FAX

Bestelldatum: **2014-01-03 10:46:49**

Bayerische StaatsBibliothek

NORMAL

Kopie

SUBITO-VF14010300379

Helmholtz Zentrum Muenchen - Dt.
Forschungszentrum f Umwelt
Zentralbibliothek / Fernleihe
Herr Rasso Ranzinger
Postfach: 1129
85758 Oberschleissheim

Ben.-Gruppe: USER-GROUP-4
Tel: +49 89 31872343
Mail: library@helmholtz-muenchen.de

Fax: +49 89 3187172484

Subito-Kundennummer:
SLS02X00668
Subito-Bestellnummer:
SUBITO-VF14010300379

Z 90.1364 Hbzs 196-31 a = Neueste Hefte

Jahrgang: 2013

Band/Heft: 23/4

Seiten: 143-152

Verfasser: Tozzi, F; Teumer, A; MunafA , M; Rawal,

Titel: A genomewide association study of smoking relapse in four European population-based samples.

Psychiatric genetics
ISSN: 0955-8829

Bemerkung: Paulini

Beschreibung:

Die Abrechnung dieser Lieferung erfolgt über die subito-Zentralregulierung

Bei Rückfragen wenden Sie sich bitte innerhalb von 10 Tagen an die Bayerische Staatsbibliothek, Direktlieferdienste Tel. ++49 89 28 638-26 43, doklief@bsb-muenchen.de

A genomewide association study of smoking relapse in four European population-based samples

Federica Tozzi[a,b*], Alexander Teumer[g*], Marcus Munafò[m*], Rajesh Rawal[e*], Gbenga Kazeem[l], Marcel Gerbaulet[f], Wendy McArdle[n], Howard Chilcoat[c], Angela Döring[e], Norbert Dahmen[f], Vincent Mooser[d], Matthias Nauck[h], Susan M Ring[n], Justin P. Rubio[l], Peter Vollenweider[o], Gérard Waeber[o], Ulrich John[i], Henry Völzke[j], Georg Homuth[g], Harald J. Freyberger[k], Uwe Völker[g], George Davey-Smith[n], Christian Gieger[e], Martin Preisig[p] and Hans J. Grabe[k]

Objectives **Genomewide association studies (GWAS) have identified clear evidence of genetic markers for nicotine dependence. Other smoking phenotypes have been tested, but the results are less consistent. The tendency to relapse versus the ability to maintain long-term abstinence has received little attention in genetic studies; thus, our aim was to provide a better biological understanding of this phenotype through the identification of genetic loci associated with smoking relapse.**

Methods **We carried out a GWAS on data from two European population-based collections, including a total of 835 cases (relapsers) and 990 controls (abstainers). Top-ranked findings from the discovery phase were tested for replication in two additional independent European population-based cohorts.**

Results **Of the seven top markers from the discovery phase, none were consistently associated with smoking relapse across all samples and none reached genomewide significance. A single-nucleotide polymorphism rs1008509, within the *Xylosyltransferase II* (*XYLT2*) gene, was suggestively associated with smoking relapse in the discovery phase ($\beta = -0.504$; $P = 5.6E-06$) and in the first replication sample (ALSPAC) ($\beta = -0.27$; $P = 0.004$; $n = 1932$), but not in the second sample (KORA) ($\beta = 0.19$; $P = 0.138$; $n = 912$). We failed to identify an association between loci implicated previously in other smoking phenotypes and smoking relapse.**

Conclusion **Although no genomewide significant findings emerged from this study, we found that loci implicated in other smoking phenotypes were not associated with smoking relapse, which suggests that the neurobiology of smoking relapse and long-term abstinence may be distinct from biological mechanisms implicated in the development of nicotine dependence.** ***Psychiatr Genet*** **23:143–152 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Psychiatric Genetics 2013, **23**:143–152

