced the difference in waist to hip ratio between A/G and G/G carriers ($p = 0.0294$). In this model, gender also significantly influenced the difference in waist to hip ratio between A/G and G/G carriers ($p = 0.0314$).

Third, we tested for gene $\times$ age interactions in the association between rs2424577 and BMI, waist circumference and waist to hip ratio in our replication cohorts. In the group of Danish lean men, age also significantly influenced the difference in BMI between A/A and A/G carriers and between A/A and G/G carriers ($p = 0.0007$ and $p = 0.022$, respectively). In the group of Swedish women, age influenced the

difference in BMI between A/A and G/G carriers and between A/G and G/G carriers ($p = 0.0261$ and $p = 0.0088$, respectively). Age influenced the difference in waist circumference between A/A and A/G carriers ($p = 0.032$) in the group of Danish lean women and the difference in waist circumference between A/A and A/G carriers ($p = 0.007$) and between A/A and G/G carriers ($p = 0.03$) in the group of Danish obese women.

Meta-Analysis of the Association of rs2424577 and BMI

To substantiate our results, we carried out a meta-analysis on the difference in BMI between A/A and G/G carriers for rs2424577 in our six cohorts (fig. 3). The association between BMI (at each available time point in each population of our study) and rs2424577 remains significant, and the G/G genotype is associated with a lower BMI (meta p value = 0.0312 , meta estimate = -0.18 , 95% CI = -0.37 to 0.02).

Discussion

In our study we have identified a relationship between a variant in the *CST3* gene – rs2424577 – and corpulence related phenotypes in four independent populations. Nevertheless the contribution of this SNP remains unclear since G/G genotype was associated with a protective effect in the French SUVIMAX, Danish lean men and Danish obese women groups and a deleterious effect in the Swedish female SPAWN cohort. G/G carriers had lower BMI, waist circumference and waist to hip ratio in the French SUVIMAX and the Danish lean men groups, lower waist circumference in the group of Danish obese women (DNBC), but they had higher BMI and waist circumference in the group of Swedish women (SPAWN).

This type of result is described in the literature as a 'flip-flop phenomenon'. Lin et al. [36] demonstrated that such findings may arise from a multi-locus effect, implying a change in LD pattern between the two markers in the different studied populations. Nevertheless Zaykin et al. [37] demonstrated that such findings could simply be due to a change in haplotype frequencies in the different populations. Finally, our observations could simply be random findings. However, Clarke et al. [38] calculated the probability of obtaining such findings due to a true association or at random. They demonstrated that under the null hypothesis of no association, the probability of a significant allele flip is always $\alpha^2/2 = 0.00125$, whereas, in the case of a true association, the probability of such observation depends on the power of the study. In other words, it appeared that the probability to randomly obtain such findings is negligible [38]. Such flip-flop associations have already been reported for several other complex diseases including autism and late onset Alzheimer's disease. In autism, both the long and short allele at the HTTLR locus of the serotonin

transporter gene have been reported as risk alleles for autistic disorders [39–44]. In late onset Alzheimer's disease, two independent studies report the opposite allele at the same SNP in the glutathione-S-transferase omega-1 gene (*GSTO1*) to be positively associated with age at onset of the disease [45, 46]. Based on these previous studies, our observation could therefore be considered as relevant. Importantly, we found significant gene $\times$ age interactions in the association with corpulence traits in the different and heterogeneous populations we explored. Age might influence the association between *CST3* rs2424577 and corpulence related phenotypes. However, a gene $\times$ age interaction seems quite unlikely to explain the flip-flop phenomenon observed in this study since, when considering the age fitted values of BMI, we still retrieved the flip-flop effect, i.e. G/G carriers were still more corpulent in the group of Swedish women and less corpulent in the other groups. We carried out a meta-analysis to get an overview of the global significance of our findings. Since there is some evidence that *CST3* could undergo alternate expression (see below and [47]), we decided to carry out this analysis on the results that we obtained for *CST3* rs2424577 A/A versus G/G (homozygotes subjects only) and searched for an overall association with BMI in our six longitudinal studies. According to this meta-analysis, the overall association of *CST3* rs2424577 and BMI was significant with G/G genotype associated to a lower BMI.

