Genetic analysis of recently identified type 2 diabetes loci in 1,638 unselected patients with type 2 diabetes and 1,858 control participants from a Norwegian population-based cohort (the HUNT study)

J. K. Hertel · S. Johansson · H. Ræder · K. Midthjell ·
V. Lyssenko · L. Groop · A. Molven · P. R. Njølstad

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Abstract

Aims/hypothesis Recent genome-wide association studies performed in selected patients and control participants have provided strong support for several new type 2 diabetes susceptibility loci. To get a better estimation of the true risk conferred by these novel loci, we tested a completely unselected population of type 2 diabetes patients from a Norwegian health survey (the HUNT study).

Methods We genotyped single nucleotide polymorphisms (SNPs) in *PKN2*, *IGFBP2*, *FLJ39370* (also known as *C4ORF32*), *CDKAL1*, *SLC30A8*, *CDKN2B*, *HHEX* and *FTO* using a Norwegian population-based sample of 1,638 patients with type 2 diabetes and 1,858 non-diabetic control participants (the HUNT Study), for all of whom data on

BMI, WHR, cholesterol and triacylglycerol levels were available. We used diabetes, measures of obesity and lipid values as phenotypes in case-control and quantitative association study designs.

Results We replicated the association with type 2 diabetes for rs10811661 in the vicinity of *CDKN2B* (OR 1.20, 95% CI: 1.06–1.37, $p=0.004$), rs9939609 in *FTO* (OR 1.14, 95% CI: 1.04–1.25, $p=0.006$) and rs13266634 in *SLC30A8* (OR 1.20, 95% CI: 1.09–1.33, $p=3.9\times 10^{-4}$). We found borderline significant association for the *IGFBP2* SNP rs4402960 (OR 1.10, 95% CI: 0.99–1.22). Results for the *HHEX* SNP (rs1111875) and the *CDKAL1* SNP (rs7756992) were non-significant, but the magnitude of effect was similar to previous estimates. We found no

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J. K. Hertel · S. Johansson · H. Ræder · P. R. Njølstad
Department of Clinical Medicine, University of Bergen,
Bergen, Norway

J. K. Hertel · S. Johansson
Center for Medical Genetics and Molecular Medicine,
Haukeland University Hospital,
Bergen, Norway

H. Ræder · P. R. Njølstad (✉)
Department of Pediatrics, Haukeland University Hospital,
N-5021 Bergen, Norway
e-mail: pal.njolstad@uib.no

K. Midthjell
HUNT Research Center,
Department of Public Health and General Practice,
Norwegian University of Science and Technology,
Verdal, Norway

V. Lyssenko · L. Groop
Department of Clinical Sciences, Diabetes and Endocrinology
Research Unit, University Hospital Malmö, Lund University,
Malmö, Sweden

L. Groop
Department of Medicine, Helsinki University Hospital,
University of Helsinki,
Helsinki, Finland

A. Molven
The Gade Institute, University of Bergen,
Bergen, Norway

A. Molven
Department of Pathology, Haukeland University Hospital,
Bergen, Norway

support for an association with the less consistently replicated *FLJ39370* or *PKN2* SNPs. In agreement with previous studies, *FTO* was most strongly associated with BMI ($p=8.4\times 10^{-4}$).

Conclusions/interpretation Our data show that SNPs near *IGFBP2*, *CDKAL1*, *SLC30A8*, *CDKN2B*, *HHEX* and *FTO* are also associated with diabetes in non-selected patients with type 2 diabetes.

