Anthropometry, Carbohydrate and Lipid Metabolism in the East Flanders Prospective Twin Survey: Linkage of Candidate Genes Using Two Sib-Pair Based Variance Components Analyses

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Insulin resistance and obesity are underlying causes of type 2 diabetes and therefore much interest is focused on the potential genes involved. A series of anthropometric and metabolic characteristic were measured in 240 MZ and 112 DZ twin pairs recruited from the East Flanders Prospective Twin Survey. Microsatellite markers located close to ABCC8, ADIPOQ, GCK, IGF1, IGFBP1, INSR, LEP, LEPR, PPARγ and the RETN gene were genotyped. Univariate single point variance components linkage analyses were performed using two methods: (1) the standard method, only comprising the phenotypic and genotypic data of the DZ twin pairs and (2) the extended method, also incorporating the phenotypic data of the MZ twin pairs. Suggestive linkages (LOD > 1) were observed between the ABCC8 marker and waist-to-hip ratio and HDL-cholesterol levels. Both markers flanking ADIPOQ showed suggestive linkage with triglycerides levels, the upstream marker also with body mass and HDL-cholesterol levels. The IGFBP1 marker showed suggestive linkage with fat mass, fasting insulin and leptin levels and the LEP marker showed suggestive linkage with birth weight. This study suggests that DNA variants in ABCC8, ADIPOQ, IGFBP1 and LEP gene region may predispose to type 2 diabetes. In addition, the two methods used to perform linkage analyses yielded similar results. This was however not the case for birth weight where chorionicity seems to be an important confounder.

Keywords: East Flanders Prospective Twin Survey, Type 2 diabetes, Obesity, Dyslipidaemia, Linkage, Candidate Genes

Type 2 diabetes is a complex disorder that represents a major international public health threat (O'Rahilly et al., 2005). Although lifestyle factors play a prominent role in the development of the disease, the high prevalence of type 2 diabetes in specific ethnic groups and the much higher concordance rates of traits associated with the disorder in monozygotic (MZ) twins compared to dizygotic (DZ) twins indicate that genetic predisposition is also important (Krosnick, 2000; Souren et al., 2007). To uncover genes involved in type 2 diabetes, numerous genome-wide linkage scans have been carried out and evidence for linkage at several chromosomal regions has been reported (Arya et al., 2002; Loos et al., 2003; Rankinen et al., 2006). Usually, these regions are large and contain many genes and therefore candidate genes in subsequent studies are often selected based on pre-existing knowledge. Currently, much interest is focused on genes involved in insulin resistance and obesity, since these two intertwined factors are considered to be underlying causes of type 2 diabetes (Golay & Ybarra, 2005; Grundy, 2006).

In the condition of insulin resistance, normal insulin concentrations fail to trigger a normal metabolic

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response. To resist diabetes, pancreatic beta cells then need to overcompensate with sufficient insulin release (Saltiel, 2001).

Pancreatic beta cells produce and secrete insulin mainly in response to blood glucose, which is taken up into the cells through the glucose transporter isotype 2 (GLUT2) and subsequently phosphorylated into glucose-6-phosphate by glucokinase (GCK) (Kulkarni, 2004; Saltiel, 2001). This metabolic activity in the pancreatic beta cell leads to an increase in the ATP/ADP ratio and closure of the ATP-sensitive potassium (K_{ATP}) channel, which is composed of the inward rectifying K^+ channel (Kir6.2) and the sulfonylurea receptor (SUR1; encoded by *ABCC8*). The closure is followed by membrane depolarization, which activates voltage gated calcium channels and leads to intracellular Ca^{2+} rise triggering insulin secretion (Ashcroft, 2005; Yan et al., 2004).

In addition to insulin there are also other hormones with insulin-like effects. The most important of these is insulin-like growth factor 1 (IGF1), which is the second most powerful peptide with glucose lowering effects (Janssen & Lamberts, 2002). The effects of insulin and IGF1 on carbohydrate, lipid and protein metabolism are mediated by their receptors (INSR, IGF1R). Insulin and IGF1 are able to bind to each others receptor, although the affinity is considerably lower (Bluher et al., 2005). IGF1 circulates almost entirely bound to one of the six members of the IGF binding proteins (IGFBPs) family. Although the majority of IGF1 is bound to IGFBP3, IGFBP1 has been suggested to be the short-term regulator of IGF1 bioactivity and is negatively associated with risk factors of cardiovascular disease and positively with insulin sensitivity (Heald et al., 2001; Janssen & Lamberts, 2002; Lee et al., 1997).

Obesity, another major factor underlying type 2 diabetes, is defined as excess accumulation of adipose tissue. Adipose tissue, formerly considered to be an inert energy depository, is now also known as an important endocrine organ producing active molecules called adipocytokines including adiponectin, leptin and resistin, which are encoded by *ADIPOQ*, *LEP* and *RETN*, respectively (Gil-Campos et al., 2004). Increased adiponectin levels are associated with weight loss and increased insulin sensitivity (Koerner et al., 2005; Meier & Gressner, 2004). Additionally, in prepubertal children, adiponectin is negatively associated with plasma lipid markers, except for HDL-cholesterol that shows a positive relation (Gil-Campos et al., 2004). Leptin, which biological activities are carried out through selective binding to its receptor (LEPR), is mainly produced by adipocytes, but also expressed in other tissues including placenta, ovaries, skeletal muscle, stomach, pituitary and liver (Koerner et al., 2005; Paracchini et al., 2005). Leptin acts as a satiety signal affecting central circuits in the hypothalamus resulting in suppressed food intake and increased energy expenditure (Koerner et al., 2005). Furthermore peripheral leptin seems to play a role in controlling cellular lipid balance, glucose homeostasis and insulin sensitivity (Koerner et al., 2005; Muoio & Lynis Dohm, 2002). Resistin has been linked to insulin resistance in murine models (Gil-Campos et al., 2004; Koerner et al., 2005). In humans, however, the relationship between resistin and markers of the metabolic syndrome is still controversial (Koerner et al., 2005; Meier & Gressner, 2004; Rea & Donnelly, 2004). Besides secreting active molecules adipose tissue has the capacity to expand drastically during life (Bluher et al., 2005), which is correlated with many unfavourable side effects. Therefore the role of proteins such as the peroxisome proliferator-activated receptor γ (PPARγ) and IGF1, which among other processes regulate fat cell formation and functioning, are also of great interest in the pathology of obesity (Bluher et al., 2005; Gurnell, 2005).