While the effect of the actual causal variant might be small, our study may nevertheless highlight an implication of *CST3* – the gene that codes for cystatin C – on the level of corpulence during life time. Since cystatin C is the most powerful endogenous inhibitor of cathepsin S, K and L [14], one might speculate that the causal variant could influence corpulence by affecting the ability of cystatin C to inhibit cathepsin activity.

In our study, we used tag SNPs, which actually reflect genetic variation of the entire *CTS3* region. It is now crucial to identify the variants with functional consequences in *CST3* gene that are in LD with rs2424577 in order to confirm their role in corpulence related phenotypes. The contribution of *CST3* gene variants has been previously described, mainly in neurological diseases and cardiovascular pathologies. The functional G73A polymorphism in *CST3* gene that codes for an alanine to threonine change in position 25 was associated to Alzheimer's disease, with homozygosity for the B haplotype (containing the A nucleotide) as a risk factor [48]. Benussi et al. [47] carried out an in vitro analysis with this SNP, and it appeared that B/B human primary epithelial fibroblasts displayed a higher intracellular retention of the protein through a less efficient cleavage of the signal peptide than A/A cells (homozygous for the G nucleotide) which is possibly due to a change in hydrophobicity in the signal peptide. However, they report that in A/B cells, while expected to have an intermediate phenotype, two out of the four analyzed cultures had A/A cells phenotype (low intracellular retention)

and the two other cultures a B/B phenotype (high intracellular retention). This result suggested that only one allele of *CST3* could be alternatively expressed [47]. The G73A variant was also associated with differences in cystatin C circulating levels, with the B/B genotype associated with lower cystatin C circulating levels [49, 50]. This polymorphism was also identified as associated to frontotemporal lobar degeneration [51] and to age related macular degeneration [52]. Importantly and similarly to our findings with corpulence related parameters, Crawford et al. [53] also found an association between this SNP and Alzheimer's disease, but in the opposite direction. A/A genotype was associated with an increased risk of developing Alzheimer's disease [53]. These findings bring evidence of association between *CST3* genotypes and Alzheimer's disease, but in different ways depending on the studied populations. The G73A variant is referenced in dbSNP (www.ncbi.nlm.nih.gov) as rs1064039. This variant has not been genotyped in any of the HapMap populations; therefore, we have no information concerning LD between this variant and the SNPs that are investigated in our study. Two other SNPs located in *CST3* promoter, –82G/C and –78T/G, have been described by Eriksson et al. [54] and are associated with cystatin C circulating levels in healthy individuals and patients with recent myocardial infarctions. The authors showed that in vitro these two polymorphisms influenced the binding of nuclear factors and affected the basal rate of gene transcription in an allele specific manner. Additional SNPs such as –82G/C, –5G/A and +4A/C were also associated with cystatin C circulating levels [55]. These polymorphisms were in LD in the population studied, and individuals who carry two copies of the major haplotype (–82G –5G +4A) had 12.4% higher circulating levels of cystatin C than non-carriers. Individuals who were heterozygous for this haplotype displayed an intermediate phenotype.

Unfortunately, none of these SNPs are referenced in HapMap. Thus no information concerning LD between these SNPs and the SNPs investigated in our study is available. We cannot exclude that other functional variants, affecting other genes located nearby *CST3*, might explain our findings. More explorations are necessary to understand the functional relevance of our findings.

In conclusion, we found an association between a *CST3* variant and corpulence during life. The complexity of this association can be explained by a combination of variants with functional effects and by the fact that in vivo *CST3* could undergo an alternative expression.

Acknowledgments

We would like to thank Dr Audrey Sabbagh for her precious advice and re-read of the manuscript.