Keywords Association study · BMI · GWA · Replication · Single nucleotide polymorphism · SNP · Susceptibility gene · Type 2 diabetes

Abbreviations

DGI	Diabetes Genetics Initiative
FUSION	Finland–United States Investigation of NIDDM Genetics
GWA	genome-wide association
HUNT	Nord-Trøndelag Health Study
HWE	Hardy–Weinberg equilibrium
SNP	single nucleotide polymorphism
WTCCC	Wellcome Trust Case Control Consortium

Introduction

The worldwide rise in prevalence of type 2 diabetes has led to an intense search for genetic risk factors of type 2 diabetes. Although environmental influences certainly accelerate disease development in those with genetic predisposition, it is nonetheless of great clinical importance, and indeed a formidable challenge, to elucidate the genetic variants that increase the risk of type 2 diabetes [1]. In complex diseases such as type 2 diabetes, multiple genetic and environmental factors, as well as the interplay between these factors, determine the phenotype. Recently, several studies adopting a genome-wide association (GWA) strategy revealed genomic variants, each conferring a modest risk, to be implicated in type 2 diabetes [2–6]. The aim of the present study was to examine a Norwegian population (the Nord-Trøndelag Health Study [7]) for the association of loci with susceptibility for type 2 diabetes, obesity and lipid measures. Hence, this report describes an attempt to validate and expand results, based on studies adopting a GWA strategy, recently published by Sladek et al. [2], the Diabetes Genetics Initiative (DGI) [3], the Wellcome Trust Case Control Consortium (WTCCC)/UK Type 2 Diabetes Genetics Consortium collection [4], the Finland–United States Investigation of NIDDM Genetics (FUSION) [5] and the previous work by deCODE Genetics [6]. We also aimed to investigate whether a previously reported variant in *FTO* [8–10] was associated with risk for obesity in the Norwegian population.

Methods

Study participants and phenotype definitions The participants were ≥ 20 years of age and drawn from an extensive population-based study (HUNT Study). The survey took place from 1995 to 1997 (HUNT2) in a Norwegian county with 127,000 inhabitants [7]. The county is representative of Norway as a whole with regard to the economy, industry and sources of income, age distribution, morbidity and mortality rates. The population is stable both ethnically and geographically, with less than 3% of people of non-white origin and a net emigration level of 0.3% per year (1996–2000), making it suitable for epidemiological studies. Of the 92,434 individuals considered eligible for the study, the participation rate was 71.3%. The survey methods have been described in detail elsewhere [7, 11]. Diabetic participants were identified through a self-administered questionnaire, which has been shown to be a reliable source of information for epidemiological studies focusing on diabetes mellitus [12].

Genomic DNA was available for 1,850 diabetic participants, for 1,391 of whom more extensive clinical diabetes data were available. Participants with suspected type 1 diabetes were excluded from the study. Inclusion and exclusion criteria for the diabetic participants have been described previously [13]. In total, 1,638 type 2 diabetes participants and 1,858 non-diabetic participants (self-reported) were enrolled in the present study. The control participants were drawn from the same study population.

The study, which was approved by the Regional Committee for Research Ethics and the Norwegian Data Inspectorate, was performed according to the Helsinki Declaration, and all participants gave written informed consent.

Single nucleotide polymorphism selection The selection of single nucleotide polymorphisms (SNPs) prioritised for genotyping in the present study was based on publicly available GWA results as of June 2007, i.e. Sladek et al., the DGI–FUSION–WTCCC collaboration and deCODE Genetics [2–6, 8]. We included only SNPs that were robustly replicated in at least two of the GWA studies, i.e. *IGFBP2*, *CDKAL1*, *SLC30A8*, *CDKN2B*, *HHEX* and *FTO*. Because of the close relation between the Norwegian and Swedish populations, we also included two SNPs in *PKN2* and *FLJ39370* (also known as *C4ORF32*), which had shown rather strong evidence of association in the DGI Scandinavian data set [3], although not in the UK sample sets [4].

Genotyping The genotyping was completed using MassARRAY iPLEX system (Sequenom, San Diego, CA, USA). DNA from both patients and control participants

were mixed on each 384-assay plate along with 20 internal controls and 12 blank samples uniquely distributed within each plate. Genotype concordance rate for the internal controls for the eight markers included was >99.8% ($n=1,703$ genotypes). We genotyped one 384-plate on two separate occasions and found a 100% concordance rate between the two runs ($n=2,878$ duplicate genotypes). We successfully obtained genotypes from 3,496 individuals after removing 41 individuals due to low genotyping (maximum individual missingness rate >0.2), the total genotyping rate being >98% in the remaining individuals.