Previously we estimated the heritability of 18 anthropometric and metabolic characteristics associated with type 2 diabetes measured in 378 healthy twin pairs recruited from the East Flanders Prospective Twin Survey (EFPTS) (Souren et al., 2007). In general, heritability estimates of the traits studied in our twin sample were high. In order to explain some of the genetic variance observed, we performed univariate single point variance components linkage analyses using microsatellite markers located within or near candidate genes including *ABCC8*, *ADIPOQ*, *GCK*, *IGF1*, *IGFBP1*, *INSR*, *LEP*, *LEPR*, *PPARγ* and *RETN*. Linkage to metabolic traits in these candidate gene regions provides evidence for the possible presence of genetic variation in that region influencing these traits and is an incentive for further fine mapping the region. Several software packages are available to carry out sib-pair based variance components linkage analyses. The statistical package Mx is especially designed to analyse twin data (Neale et al., 2002), but another commonly used program is MERLIN (Abecasis et al., 2002). The advantage of Mx compared to MERLIN is that Mx also incorporates the phenotypic data of the MZ twins. This allows to partition the total variation into effects due to the quantitative trait locus (QTL; Q), additive genetic (A), nonadditive genetic (D), or common environmental (C) and unique environmental effects (E), resulting in a very accurate estimation of the variance components. In addition, when the distribution of a trait differs among men and women it is possible to model these sex differences by using a scalar model. By comparing the fit-functions of the different models one can assess which model provides the best fit to the data and use that model to conduct the linkage analysis. MERLIN, on the contrary simply incorporates the phenotypic and genotypic data of the DZ twins and decomposes the total phenotypic variance into effects due to the QTL (Q), additive genetic factors (A) and unique environmental factors (E). In the present study, sib-pair variance component linkage analyses were conducted using both the

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'MERLIN method', which we call the standard method, and the 'Mx method', which we call the extended method.

Materials and Methods

Subjects

The EFPTS is a population-based twin register, which started in 1964 and has been recording all multiple births in the Belgian Province of East Flanders until the present (Loos et al., 1998). For the current study, a series of anthropometric and metabolic phenotypes of 378 complete twin pairs were available, divided over 138 DZ pairs (46 male, 49 female and 43 mixed pairs) and 240 MZ pairs (113 male and 127 female pairs). Since not all participants were willing to provide DNA, DNA was available from 288 complete twin pairs comprising 112 DZ pairs (34 male, 43 female and 35 mixed pairs) and 176 MZ pairs (78 male and 98 female pairs). In the present analysis, if genotypic data were absent then the phenotypic data could only be used if the twins were MZ. As a result, in the current analysis phenotypic data of 352 complete pairs were used, divided over 112 DZ pairs and 240 MZ pairs, of which 138 pairs were monochorionic (MC) and 102 were dichorionic (DC). Birth weights were obtained from obstetric records, and gestational age reported by the obstetrician was calculated as the number of completed weeks of pregnancy based on the last menstrual period. The methods used to measure the phenotypes at adult age and a detailed description of the twin sample has been described earlier (Loos et al., 1998; Souren et al., 2007). The Ethics Committee of the Faculty of Medicine of the Katholieke Universiteit Leuven approved the project and all participants gave informed consent.

DNA Extractions and Marker Selection

Genomic DNA was extracted initially from available placental tissue collected at birth. If placental tissue was unavailable, twin pairs were contacted and whole blood or mouth swabs were taken for DNA extraction. The DNA mini kit (Qiagen) was used for placental and mouth swab DNA extraction, the Wizard kit (Promega) was used for blood DNA extraction, both according to manufacturer's instructions. Microsatellite markers (di- and tetranucleotide repeats) were selected from the human UniSTS map (http://www.ncbi.nlm.nih.gov/genome/sts). Markers were chosen based on distance to the candidate gene and level of heterozygosity. The location of the markers and candidate genes as well as marker heterozygosity is presented in Table 1.

Polymerase Chain Reactions (PCRs)

Ten PCR protocols were used to amplify 14 markers of 10 candidate genes. Six markers could be optimized to fit in two multiplexed PCR protocols (multiplex 1: markers D3S1602, GCK, D7S2427 and D1S198; multiplex 2: markers D3S3686 and D11S902) and the others were amplified in singleplex PCR protocols. Primer sequences including fluorescent labels, $MgCl_2$ concentrations, PCR annealing temperature and the number of cycles are listed in Table 1. Further PCR ingredients (15 or 20 µl) consisted of 1x PCR buffer (Invitrogen, Breda, the Netherlands), 0.33 mM dNTPs (GE Healthcare, Eindhoven, the Netherlands), 4-8 pmol primers (Eurogentec, Seraing, Belgium), 5% DMSO and 0.5-1 U *Taq* DNA polymerase (Invitrogen). All PCRs were performed in Biometra T1 thermal cyclers (Westburg, Leusden, the Netherlands), starting with an initial denaturation at 95°C for 5 min and ending with a final extension of 10 min at 72°C.

Genotyping

All TET-labeled PCR products were diluted 10 times, FAM-labeled products 5-10 times and HEX-labeled products 0-5 times. Labeled fragments were size-resolved by capillary electrophoresis on the ABI3100 Genetic Analyzer and size calling was performed with Genescan Software version 3.7 (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). The TAMRA 350-size standard was used for fragment length calling. Two experienced researchers scored the final genotypes independently and a third expert was consulted for discrepant results.

General Statistical Analysis

Anthropometric and metabolic characteristics are expressed as mean ± *SD*. Traits with a skewed distribution were converted to a normal distribution by log transformation. Differences between men and women according to zygosity were tested using the PROC MIXED method, in which adjustment for the intra-twin pair relationship was possible. Differences were considered significant if $p < .05$. For all traits effects of potential covariates were also checked using PROC MIXED, which has been described in detail before (Souren et al., 2007). General statistical analysis were conducted using the statistical package SAS (version 9.1, SAS Institute).