This work was supported by a Programme Hospitalier de Recherche Clinique, Assistance Publique des Hôpitaux de Paris (AOR 02076), two grants from the French National Agency of Research (OBCAT, program

ANR-05-PCOD-026-01, and COMPALIMAGE), a prize DANONE/Fondation pour la Recherche Médicale to KC, AFA insurance company (Sweden), by the Commission of the European Communities (Collaborative Project ADAPT, contract number HEALTH-F262008-201100). Support was obtained by region Ile de France and by the EU FP6 program HEPADIP. The Danish National Research Foundation established the Danish Epidemiology Science Centre, which initiated and created the Danish National Birth Cohort. The cohort is furthermore a result of a major grant from this Foundation. Additional support for the Danish

National Birth Cohort was obtained from the Pharmacy Foundation, the Egmont Foundation, the March of Dimes Birth Defects Foundation and the Augustinus Foundation.

Disclosure Statement

None of the authors have a financial interest related to this work.

References

- Silventoinen K, Kaprio J: Genetics of tracking of body mass index from birth to late middle age: evidence from twin and family studies. *Obes Facts* 2009;2:196–202.
- Willer CJ, Speliotes EK, Loos RJF, Li S, Lindgren CM, et al: Six new loci associated with body mass index highlight a neuronal influence on body weight regulation. *Nat Genet* 2009;41:25–34.
- Thorleifsson G, Walters GB, Gudbjartsson DF, Steinthorsdottir V, Sulem P, Helgadóttir A, Styrkarsdóttir U, Gretarsdóttir S, Thorlacius S, Jonsdóttir I, Jonsdóttir T, Olafsdóttir EJ, Olafsdóttir GH, Jonsson T, Jonsson F, Borch-Johnsen K, Hansen T, Andersen G, Jorgensen T, Lauritzen T, Aben KK, Verbeek ALM, Roeleveld N, Kampman E, Yanek LR, Becker LC, Tryggvadóttir L, Rafnar T, Becker DM, Gulcher J, Kiemeneys LA, Pedersen O, Kong A, Thorsteinsdóttir U, Stefansson K: Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity. *Nat Genet* 2009;41:18–24.
- Lindgren CM, Heid IM, Randall JC, Lamina C, Steinthorsdóttir V, et al: Genome-wide association scan meta-analysis identifies three loci influencing adiposity and fat distribution. *PLoS Genet* 2009;5:e1000508.
- Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, et al: Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. *Nat Genet* 2010;42:937–948.
- Naukkarinen J, Surakka I, Pietiläinen KH, Rissanen A, Salomaa V, Ripatti S, Yki-Järvinen H, van Duijn CM, Wichmann H, Kaprio J, Taskinen M, Peltonen L: Use of genome-wide expression data to mine the ‘gray zone’ of GWA studies leads to novel candidate obesity genes. *PLoS Genetics* 2010;6:e1000976.
- Liu Y, Guo Y, Zhang L, Pei Y, Yu N, Yu P, Pappasian CJ, Deng H: Biological pathway-based genome-wide association analysis identified the vasoactive intestinal peptide (VIP) pathway important for obesity. *Obesity (Silver Spring)* 2010;18:2339–2346.
- Moonesinghe R, Liu T, Khoury MJ: Evaluation of the discriminative accuracy of genomic profiling in the prediction of common complex diseases. *Eur J Hum Genet* 2010;18:485–489.
- van den Berg SW, Dollé MET, Imholz S, van der A DL, van 't Slot R, Wijmenga C, Verschuren WMM, Strien C, Siezen CLE, Hoebee B, Feskens EJM, Boer JMA: Genetic variations in regulatory pathways of fatty acid and glucose metabolism are associated with obesity phenotypes: a population-based cohort study. *Int J Obes* 2009;33:1143–1152.
- Jess T, Zimmermann E, Kring SII, Berentzen T, Holst C, Toubro S, Astrup A, Hansen T, Pedersen O, Sørensen TIA: Impact on weight dynamics and general growth of the common FTO rs9939609: a longitudinal Danish cohort study. *Int J Obes* 2008;32:1388–1394.
- Luan J, Kerner B, Zhao J, Loos RJ, Sharp SJ, Muthén BO, Wareham NJ: A multilevel linear mixed model of the association between candidate genes and weight and body mass index using the Framingham longitudinal family data. *BMC Proc* 2009;3(suppl 7):S115.
- Taleb S, Lacasa D, Bastard J, Poitou C, Cancellor R, Pelloux V, Viguerie N, Benis A, Zucker J, Bouillot J, Coussieu C, Basdevant A, Langin D, Clément K: Cathepsin S, a novel biomarker of adiposity: relevance to atherogenesis. *FASEB J* 2005;19:1540–1542.
- Lafarge J, Naour N, Clément K, Guerre-Millo M: Cathepsins and cystatin C in atherosclerosis and obesity. *Biochimie* 2010;92:1580–1586.