Statistical analysis Each SNP was assessed for association with type 2 diabetes, BMI, WHR, and cholesterol and triacylglycerol levels using the software package PLINK [14]. The allelic association of each particular SNP with type 2 diabetes was examined using χ^2 analysis. Further, we also applied a logistic regression model with age, sex and BMI as covariates. All SNPs were consistent with Hardy–Weinberg equilibrium (HWE), both in patients and control participants, and none of the markers were excluded on the basis of the HWE test ($p \leq 0.05$). Estimates of the statistical power of our study, given different risk allele frequencies and ORs, are presented in Electronic supplementary material (ESM) Table 1. We had >80% power to detect high-frequency alleles with ORs of 1.15 to 1.20, but only around 40% power if the true ORs were 1.10. These estimates were performed using the Genetic Power Calculator [15].

For association analysis of BMI, WHR, and cholesterol and triacylglycerol levels as quantitative traits, we used a linear regression model using the PLINK software [14] both with and without age, sex and diabetic status as covariates. Before our analyses, we log-transformed triacylglycerol values for each participant in the cohort. Information on lipid-lowering therapy was not available and thus not considered.

Table 1 Clinical characteristics of the HUNT2 type 2 diabetes and control participants

Variable	Type 2 diabetes ($n=1,638$)	Control ($n=1,858$)
Sex (men:women; n)	764:874	880:978
Age at diagnosis (years)	58.4 $\pm$ 15.1	–
Age at examination (years)	68.4 $\pm$ 12.1	56.3 $\pm$ 18.4
BMI (kg/m ²)	29.2 $\pm$ 4.8	26.5 $\pm$ 4.2
Serum cholesterol (mmol/l)	6.2 $\pm$ 1.3	6.1 $\pm$ 1.3
Serum triacylglycerol (mmol/l)	2.5 $\pm$ 1.6	1.9 $\pm$ 1.2
Serum HDL-cholesterol (mmol/l)	1.2 $\pm$ 0.4	1.4 $\pm$ 0.4
Random serum glucose (mmol/l)	9.6 $\pm$ 4.2	5.4 $\pm$ 1.4

Values are means $\pm$ SD

Results for association of BMI with rs9939609 (*FTO*) genotypes were obtained by applying BMI as a continuous trait using a genotypic Cochran–Armitage trend test. These analyses were performed using Stata 8.0 (Stata Corp, College Station, TX, USA). The p values presented in this report were not corrected for the number of tests performed and all CIs are presented as 95% CI.

Results

Results for the association of loci with type 2 diabetes The clinical characteristics of the participants enrolled in the present study are summarised in Table 1. Results for the association study using type 2 diabetes as phenotype are shown in Table 2. We found a significant association with type 2 diabetes for the SNPs rs10811661 in the vicinity of *CDKN2B* (OR 1.20, $p=0.004$), rs9939609 in *FTO* (OR 1.14, $p=0.006$) and rs13266634 in *SLC30A8* (OR 1.20, $p=3.9 \times 10^{-4}$). Interestingly, the *FTO* variant, which was previously shown to be associated with diabetes probably via a primary effect on obesity [8], was still significant after adjustment for BMI (OR 1.14, $p=0.02$).

We found a borderline association with type 2 diabetes for the *IGFBP2* SNP rs4402960 (OR 1.10, $p=0.074$), which persisted also after controlling for cofactors. Furthermore, the *HHEX* SNP rs1111875 (OR 1.06, $p=0.196$) and the *CDKAL1* SNP rs7756992 (OR 1.07, $p=0.192$) showed slightly lower ORs than in previous studies, and adjustment for age, sex and BMI increased the ORs only slightly. We found no association for rs17044137 (OR 1.01, $p=0.910$) and rs6698181 (OR 1.02, $p=0.690$) near *FLJ39370* and *PKN2*, respectively.