Linkage Analysis

A χ^2-test was used to check Hardy-Weinberg equilibrium for genotype frequencies of the microsatellite markers, using only one randomly selected co-twin per pair (using genotypes of MZ and DZ twin pairs). To test for linkage between marker loci and the anthropometric and metabolic characteristics, univariate single point variance components linkage analyses were performed using the statistical package Mx (Neale et al., 2002). The script was downloaded from the GenomEUtwin Mx-script library (http://www.psy.vu.nl/mxbib/). Linkage analysis was performed with and without adjusting for significant covariates. In addition we compared two methods: (1) standard sib-pair variance components linkage analysis, only comprising the phenotypic and genotypic data of the DZ twin pairs and (2) extended sib-pair variance components linkage analysis, also incorporating the phenotypic data of the MZ twin pairs. In the standard method the phenotypic variation is decomposed into

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Table 1

Gene and Marker Positioning, Primer Sequences and PCR Conditions

Gene	Marker	Physical Position[a]	Genetic Position[b]	Forward primer sequence (5′ → 3′)[c]	Reverse primer sequence (5′ → 3′)	Product	Cycles	T	$MgCl_2$[d]	Alleles	H
ABCC8		17.371.009-17.455.025									
	D11S902	17.445.082-17.445.228	21.5	HEX-CGGCTGTGAATATACTTAATGC	CAACAGCAATGGGAAGTT	137-159	33	55	2.25	12	82
	D3S1602	187.514.441-187.514.733	201.1	TET-AGAGCCTTCTATGGGTCTACAT	AGCTCAACCTTCAAACATACATT	271-297	31	55	2.25	13	84
ADIPOQ		188.043.157-188.058.946									
	D3S3686	Not available	203.8	HEX-AGGGTATTTCATTCCCATTG	CCAGGTTACGCCAAGTG	105-135	33	55	2.25	15	81
	GCK	44.142.647-44.142.841	67.4	TET-CACACCAAAACTGCCTGTATTAG	TTGGTCAGTGTAGGCTGAACTC	194-204	31	55	2.25	5	49
GCK		44.150.395-44.195.563									
	D7S2427	44.931.275-44.931.510	68.5	TET-AGGATTGTTTGAGCCCAGA	TTAGATTCCCATCAGCAGC	207-245	31	55	2.25	17	82
IGF1		101.313.806-101.398.454									
	IGF1	101.399.183-101.399.376	109.5	FAM-GCTAGCCAGCTGGTGTTATT	ACCACTCTGGGAGAAGGGTA	174-196	35	60	2.25	7	53
	D7S478	44.898.728-44.898.851	69	HEX-TGTGTCATTACGCTTTTCATC	TCAAATGGTTCAGGAGAAAGA	108-130	40	60	2.25	10	70
IGFBP1		45.894.484-45.899.792									
	D7S2506	47.646.979-47.647.146	69.6	TET-CAGCAGGGCTTGAAATGAAC	ACACAGTGGAGCTGGCATAG	150-186	35	60	1.69	18	84
	D19S1034	6.064.256-6.064.482	20.8	FAM-AGGCTGTGGTGAGCTATGAC	GTGTCCCTAGCACCTAGCAA	217-239	35	55	1.5	7	70
INSR		7.067.638-7.245.011									
LEP		127.668.567-127.684.917									
	D7S530	128.989.684-128.989.791	134.6	FAM-TGCATTTTAGTGGAGCACAG	CAGGCATTGGGAACTTTG	98-118	32	55	2.25	11	76
	D1S2638	63.325.557-63.325.776	96	HEX-CTTGGATTGGTGGGTACTA	TGAGGTTTCAGGGTGGCT	223-245	33	55	2.25	12	82
LEPR		65.658.906-65.875.410									
	D1S198	66.783.773-66.784.080	99.3	TET-GACTTCACCATCAACGCCTG	CAGGAAAGTGGATGTGACGA	307-325	31	55	2.25	10	81
	D3S1263	11.492.252-11.492.482	36.1	FAM-CTGTTGACCCATTGATACCC	GATGCCTTGCGGCTATGA	253-297	32	55	2.25	19	87
PPARγ		12.304.359-12.450.843									
RETN		7.639.972-7.641.340									
	D19S413	9.040.462-9.040.546	32.4	TET-GTTTATTTTAAATGCTCTTACCACA	CCATCAACTCACCTACTTATC	65-85	31	55	2.25	11	79

Note: [a]Physical map positions (bp) are according to the sequence map accessible on the NCBI website (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9606 and http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene); [b]Genetic map positions (cM) are according to Marshfield; [c]Forward primers were 5′-labeled with fluorescent dyes (in bold); [d]$MgCl_2$ in mM. T: annealing temperature (°C), H: heterozygosity (%).

variance due to the QTL (Q), additive genetic factors (A) and unique environmental factors (E). In the extended method, the phenotypic data of the MZ twin pairs were also included, having the advantage of partitioning the phenotypic variance into effects due to the QTL (Q), additive genetic (A), nonadditive genetic (D), or common environmental (C) and unique environmental effects (E). For the latter method, optimisation of the univariate model of the different phenotypes has been carried out. A detailed description of this optimisation procedure has been reported elsewhere (Souren et al., 2007), and a short summary is presented in columns 2, 3 and 16 of Table 4. Our sample size is too small to conduct sex-specific linkage analyses, therefore we used a scalar model (allowing the total variances to differ between men and women) for the traits (BMI, body mass, fat body mass) that had a model with different variance components estimates for men and women as the best fitting model (Souren et al., 2007). In the unadjusted analysis sex effects were not modeled. In these instances only simple univariate models without scalar sex effects were used. For birthweight the intrapair correlations differ between MZ MC and MZ DC twins. Since data on chorionicity were available, extended variance component linkage analysis for birth weight was performed in three different ways: (1) using only phenotypic data of MZ DC twins, (2) using only phenotypic data of MZ MC twins, and (3) incorporating phenotypic data of both MZ MC and MZ DC twins.