- Türk V, Bode W: The cystatins: protein inhibitors of cysteine proteinases. *FEBS Lett* 1991;285:213–219.
- Wasén E, Isoaho R, Mattila K, Vahlberg T, Kiveli S, Irjala K: Serum cystatin C in the aged: relationships with health status. *Am J Kidney Dis* 2003;42:36–43.
- Luc G, Bard J, Lesueur C, Arveiler D, Evans A, Amouyel P, Ferrières J, Juhan-Vague I, Fruchart J, Ducimetière P: Plasma cystatin-C and development of coronary heart disease: the PRIME study. *Atherosclerosis* 2006;185:375–380.
- Knight EL, Verhave JC, Spiegelman D, Hillege HL, de Zeeuw D, Curhan GC, de Jong PE: Factors influencing serum cystatin C levels other than renal function and the impact on renal function measurement. *Kidney Int* 2004;65:1416–1421.
- Ognibene A, Mannucci E, Caldini A, Terreni A, Brogi M, Bordini G, Sposato I, Mosconi V, Salvadori B, Rotella CM, Messeri G: Cystatin C reference values and aging. *Clin Biochem* 2006;39:658–661.
- Ichihara K, Saito K, Itoh Y: Sources of variation and reference intervals for serum cystatin C in a healthy Japanese adult population. *Clin Chem Lab Med* 2007;45:1232–1236.
- Muntner P, Winston J, Uribarri J, Mann D, Fox CS: Overweight, obesity, and elevated serum cystatin C levels in adults in the United States. *Am J Med* 2008;121:341–348.
- Taglieri N, Koenig W, Kaski JC: Cystatin C and cardiovascular risk. *Clin Chem* 2009;55:1932–1943.
- Naour N, Fellahi S, Renucci J, Poitou C, Rouault C, Basdevant A, Dutoir A, Alessi M, Bastard J, Clément K, Guerre-Millo M: Potential contribution of adipose tissue to elevated serum cystatin C in human obesity. *Obesity (Silver Spring)* 2009;17:2121–2126.
- Taleb S, Cancellor R, Clément K, Lacasa D: Cathepsin S promotes human preadipocyte differentiation: possible involvement of fibronectin degradation. *Endocrinology* 2006;147:4950–4959.
- Hercberg S, Preziosi P, Briançon S, Galan P, Triol I, Malvy D, Roussel AM, Favier A: A primary prevention trial using nutritional doses of antioxidant vitamins and minerals in cardiovascular diseases and cancers in a general population: the SU.VI.MAX study – design, methods, and participant characteristics. *Supplementation en Vitamines et Minéraux Antioxydants. Control Clin Trials* 1998;19:336–351.
- Dolley G, Bertrais S, Frochot V, Bebel J, Guerre-Millo M, Tores F, Rousseau F, Hager J, Basdevant A, Hercberg S, Galan P, Oppert J, Lacorte J, Clément K: Promoter adiponectin polymorphisms and waist/hip ratio variation in a prospective French adults study. *Int J Obes* 2008;32:669–675.
- Linné Y, Rössner S: Interrelationships between weight development and weight retention in subsequent pregnancies: the SPAWN study. *Acta Obstet Gynecol Scand* 2003;82:318–325.
- Olsen J, Melbye M, Olsen SF, Sørensen TI, Aaby P, Andersen AM, Taxbøl D, Hansen KD, Juhl M, Schow TB, Sørensen HT, Andresen J, Mortensen EL, Olesen AW, Sørensgaard C: The Danish national birth cohort – its background, structure and aim. *Scand J Public Health* 2001;29:300–307.
- Devlin B, Risch N: A comparison of linkage disequilibrium measures for fine-scale mapping. *Genomics* 1995;29:311–322.
- Becker T, Knapp M: Maximum-likelihood estimation of haplotype frequencies in nuclear families. *Genet Epidemiol* 2004;27:21–32.
- Lin DY, Huang BE: The use of inferred haplotypes in downstream analyses. *Am J Hum Genet* 2007;80:577–579.
- R Development Core Team: R: A language and environment for statistical computing. 2008, available from www.R-project.org.
- Fox J: car: Companion to Applied Regression. 2009, available from www.r-project.org, <http://socserv.socsci.mcmaster.ca/jfox/>.
- Pinheiro J, Bates D, DebRoy S, Deepayan Sarkar D; R Core team: nlme: Linear and Nonlinear Mixed Effects Models. 2008, available from www.r-project.org.
- Crawley MJ: The R Book. West Sussex, Wiley and Sons, 2007.
- Lumley T: Rmeta: meta-analysis. R package version 2.16, available from www.r-project.org.
- Lin P, Vance JM, Pericak-Vance MA, Martin ER: No gene is an island: the flip-flop phenomenon. *Am J Hum Genet* 2007;80:531–538.
- Zaykin DV, Shibata K: Genetic flip-flop without an accompanying change in linkage disequilibrium. *Am J Hum Genet* 2008;82:794–796; author reply 796–797.
- Clarke GM, Cardon LR: Aspects of observing and claiming allele flips in association studies. *Genet Epidemiol* 2010;34:266–274.