It is possible that some SNPs may have a more pronounced effect on type 2 diabetes in obese participants than in non-obese participants. We therefore stratified the patients and control participants on the basis of BMI, generating one group of participants with BMI < 30 kg/m² and one with BMI ≥ 30 kg/m² (ESM Tables 2 and 3). Consistent with the regression-based results (with BMI as cofactor), results were not significantly different between the obese and the non-obese groups. However, the statistical confidence of the association between the *FTO* SNP and type 2 diabetes was further increased (OR 1.27, $p=4.5 \times 10^{-4}$), when performing a case-control study involving obese type 2 diabetes participants and non-obese controls.

Association results for the obesity and lipid measures The results for the association study using BMI, WHR, and cholesterol and triacylglycerol levels as alternative phenotypes are shown in Table 3. The *FTO* rs9939609 was associated with BMI ($p=8.4 \times 10^{-4}$), both for men ($p=5.1 \times$

Table 2 Association results for type 2 diabetes as phenotype in type 2 diabetic and control participants from the HUNT Study

Nearest gene	SNP	Chr	Position ^a	Risk allele ^a	Freq ^b	χ^2 test	OR (95% CI)	<i>p</i> value	Adjusted for age and sex ^c	
									OR (95% CI)	<i>p</i> value
<i>PKN2</i>	rs6698181	1	88,915,893	T	0.3427	0.1587	1.02 (0.92–1.13)	0.690	1.03 (0.92–1.14)	0.646
<i>IGFBP2</i>	rs4402960	3	186,994,381	T	0.3005	3.203	1.10 (0.99–1.22)	0.074	1.07 (0.96–1.19)	0.224
<i>FLJ39370</i>	rs17044137	4	113,014,746	A	0.2278	0.0127	1.01 (0.90–1.12)	0.910	1.04 (0.92–1.17)	0.520
<i>CDKALI</i>	rs7756992	6	20,787,688	G	0.2718	1.702	1.07 (0.97–1.19)	0.192	1.08 (0.97–1.20)	0.181
<i>SLC30A8</i>	rs13266634	8	118,253,964	C	0.6902	12.56	1.20 (1.09–1.33)	3.9×10^{-4}	1.18 (1.08–1.26)	7.0×10^{-4}
<i>CDKN2B</i>	rs10811661	9	22,124,094	T	0.8235	8.198	1.20 (1.06–1.37)	0.004	1.13 (1.00–1.25)	0.048
<i>HHEX</i>	rs1111875	10	94,452,862	C	0.5614	1.671	1.06 (0.97–1.18)	0.196	1.09 (0.99–1.18)	0.078
<i>FTO</i>	rs9939609	16	52,378,028	A	0.4267	7.58	1.14 (1.04–1.25)	0.006	1.19 (1.07–1.31)	0.001
									1.14 (1.03–1.28)	0.015

^a Positions and alleles are indexed from the forward strand of the human reference sequence NCBI Build 36.2^b Frequency (Freq) of risk allele based on controls in the HUNT study^c Logistic regression model

Chr, chromosome

Table 3 Quantitative trait association results (*p* values) of BMI, WHR, cholesterol and log triacylglycerol levels as alternative phenotypes in HUNT