Estimates of the variance component associated with the QTL were obtained using the *pi-hat* approach, in which the covariance due to the marker or QTL for a sib-pair is modelled as a function of the proportion of alleles shared identical by descent (IBD). Since parental data were not available, the probabilities of sharing 0, 1 or 2 alleles IBD were estimated in MERLIN (Abecasis et al., 2002) using allele frequencies based on one randomly selected co-twin per pair (using genotypes of MZ and DZ twin pairs). The effect of the QTL was evaluated by comparing a full model, in which the genetic variance caused by Q for a given phenotype was free, with a restricted model, in which the effect of Q was equal to zero. Logarithm of the odds (LOD) scores, computed as the difference in –2log likelihood divided by 4.6, were used to evaluate the QTL effect. Because we used a candidate gene approach, the multiple testing problem associated with genome-wide search does not apply (Lander & Kruglyak, 1995). Suggestive evidence for linkage was defined by LOD>1 and significant evidence for linkage by LOD>3.

Results

General Statistical Analysis

In Table 2 anthropometric and metabolic characteristics of the MZ and DZ twins are expressed as mean ± SD according to sex. In both MZ and DZ twins, men had a higher body height, body mass, WHR, lean body mass and fasting glucose levels, but lower sum of four skinfolds, fat mass, IGFBP1, fasting insulin, leptin, total cholesterol, HDL-cholesterol and non-esterified fatty acids levels than women ($p < .05$). In DZ twins only, men had a higher weight at birth ($p < .05$).

Linkage Analysis

Microsatellite markers D7S2427 (*GCK*) and D7S478 (*IGFBP1*) deviated from the assumptions of Hardy-Weinberg equilibrium ($p < .05$) and were excluded from further linkage analyses.

Results of the univariate single-point variance component linkage analysis performed using the standard and the extended method, unadjusted and adjusted for significant covariates are listed in Table 3 and Table 4. After adjustment, the D11S902 (*ABCC8*) marker showed suggestive linkage with WHR, and with HDL-cholesterol levels using the extended method only. Before adjustment, linkage with HDL-cholesterol levels was nonsignificant. The D3S1602 (*ADIPOQ*) marker showed in the adjusted analysis suggestive linkage with body mass and HDL-cholesterol levels using both methods, and with triglycerides levels using the extended method only. Before controlling for covariates linkage with triglycerides levels was nonsignificant and suggestive linkage with body height and lean body mass was observed. The downstream marker D3S3686 showed also suggestive linkage with triglycerides levels using both methods, while before adjustment this linkage was nonsignificant and linkage with HDL-cholesterol levels seemed suggestive.

After controlling for covariates, the D7S2506 marker (*IGFBP1*) showed suggestive linkage with leptin levels using both methods, with fat mass using the standard method only and with fasting insulin using the extended method only. Before adjusting, this marker showed also suggestive linkage with the sum of four skinfolds, and with fat mass it showed significant linkage.

After adjustment, the D7S530 (*LEP*) marker showed suggestive linkage with birth weigh using the standard method only. Before controlling for covariates, also suggestive linkage with fasting insulin levels was observed.

The D1S2638 (*LEPR*) and the D19S1034 (*INSR*) marker showed in the unadjusted analysis suggestive linkage with IGFBP1 and with fasting glucose levels, respectively. However, after adjustment these linkages became nonsignificant.

Discussion

In the current study, a series of anthropometric and metabolic characteristics were measured in 240 MZ and 112 DZ twin pairs recruited from the EFPTS. Fourteen microsatellite markers located within 10 candidate gene regions were genotyped and univariate single point variance components linkage analyses were performed. The initial DZ twin sample comprises 138

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Table 2

Anthropometric and Metabolic Characteristics of the MZ and DZ Twins According to Sex

Characteristic	MZ Men	MZ Women	*p*	DZ Men	DZ Women	*p*
N	226	254		104	120	
Gestational age (wks)[a]	36.8 ± 2.33	37.0 ± 2.59	0.33	37.4 ± 2.3	37.5 ± 2.2	.77
Birth weight (g)	2531 ± 471	2451 ± 485	0.17	2694 ± 472	2581 ± 470	.005
Age (yrs)[a]	25.2 ± 4.6	25.1 ± 4.6	0.83	25.1 ± 4.6	25.5 ± 4.6	.54
Body height (cm)	178.1 ± 6.3	165.2 ± 6.2	< .0001	178.5 ± 6.6	166.7 ± 6.5	< .0001
Body mass (kg)	70.3 ± 9.7	60.5 ± 10.1	< .0001	70.1 ± 10.0	60.8 ± 9.6	< .0001
BMI (kg/m^2)[b]	22.0 ± 1.1	21.9 ± 1.2	0.97	21.9 ± 1.1	21.7 ± 1.1	.87
WHR (%)	83.4 ± 5.5	73.4 ± 4.6	< .0001	82.6 ± 5.4	72.2 ± 4.1	< .0001
Sum of four skin folds (mm)[b]	35.4 ± 1.5	56.4 ± 1.4	< .0001	33.9 ± 1.1	52.3 ± 1.4	< .0001
Fat mass (kg)[b]	11.6 ± 1.5	16.8 ± 1.3	< .0001	12.1 ± 1.5	16.6 ± 1.3	< .0001
Lean body mass (kg)	57.7 ± 6.5	43.0 ± 5.3	< .0001	56.9 ± 6.6	43.6 ± 5.8	< .0001
IGFBP1 (ng/ml)[b]	10.7 ± 1.9	16.7 ± 2.1	< .0001	11.9 ± 1.9	18.5 ± 2.2	< .0001
Fasting insulin (pmol/l)[b]	33.2 ± 1.5	37.3 ± 1.6	0.02	32.0 ± 1.6	40.8 ± 1.5	.0001
Fasting glucose (mmol/l)	5.0 ± 0.4	4.5 ± 0.4	< .0001	4.8 ± 0.4	4.6 ± 0.4	.0001
Leptin (ng/ml)[b]	1.6 ± 2.9	11.4 ± 2.1	< .0001	1.6 ± 2.8	11.6 ± 1.9	< .0001
Total cholesterol (mmol/l)	4.8 ± 1.0	5.1 ± 0.9	0.0008	4.9 ± 1.1	5.2 ± 0.9	.03
LDL-cholesterol (mmol/l)	3.0 ± 0.9	2.9 ± 0.8	0.54	3.1 ± 1.0	2.9 ± 0.8	.06
HDL-cholesterol (mmol/l)	1.3 ± 0.3	1.8 ± 0.4	< 0.0001	1.4 ± 0.4	1.9 ± 0.4	< .0001
Triglycerides (mmol/l)[b]	0.9 ± 1.5	0.9 ± 1.5	0.32	0.9 ± 1.5	0.9 ± 1.3	.85
Non-esterified fatty acids (mmol/l)	0.5 ± 0.2	0.7 ± 0.2	< .0001	0.5 ± 0.2	.7 ± 0.2	< .0001