- 39 Devlin B, Cook EH, Coon H, Dawson G, Grigorenko EL, McMahon W, Minshew N, Pauls D, Smith M, Spence MA, Rodier PM, Stodgell C, Schellenberg GD: Autism and the serotonin transporter: the long and short of it. *Mol Psychiatry* 2005;10:1110–1116.
- 40 Betancur C, Corbex M, Spielow C, Philippe A, Laplanche JL, Launay JM, Gillberg C, Mouren-Siméoni MC, Hamon M, Giros B, Nosten-Bertrand M, Leboyer M: Serotonin transporter gene polymorphisms and hyperserotonemia in autistic disorder. *Mol Psychiatry* 2002;7:67–71.
- 41 Maestrini E, Lai C, Marlow A, Matthews N, Wallace S, Bailey A, Cook EH, Weeks DE, Monaco AP: Serotonin transporter (5-HTT) and gamma-aminobutyric acid receptor subunit beta3 (GABRB3) gene polymorphisms are not associated with autism in the IMGSA families. The International Molecular Genetic Study of Autism Consortium. *Am J Med Genet* 1999;88:492–496.
- 42 McCauley JL, Olson LM, Dowd M, Amin T, Steele A, Blakely RD, Folstein SE, Haines JL, Sutcliffe JS: Linkage and association analysis at the serotonin transporter (SLC6A4) locus in a rigid-compulsive subset of autism. *Am J Med Genet B Neuropsychiatr Genet* 2004;127B:104–112.
- 43 Tordjman S, Gutknecht L, Carlier M, Spitz E, Antoine C, Slama F, Carsalade V, Cohen DJ, Ferrari P, Roubertoux PL, Anderson GM: Role of the serotonin transporter gene in the behavioral expression of autism. *Mol Psychiatry* 2001;6:434–439.
- 44 Yirmiya N, Pilowsky T, Nemanov L, Arbelle S, Feinsilver T, Fried I, Ebstein RP: Evidence for an association with the serotonin transporter promoter region polymorphism and autism. *Am J Med Genet* 2001;105:381–386.
- 45 Li Y, Oliveira SA, Xu P, Martin ER, Stenger JE, Scherzer CR, Hauser MA, Scott WK, Small GW, Nance MA, Watts RL, Hubble JP, Koller WC, Pahwa R, Stern MB, Hiner BC, Jankovic J, Goetz CG, Mastaglia F, Middleton LT, Roses AD, Saunders AM, Schmechel DE, Gullans SR, Haines JL, Gilbert JR, Vance JM, Pericak-Vance MA, Hulette C, Welsh-Bohmer KA: Glutathione S-transferase omega-1 modifies age-at-onset of Alzheimer disease and Parkinson disease. *Hum Mol Genet* 2003;12:3259–3267.
- 46 Kölsch H, Linnebank M, Lütjohann D, Jessen F, Wüllner U, Harbrecht U, Thelen KM, Kreis M, Hentschel F, Schulz A, von Bergmann K, Maier W, Heun R: Polymorphisms in glutathione S-transferase omega-1 and AD, vascular dementia, and stroke. *Neurology* 2004;63:2255–2260.