Nearest gene	SNP	Chr	Position	BMI	WHR			Triacylglycerol			Cholesterol		
					Unadj ^a	Adj ^b	Adj ^c	Unadj ^a	Adj ^b	Adj ^c	Unadj ^a	Adj ^b	Adj ^c
<i>PKN2</i>	rs6698181	1	88,915,893	0.136	0.145	0.110	0.597	0.352	0.281	0.518	0.786	0.830	0.848
<i>IGFBP2</i>	rs4402960	3	186,994,381	0.555	0.298	0.162	0.219	0.396	0.227	0.579	0.764	0.233	0.267
<i>FLJ39370</i>	rs17044137	4	113,014,746	0.637	0.749	0.615	0.774	0.971	0.840	0.192	0.379	0.639	0.667
<i>CDKALI</i>	rs7756992	6	20,787,688	0.846	0.828	0.818	0.735	0.769	0.453	0.143	0.852	0.949	0.882
<i>SLC30A8</i>	rs13266634	8	118,253,964	0.185	0.338	0.927	0.502	0.879	0.338	0.701	0.689	0.781	0.947
<i>CDKN2B</i>	rs10811661	9	22,124,094	0.524	0.717	0.906	0.001	0.009	0.023	0.072	0.035	0.143	0.112
<i>HHEX</i>	rs1111875	10	94,452,862	0.671	0.640	0.978	0.919	0.667	0.926	0.127	0.314	0.474	0.546
<i>FTO</i>	rs9939609	16	52,378,028	8.4×10^{-4}	8.4×10^{-4}	0.010	0.387	0.035	0.179	6.4×10^{-4}	0.904	0.939	0.899

^a Without adjustment for covariates^b Adjusted for age and sex^c Adjusted for age, sex and diabetic status

Adj, adjusted; Chr, chromosome; Unadj, unadjusted

10^{-4}) and women ($p=0.04$; ESM Table 4). We also demonstrated a strong association with triacylglycerol levels for the *FTO* SNP ($p=1.4\times 10^{-4}$), which remained after controlling for age, sex and diabetes status.

Both BMI and WHR have been shown to be associated with type 2 diabetes, but from a clinical perspective, central obesity is suggested to generate a stronger type 2 diabetic risk than general obesity (BMI). Interestingly, the strong association with obesity shown for the *FTO* SNP using BMI as phenotype could not be demonstrated using WHR as a quantitative trait for obesity (Table 3). However, the SNP in the vicinity of *CDKN2B* indicated association with WHR and also a nominal association with cholesterol. The SNPs in or near *PKN2*, *IGFBP2*, *FLJ39370*, *CDKAL1*, *SLC30A8* and *HHEX* showed no association with quantitative metabolic traits in our study (Table 3).

Discussion

This is the first replication study of the recently identified type 2 diabetes risk variants using a large population-based body of material. The HUNT samples have been validated by genotyping of known type 2 diabetes risk variants in *TCF7L2* and *KCNJ11*. We found a significant association with type 2 diabetes for the SNPs rs7903146 and rs12255372 in *TCF7L2* and rs5219 in *KCNJ11*, similar to recent data (P. Thorsby, unpublished results) and with an OR similar to other studies [16, 17], indicating that the HUNT population contains a representative diabetes cohort and that our genotyping strategy was robust.

In the present study, we confirmed the diabetes association at the *SLC30A8* locus. Our data were less compelling with regard to the SNPs tested near *IGFBP2*, *HHEX* and *CDKAL1*. However, there was a trend in the same direction and of the same magnitude as in previous reports [2–6]. These genetic variants have been confirmed to be associated with type 2 diabetes [3–6], although in some cases with modest evidence in the initial stages and strengthened evidence only in combined analyses [5]. Our findings probably reflect the possibility that the associations are stronger in certain subgroups and that very large sample sizes are needed to formally replicate the *IGFBP2*, *HHEX* and *CDKAL1* data. It should also be emphasised that the risk variants have not been fine-mapped and that even subtle differences between different populations might affect linkage disequilibrium between test and disease variants.

We were able to confirm that the rs10811661 located on chromosome 9p, 125 kb from the nearest gene *CDKN2B*, is associated with type 2 diabetes risk [3–5]. SNPs near this variant are associated with coronary artery disease [18–20].

In this regard it is interesting that we obtained evidence of nominal association between rs10811661 and both WHR and cholesterol levels. Thus, extensive studies of the region on chromosome 9 may provide more insight into the connections between type 2 diabetes and cardiovascular diseases.