Note: Data are expressed as mean ± *SD*; [a]*p*-value calculated using standard linear regression, because convergence criteria could not be met in the random intercept model; [b]Geometric mean ± *SD*.

twin pairs, of which 26 DZ twin pairs did not provide DNA. As differences in mean age (25.4 vs. 25.7), weight (65.1 vs. 65.8) and height (172.1 vs. 172.1) are negligible between the two samples, the genotyped sample is still representative for the initial DZ twin sample. Two microsatellite markers, one located near *GCK* (D7S2427) and the other near *IGFBP1* (D7S478), deviated from the assumptions of Hardy-Weinberg equilibrium (HWE). Deviation from HWE can occur due to genotyping errors, inbreeding, genetic drift, selection and population stratification. Because the last four causes have impact on the whole genome and in the current study only 2 of the 14 markers deviated from HWE, the most likely reason for deviation from HWE is genotyping errors, presumably systematic mistyping of heterozygotes as homozygotes (Gomes et al., 1999). To avoid false positive linkage results, these markers were excluded from further linkage analyses.

Linkage analyses were carried out unadjusted as well as adjusted for significant covariates, resulting in considerable differences between the linkage results (Table 3 and Table 4). In many occasions, unadjusted LOD scores were larger than 1 and gave therefore suggestive evidence for linkage, but after adjusting for covariates LOD scores were non-significant. The D7S2506 marker (*IGFBP1*) for example showed suggestive linkage with the sum of four skinfolds (LOD_S = 2.03; LOD_E = 2.19) in the unadjusted analysis, but after adjusting for sex and age the LOD scores were non-significant (LOD_S = 0.74; LOD_E = 0.66). The opposite also occurred, where LOD scores were smaller than 1 in the unadjusted analysis, but gave suggestive evidence for linkage after controlling for significant covariates. For example, linkage with the D3S3686 marker (*ADIPOQ*) and triglycerides levels was not significant in the unadjusted analysis (LOD_S = 0.60; LOD_E = 0.63), but after adjusting for covariates linkage was suggestive (LOD_S = 1.09; LOD_E = 1.42). These data indicate that controlling for covariates has great impact on the final linkage results and without adjustment false positive and false negative outcomes are eminent and may lead to futile follow up studies.

In the present study DNA was only available of 112 DZ twin pairs, and consequently our power to detect QTLs is rather low. In addition, we recognise that due to the large number of statistical tests performed some of the results reported here may represent false–positive findings and therefore our results must be interpreted cautiously. Nevertheless, five of the 12 microsatellite markers located in four candidate gene regions (*ABCC8, ADIPOQ*, *IGFBP1* and *LEP*) showed after adjusting for covariates suggestive linkage with several anthropometric and metabolic characteristics. The D11S902 marker close

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to the *ABCC8* gene showed suggestive linkage with WHR and HDL-cholesterol levels. 4.5 kb downstream of *ABCC8*, the *KCNJ11* gene encoding the other subunit of the K_{ATP} channel (Kir6.2) is located. This gene contains the E23K single nucleotide polymorphism (SNP), which is a well-established genetic risk factor for type 2 diabetes and which is in strong linkage disequilibrium with the coding SNP A1369S (rs757110) in exon 33 of *ABCC8* (Florez et al., 2004; Scott et al., 2007). However, the E23K SNP has been genotyped in our twin sample and association analysis revealed that this SNP did not explain the observed linkage signal (data not shown). This indicates that besides the *KCNJ11* E23K SNP, also other genetic variants predisposing to type 2 diabetes are located in the *ABCC8* gene region. For example, SNPs in the promoter of *ABCC8* that have in a Finnish sample been associated with an increased type 2 diabetes risk (Laukkanen et al., 2004). Nevertheless, the linkage signal might also be the consequence of rare population specific variants.

The D3S1602 marker, upstream the *ADIPOQ* gene, showed evidence for suggestive linkage with body mass, HDL-cholesterol and triglycerides levels. The D3S3686 marker, located downstream of *ADIPOQ*, also showed linkage with triglycerides levels, suggesting that a genetic variant close to the markers influences this particular trait. Several genome wide scans reported evidence for linkage with the 3q27 locus and anthropometric and metabolic characteristics (Kissebah et al., 2000; Luke et al., 2003; Pollin et al., 2005). Moreover, a genome-wide linkage scan of plasma adiponectin levels in the Amish Family Diabetes Study reported the highest LOD score between markers D3S1602 and D3S1580, which also flank the *ADIPOQ* gene, as in our study (Pollin et al., 2005). Several polymorphisms in *ADIPOQ* have already been associated with circulating adiponectin levels, obesity, insulin resistance and cardiovascular disease risk (Bouatia-Naji et al., 2006; Gable et al., 2007; Hara et al., 2002; Menzaghi et al., 2002; Pollin et al., 2005; Qi et al., 2006; Sutton et al., 2005; Vasseur et al., 2006). However, more type 2 diabetes candidate genes are located in the *ADIPOQ* gene region and accordingly the observed linkage signals might also be the result of genetic variation in one of these other genes. For example, the somatostatin (*SST*) gene which is in the close vicinity of the D3S3686 marker, and the diacylglycerol kinase gamma (*DGKG*) gene or the insulin-like growth factor 2 mRNA binding protein 2 (*IGF2BP2*) gene that are located near the D3S1602 marker.