- 47 Benussi L, Ghidoni R, Steinhoff T, Alberici A, Villa A, Mazzoli F, Nicosia F, Barbiero L, Broglio L, Feudatari E, Signorini S, Finckh U, Nitsch RM, Binetti G: Alzheimer disease-associated cystatin C variant undergoes impaired secretion. *Neurobiol Dis* 2003;13:15–21.
- 48 Finckh U, von der Kammer H, Velden J, Michel T, Andresen B, Deng A, Zhang J, Müller-Thomsen T, Zuchowski K, Menzer G, Mann U, Papassotiropoulos A, Heun R, Zurdell J, Holst F, Benussi L, Stoppe G, Reiss J, Miserez AR, Staehelin HB, Rebeck GW, Hyman BT, Binetti G, Hock C, Growdon JH, Nitsch RM: Genetic association of a cystatin C gene polymorphism with late-onset Alzheimer disease. *Arch Neurol* 2000;57:1579–1583.
- 49 Noto D, Cefalu AB, Barbagallo CM, Pace A, Rizzo M, Marino G, Caldarella R, Castello A, Pernice V, Notarbartolo A, Averna MR: Cystatin c levels are decreased in acute myocardial infarction: effect of cystatin c g73a gene polymorphism on plasma levels. *Int J Cardiol* 2005;101:213–217.
- 50 Chuo L, Sheu WHH, Pai M, Kuo Y: Genotype and plasma concentration of cystatin C in patients with late-onset Alzheimer disease. *Dement Geriatr Cogn Disord* 2007;23:251–257.
- 51 Benussi L, Ghidoni R, Galimberti D, Boccardi M, Fenoglio C, Scarpini E, Frisoni GB, Binetti G: The cst3 b haplotype is associated with frontotemporal lobar degeneration. *Eur J Neurol* 2010;17:143–146.
- 52 Zurdell J, Finckh U, Menzer G, Nitsch RM, Richard G: CST3 genotype associated with exudative age related macular degeneration. *Br J Ophthalmol* 2002;86:214–219.
- 53 Crawford FC, Freeman MJ, Schinka JA, Abdullah LI, Gold M, Hartman R, Krivian K, Morris MD, Richards D, Duara R, Anand R, Mullan MJ: A polymorphism in the cystatin C gene is a novel risk factor for late-onset Alzheimer's disease. *Neurology* 2000;55:763–768.
- 54 Eriksson P, Deguchi H, Samnegård A, Lundman P, Boquist S, Tornvall P, Ericsson C, Bergstrand L, Hansson L, Ye S, Hamsten A: Human evidence that the cystatin C gene is implicated in focal progression of coronary artery disease. *Arterioscler Thromb Vasc Biol* 2004;24:551–557.
- 55 Loew M, Hoffmann MM, Koenig W, Brenner H, Rothenbacher D: Genotype and plasma concentration of cystatin C in patients with coronary heart disease and risk for secondary cardiovascular events. *Arterioscler Thromb Vasc Biol* 2005;25:1470–1474.