The DGI defined two loci, *FLJ39370* and *PKN2*, as interesting for follow-up studies [3], whereas the WTCCC and FUSION studies showed conflicting results [4, 5]. We were not able to detect any association between type 2 diabetes and *FLJ39370* or *PKN2* candidate SNPs in the Norwegian sample, which is supposed to be genetically closely related to other Scandinavian populations. Hence, we suggest that these two SNPs probably represent false positive results in the DGI whole-genome scan.

As part of the WTCCC, Frayling et al. [8] reported that SNPs in the *FTO* gene region were highly associated with type 2 diabetes and BMI, suggesting that the *FTO* locus exerts its primary effect on adiposity and that it subsequently has an impact on type 2 diabetes [8]. In our study, we replicated the association between *FTO* and both an increased type 2 diabetes risk (OR 1.14 and $p=0.006$) and BMI ($p=8.4\times 10^{-4}$; Table 3). Interestingly, the association between rs9939609 and both type 2 diabetes and BMI remained significant after adjustment for BMI and diabetes status, respectively. It is also noteworthy that rs9939609 demonstrated a strong association with triacylglycerol levels ($p=1.4\times 10^{-4}$), which was not abolished after correcting for diabetes status. Thus, our data suggest that the relation between *FTO* and both BMI and diabetes is more complex than initially thought. Our results indicate that carriers of two A alleles at rs9939609 are at greater risk of being overweight, confirming results reported by Frayling et al. [8].

In contrast, using WHR as a quantitative measure of obesity, *FTO* showed only a nominal association after adjustment for sex and age, and no association when corrected for diabetes status. Moreover, the variant near *CDKN2B*, for which we observed no association with BMI, showed evidence of a nominal association with WHR. However, these results should be regarded as explorative; indeed, the lack of association between *FTO* and WHR in our sample could also be due to limited power. Larger studies are therefore needed to determine the role of *FTO* in central obesity.

Our study has both strengths and limitations. The HUNT study includes a well-characterised population from a clearly defined region of Norway where participants were recruited without regard to disease status and where the controls were drawn from the same population. Thus, there was no selection bias that could arise when conducting genetic studies [21]. Although we did not have the opportunity to formally test for possible population sub-

structures with the limited numbers of markers genotyped, we believe that this population is more, rather than less homogeneous than other type 2 diabetes sample collections. Furthermore, the allele frequencies and the size effects are similar to previous publications, arguing against problems with population stratification. As a replication study, the sample size of ~3,500 patients and control participants was only powered to detect these relatively small ORs at a nominal significance. We argue that it is not necessary to correct for multiple comparisons when using diabetes as a trait, since this could be considered a pure replication study. However, for the additional phenotypic traits, results are more explorative and further studies are needed to address whether the observed additional associations with WHR and triacylglycerol levels represent true effects or spurious associations. Another point to note is that we tested only those SNPs that had been used in the initial reports [2–6, 8]. Hence, another explanation for the lack of statistical support for some of the best GWA loci may be that the most highly associated SNPs vary from one population to another.

The whole-genome scan, in combination with large data sets, has recently shown its promise and delivered a whole set of new susceptibility loci for type 2 diabetes. The HUNT study with its unselected population may provide important insights, as it confirms most, but not all, previously identified loci associated with type 2 diabetes. Our results show that these findings can be generalised to a completely unselected population such as the Norwegian HUNT study (ESM Table 5). Importantly, the size effects that we observed are very similar to the estimates from the previous studies, indicating that the design of the first round of the whole-genome scans seems to pinpoint risk-alleles that show generality, at least in other Northern European populations. It is, nevertheless, crucial to continue to perform in-depth follow-up studies for these and future susceptibility loci in unselected samples of patients and control participants, since inclusion criteria based on age of onset, family history of the trait and BMI may affect the type of loci detected. Although speculative at this early stage, most loci detected so far seem to primarily affect insulin release rather than insulin resistance [22, 23]. However, before making such statements, researchers need to achieve a better understanding of how variation in the function of these loci leads to clinical disease.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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