The D7S2506 marker located in the *IGFBP1* gene region showed suggestive linkage with fat mass, fasting insulin and leptin levels. Until now, just one genome wide linkage scan reported suggestive linkage with this region (D7S1818; LOD = 2.20) and trends in BMI from childhood to adulthood (Chen et al., 2004). Probably as a consequence, only few association studies with *IGFBP1* have been carried out. Ukkola et al. (2001) reported association with an intronic SNP and overfeeding induced changes in abdominal visceral fat, OGTT (oral glucose tolerance test) insulin area and total cholesterol levels in twins, and a coding SNP (A4403G) has been associated with impaired renal function in type 2 diabetes (Stephens et al., 2005). Although *IGFBP1* has not been studied very intensively, our linkage results and the strong phenotypic correlation of IGFBP1 levels with other metabolic risk factors suggest an interesting role of the *IGFBP1* gene in the development of the metabolic syndrome (Heald et al., 2001; Wolk et al., 2004). The fact that we did not observe linkage between the D7S2506 marker and IGFBP1 levels is remarkable. However, in a genome wide linkage analysis using expression phenotypes, more *trans*-acting QTLs than *cis*-acting QTLs (mapped within 5Mb of the target gene) were observed (Morley et al., 2004). This indicates that even gene expression levels are complex traits and transcription regulators are not by definition closely located to the gene. In addition, it should be noted that the *IGFBP1* gene is located closely to the *IGFBP3* gene, and therefore *IGFBP3* could also be responsible for the observed linkage signal.

The D7S530 marker located near the *LEP* gene (7q32.2) showed suggestive linkage with birthweight. Linkage of the 7q32.3 locus with birthweight has, as far as we know, not been reported previously. However, a growth-promoting role for leptin during fetal development has been suggested before (Domali & Messinis, 2002), because umbilical cord leptin levels are positively correlated with birthweight (Schulz et al., 2000). Moreover, we recently showed that two polymorphisms in the leptin receptor (*LEPR*) gene predispose to a higher weight at birth (Souren et al., 2008). Interestingly, in the present study the two microsatellite markers located near the *LEPR* gene showed no linkage with birthweight. To detect a QTL for a complex trait like birthweight, which is controlled by many loci with small effects, association studies are more powerful than linkage studies. Hence, linkage signals are probably not the result of one polymorphism, but represent a joint effect of several (common and rare) variants.

In the literature the *GCK*, *IGF1*, *INSR*, *LEP*, *LEPR*, *PPARγ* and *RETN* gene regions have been associated with type 2 diabetes or traits related to this disease (Rankinen et al., 2006). In the current study we did not detect linkage with traits related to type 2 diabetes and markers located within these gene regions. This does not imply that these regions do not contribute to the development of type 2 diabetes in the current sample, but the studied sample or the contribution of these loci to the traits may be too small to detect them in this linkage study.

We compared two methods when performing univariate single point variance components linkage analysis. The standard method comprised only pheno-

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typic and genotypic data of the DZ twin pairs and is therefore equal to the sib-pair based variance component linkage analysis implemented in MERLIN (Abecasis et al., 2002). In the extended method, which is implemented in Mx (Neale et al., 2002), the phenotypic data of the MZ twin pairs were also incorporated and model fitting was carried out to obtain the best-fitting model. This results in a more accurate estimation of the variance components compared to the standard method. Despite the differences between the two methods used, they gave fairly similar linkage results. However, for fasting insulin, of which the variance is partly explained by nonadditive genetic factors (DE model), LOD scores were remarkably higher using the extended method compared to the standard method (see Table 3 and Table 4). This might be the result of the low DZ twin correlations, because when non-additive genetic factors are present DZ twin correlations are less than half of the MZ twin correlations. In the standard method the size of the genetic component is estimated based on data of DZ twins only, and we observed that the size of the genetic component in the standard method was lower than in the extended method. Our data might suggest that the underestimation of the genetic effect size in the standard method also results in an underestimation of the QTL effect. This indicates, that when nonadditive genetic factors are present the extended method is more powerful than the standard method. However, nonadditive genetic factors also explain part of the variance of IGFBP1 levels and for this trait LOD scores were not consistently higher using the extended method. Moreover, our sample size and the number of microsatellite markers tested are limited, and therefore these results should be interpreted carefully.

We also performed linkage analyses with birth weight. Because MZ MC twins suffer a more adverse intrauterine environment than MZ DC twins, intra-pair correlations for birthweight are significantly lower for MZ MC compared to MZ DC twins. Since the EFPTS has data on chorionicity available, we were able to compare four different methods for birthweight: (1) the standard method, (2) the extended method incorporating only phenotypic data of MZ DC twins, (3) the extended method incorporating only phenotypic data of MZ MC twins, and (4) the extended method in which phenotypic data of both MZ MC and MZ DC were incorporated (see Tables 3 and 4). In general, the four methods gave similar results, except for the D7S530 marker that showed suggestive linkage using the standard method ($LOD_{Sunadjusted}$ = 1.13, $LOD_{Sadjusted}$ = 1.02) and the extended method in which only the phenotypic data of the MZ DC twins were incorporated ($LOD_{E(MZDConly)unadjusted}$ = 1.12, $LOD_{E(MZDConly)adjusted}$ = 0.97). However, LOD scores for the D7S530 marker were considerably lower in the methods incorporating phenotypic data of the MZ MC twins ($LOD_{E(MZMConly)unadjusted}$ = 0.41, $LOD_{E(MZMConly)adjusted}$ = 0.26; $LOD_{Eunadjusted}$ = 0.78, $LOD_{Eadjusted}$ = 0.63). Probably due to the low covariance of the MZ MC twins, the size of the genetic effect and consequently the QTL effect was underestimated. This suggests that including phenotypic data of MZ MC twins results in a reduced power to detect a QTL for birth weight using variance component linkage analyses. In addition, these results indicate that when chorionicity is an important confounder and data on chorionicity is unavailable, variance component linkage analysis should be performed using data of DZ twins only.

Although the sample size and the number of microsatellite markers tested in the present study are limited, our data suggest that genetic variation located in the *ABCC8, ADIPOQ, IGFBP1* and *LEP* gene regions influence anthropometric and metabolic characteristics in the East Flanders twin sample. In addition, our data shows that carrying out sib-pair based variance components linkage analyses by either using advanced modelling in Mx or using the straightforward standard method implemented in the MERLIN package yield similar results, at least when chorionicity is not an important confounder.

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Table 3

Results (LOD Scores) of Univariate Single-Point Variance Components Linkage Analyses Unadjusted for Significant Covariates Performed Using the Standard (S) and the Extended (E) Method

			ABCC8	*ADIPOQ*		*GCK*	*IGF1*	*IGFBP1*	*INSR*	*LEP*	*LEPR*		*PPARγ*	*RETN*
Trait	Method	Model	D11S902	D3S1602	D3S3686	GCK	IGF1	D7S2506	D19S1034	D7S530	D1S2638	D1S198	D3S1263	D19S413
Birth weight	S		0.00	0.00	0.15	0.00	0.00	0.21	0.11	**1.13**	0.00	0.07	0.13	0.52
	$E_{MZDConly}$	ACE	0.00	0.03	0.61	0.00	0.00	0.19	0.01	**1.12**	0.00	0.00	0.27	0.39
	$E_{MZMConly}$	ACE	0.00	0.02	0.31	0.00	0.00	0.11	0.01	0.41	0.00	0.01	0.13	0.30
	E	ACE	0.00	0.03	0.56	0.00	0.00	0.18	0.01	0.78	0.00	0.00	0.24	0.43
Body height	S		0.46	**1.33**	0.67	0.03	0.00	0.00	0.00	0.06	0.00	0.16	0.03	0.00
	E	AE	0.51	**1.17**	0.60	0.06	0.00	0.00	0.00	0.09	0.00	0.10	0.02	0.00
Body mass	S		0.11	**2.07**	0.20	0.00	0.00	0.00	0.00	0.08	0.53	0.00	0.50	0.00
	E	AE	0.15	**1.98**	0.16	0.00	0.00	0.00	0.00	0.04	0.60	0.00	0.32	0.00
BMI	S		0.00	0.47	0.07	0.00	0.00	0.49	0.00	0.11	0.11	0.00	0.41	0.00
	E	AE	0.00	0.56	0.08	0.00	0.00	0.76	0.00	0.20	0.00	0.00	0.62	0.00
WHR	S		**1.02**	0.21	0.19	0.28	0.00	0.00	0.00	0.32	0.10	0.00	0.00	0.00
	E	AE	**1.39**	0.05	0.09	0.23	0.00	0.00	0.00	0.29	0.34	0.00	0.00	0.00
Sum of four skin folds	S		0.00	0.58	0.29	0.00	0.00	**2.03**	0.00	0.00	0.00	0.01	0.00	0.00
	E	AE	0.00	0.53	0.29	0.00	0.00	**2.19**	0.00	0.00	0.00	0.04	0.00	0.00
Fat mass	S		0.00	0.88	0.27	0.00	0.00	**3.64**	0.00	0.00	0.44	0.00	0.54	0.00
	E	AE	0.00	1.00	0.24	0.00	0.00	**3.53**	0.00	0.00	0.22	0.00	0.53	0.00
Lean body mass	S		0.56	**1.56**	0.70	0.00	0.00	0.00	0.00	0.06	0.45	0.21	0.05	0.00
	E	AE	0.61	**1.34**	0.66	0.01	0.00	0.00	0.00	0.06	0.49	0.17	0.03	0.00
IGFBP1	S		0.00	0.03	0.10	0.02	0.25	0.11	0.00	0.00	1.00	0.00	0.01	0.00
	E	DE	0.00	0.03	0.13	0.06	0.25	0.12	0.00	0.04	**1.12**	0.00	0.03	0.04
Fasting insulin	S		0.30	0.00	0.00	0.00	0.00	0.97	0.00	**1.09**	0.59	0.09	0.00	0.00
	E	DE	0.60	0.02	0.04	0.19	0.22	**1.28**	0.03	**1.43**	0.86	0.35	0.00	0.01
Fasting glucose	S		0.00	0.00	0.00	0.11	0.00	0.17	0.92	0.30	0.00	0.00	0.00	0.48
	E	AE	0.00	0.00	0.00	0.12	0.00	0.16	**1.04**	0.32	0.00	0.00	0.00	0.42
Leptin	S		0.00	0.06	0.00	0.01	0.00	0.88	0.01	0.07	0.07	0.39	0.05	0.00
	E	AE	0.00	0.03	0.00	0.04	0.00	**1.21**	0.03	0.04	0.06	0.49	0.05	0.00
Total cholesterol	S		0.05	0.03	0.00	0.00	0.05	0.01	0.18	0.00	0.04	0.07	0.00	0.01
	E	ACE	0.03	0.03	0.00	0.00	0.18	0.00	0.14	0.00	0.13	0.11	0.00	0.04
LDL-cholesterol	S		0.23	0.00	0.00	0.00	0.19	0.00	0.03	0.00	0.00	0.02	0.00	0.00
	E	ACE	0.37	0.00	0.00	0.00	0.70	0.00	0.00	0.00	0.19	0.14	0.00	0.00
HDL-cholesterol	S		0.84	**2.95**	**2.49**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.19
	E	AE	0.92	**2.96**	**2.42**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22
Triglycerides	S		0.00	0.79	0.60	0.49	0.00	0.34	0.04	0.18	0.01	0.00	0.56	0.00
	E	AE	0.00	0.84	0.63	0.41	0.00	0.37	0.04	0.19	0.00	0.00	0.57	0.00
Non-esterified fatty acids	S		0.68	0.06	0.16	0.00	0.44	0.00	0.00	0.00	0.21	0.02	0.00	0.00
	E	AE	0.63	0.04	0.13	0.00	0.38	0.00	0.00	0.00	0.20	0.02	0.00	0.00

Note: Bold and underlined numbers: LOD>1 and LOD<3 suggestive evidence for linkage; LOD>3 significant evidence for linkage. S: standard method, E: extended method, $E_{MZDConly}$: only phenotypic data of MZ DC twins were incorporated, $E_{MZMConly}$: only phenotypic data of MZ MC twins were incorporated.

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Table 4

Results (LOD Scores) of Univariate Single-Point Variance Components Linkage Analyses Adjusted for Significant Covariates Performed Using the Standard (S) and the Extended (E) Method

			ABCC8	*ADIPOQ*		*GCK*	*IGF1*	*IGFBP1*	*INSR*	*LEP*	*LEPR*		*PPARγ*	*RETN*	
Trait	Method	Model	D11S902	D3S1602	D3S3686	GCK	IGF1	D7S2506	D19S1034	D7S530	D1S2638	D1S198	D3S1263	D19S413	Covariates
Birth weight	S		0.00	0.02	0.20	0.04	0.00	0.07	0.03	**1.02**	0.00	0.00	0.32	0.61	Sex, gestational age
	$E_{MZDConly}$	ACE	0.00	0.02	0.23	0.03	0.00	0.06	0.03	0.97	0.00	0.00	0.37	0.57	
	$E_{MZMConly}$	ACE	0.00	0.02	0.13	0.00	0.00	0.07	0.02	0.26	0.00	0.00	0.16	0.35	
	E	ACE	0.00	0.02	0.25	0.01	0.00	0.08	0.03	0.63	0.00	0.00	0.31	0.58	
Body height	S		0.00	0.95	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.67	0.00	Sex, age
	E	AE	0.00	0.86	0.11	0.00	0.00	0.01	0.00	0.00	0.00	0.07	0.66	0.00	
Body mass	S		0.00	**1.59**	0.00	0.00	0.00	0.41	0.00	0.02	0.56	0.00	0.52	0.00	Sex, age, height
	E	AE[a]	0.00	**1.50**	0.01	0.00	0.00	0.29	0.00	0.05	0.24	0.00	0.35	0.00	
BMI	S		0.00	0.47	0.05	0.00	0.00	0.50	0.00	0.16	0.12	0.00	0.41	0.00	Age
	E	AE[a]	0.00	0.50	0.09	0.00	0.00	0.47	0.00	0.42	0.00	0.00	0.33	0.00	
WHR	S		**1.02**	0.01	0.00	0.37	0.00	0.00	0.00	0.59	0.02	0.00	0.03	0.00	Sex, age
	E	AE[a]	**1.09**	0.00	0.00	0.48	0.00	0.00	0.00	0.68	0.09	0.00	0.01	0.00	
Sum of four skin folds	S		0.00	0.53	0.29	0.00	0.00	0.74	0.00	0.00	0.03	0.00	0.00	0.00	Sex, age
	E	AE[a]	0.00	0.71	0.36	0.00	0.00	0.66	0.00	0.00	0.00	0.00	0.00	0.00	
Fat mass	S		0.00	0.67	0.21	0.01	0.02	**1.20**	0.00	0.13	0.18	0.00	0.41	0.00	Sex, age
	E	AE[a]	0.00	0.75	0.20	0.05	0.00	0.85	0.00	0.23	0.12	0.00	0.46	0.00	
Lean body mass	S		0.03	0.97	0.05	0.00	0.00	0.02	0.00	0.04	0.16	0.00	0.02	0.00	Sex, age, height
	E	AE[a]	0.00	0.92	0.00	0.00	0.00	0.14	0.00	0.01	0.27	0.00	0.09	0.00	
IGFBP1	S		0.00	0.02	0.26	0.09	0.08	0.20	0.00	0.00	0.56	0.00	0.01	0.00	Sex, age, BMI
	E	DE[a]	0.00	0.01	0.40	0.09	0.07	0.13	0.00	0.00	0.55	0.00	0.04	0.03	
Fasting insulin	S		0.11	0.05	0.05	0.00	0.00	0.76	0.00	0.29	0.46	0.07	0.00	0.00	Age, S4SF
	E	DE	0.31	0.18	0.28	0.11	0.16	**1.13**	0.07	0.66	0.70	0.31	0.00	0.00	
Fasting glucose	S		0.00	0.00	0.00	0.19	0.00	0.17	0.91	0.10	0.00	0.00	0.05	0.23	Sex, BMI
	E	AE[a]	0.00	0.00	0.00	0.28	0.00	0.08	0.70	0.16	0.00	0.00	0.11	0.09	
Leptin	S		0.01	0.37	0.15	0.02	0.34	**1.54**	0.00	0.00	0.18	0.00	0.00	0.00	Sex, age, S4SF
	E	AE[a]	0.00	0.36	0.22	0.32	0.12	**1.42**	0.00	0.00	0.21	0.00	0.00	0.00	
Total cholesterol	S		0.02	0.00	0.00	0.00	0.28	0.00	0.09	0.00	0.12	0.30	0.00	0.11	Age, S4SF
	E	AE	0.03	0.00	0.00	0.00	0.04	0.00	0.15	0.00	0.00	0.12	0.00	0.04	
LDL-cholesterol	S		0.07	0.00	0.00	0.04	0.43	0.00	0.05	0.00	0.14	0.22	0.00	0.00	Sex, age, S4SF
	E	AE	0.02	0.00	0.00	0.06	0.05	0.00	0.10	0.00	0.00	0.06	0.00	0.00	
HDL-cholesterol	S		0.93	**1.83**	0.57	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.48	Sex, S4SF
	E	AE[a]	**1.09**	**1.88**	0.70	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.47	
Triglycerides	S		0.00	1.00	**1.09**	0.73	0.00	0.45	0.02	0.00	0.00	0.00	0.28	0.00	Sex, S4SF
	E	AE	0.00	**1.15**	**1.42**	0.58	0.00	0.64	0.04	0.00	0.00	0.00	0.17	0.00	
Non-esterified fatty acids	S		0.28	0.16	0.15	0.00	0.27	0.00	0.01	0.00	0.19	0.02	0.00	0.00	Sex, S4SF
	E	AE[a]	0.21	0.05	0.13	0.00	0.27	0.00	0.00	0.00	0.18	0.01	0.00	0.00	

Note: Bold and underlined numbers: LOD > 1 suggestive evidence for linkage. S: standard method, E: extended method, $E_{MZDConly}$: only phenotypic data of MZ DC twins were incorporated, $E_{MZMConly}$: only phenotypic data of MZ MC twins were incorporated. [a] Scalar effect.

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