Keywords: ALSPAC, genetics, KORA, PsyCoLaus, SHIP, smoking

[a]Genetics Division, Quantitative Sciences, GlaxoSmithKline R&D, Verona, Italy, [b]Department of Psychiatry, School of Medicine, University of North Carolina, Chapel Hill, [c]Worldwide Epidemiology, Quantitative Sciences, GlaxoSmithKline, Research Triangle Park, North Carolina, [d]Genetics Division, GlaxoSmithKline R&D, Upper Merion, Pennsylvania, USA, [e]German Research Center for Environmental Health, Institute of Epidemiology and Helmholtz Zentrum München, Neuherberg, [f]Department of Psychiatry and Psychotherapy, University of Mainz, Mainz, [g]Interfaculty Institute for Genetics and Functional Genomics, [h]Institute of Clinical Chemistry and Laboratory Medicine, [i]Institute of Epidemiology and Social Medicine, [j]Institute for Community Medicine, University of Greifswald, Greifswald, [k]Department of Psychiatry and Psychotherapy, University Medicine Greifswald, HELIOS-Hospital Stralsund, Stralsund, Germany, [l]Genetics Division, Quantitative Sciences, GlaxoSmithKline R&D, Stevenage, [m]School of Experimental Psychology, [n]MRC CAiTE, School of Social and Community Medicine, University of Bristol, Bristol, UK, Departments of [o]Internal Medicine and [p]Psychiatry, University Hospital Center and University of Lausanne, Lausanne, Switzerland

Correspondence to Hans J. Grabe, MD, Department of Psychiatry and Psychotherapy, University Medicine Greifswald, HELIOS-Hospital Stralsund, Rostocker Chaussee 70, 18437 Stralsund, Germany
Tel: +49 3831/45 2106; fax: +49 3831/45 2105;
e-mail: grabeh@uni-greifswald.de

*Federica Tozzi, Alexander Teumer, Marcus Munafò and Rajesh Rawal contributed equally to the writing of this article.

Received 23 September 2011 **Revised** 8 October 2012
Accepted 27 October 2012

Introduction

Smoking is highly prevalent and is one of the leading causes of morbidity and mortality worldwide (Murray, 2006). Pharmacological interventions currently available for the treatment of nicotine addiction are only moderately efficacious. Long-term success rates are low, and the efficacy of available drugs is mostly related to smoking cessation (initiating abstinence and sustaining it in the short term), with no proven efficacy for relapse prevention. Rates of smoking relapse after attempts to quit are high (Garvey *et al.*, 1992; Hughes *et al.*, 2004), and achievement of long-term abstinence remains the main challenge for emerging new treatments.

There is evidence for genetic influences on smoking behaviours, with a possible increasing genetic influence

from smoking initiation to downstream smoking phenotypes such as persistence and abstinence (Li *et al.*, 2003). Twin study data indicate that smoking initiation, nicotine dependence (ND), smoking persistence and ability to successfully abstain from smoking all show considerable heritability (Sullivan and Kendler, 1999; Lessov *et al.*, 2004; Maes *et al.*, 2004; Broms *et al.*, 2006; Lessov-Schlaggar *et al.*, 2006). It has been suggested that there is a modest overlap with genes identified in genomewide association studies (GWAS) of dependence on addictive substances and the ability to successfully abstain from smoking (Conti *et al.*, 2008; Uhl *et al.*, 2008). Thus, the mechanisms implicated in smoking relapse may differ from those implicated in the transition to addiction. Recent GWAS have identified clear and consistent evidence of genetic markers for smoking quantity and ND (Liu *et al.*, 2010; Thorgeirsson *et al.*, 2010; Tobacco and Genetics Consortium, 2010). The strongest association for the smoking quantity phenotype was underpinned by variants near the *CHRNA3* and *CHRNA5* genes on chromosome 15q25, which was also associated previously with the ND phenotype (Saccone *et al.*, 2007; Thorgeirsson *et al.*, 2008). Other smoking phenotypes have been tested, including smoking cessation, but the results are less consistent. Furthermore, the most significant single-nucleotide polymorphisms (SNPs) (rs1051730) within the *CHRNA3* gene, associated with ND/smoking quantity, have shown no association with other smoking phenotypes, such as willingness, attempt or preparation to quit (Marques-Vidal *et al.*, 2011).

Here, we report the results from GWAS and meta-analysis carried out in a total of 4744 individuals from four independent case–control samples selected from four European population-based cohorts, focusing on a smoking phenotype that, to the best of our knowledge, has not been studied previously: smoking relapse versus long-term abstinence. Understanding the complex genetics that underlie the smoking relapse phenotype holds the most promise for the development of biological treatments for nicotine addiction.

Methods

Participants

The present study was carried out on four independent European collections. The case–control discovery samples were derived from a population-based study from Lausanne (PsyCoLaus, Switzerland) and from the Study of Health in Pomerania (SHIP, Germany). The replication sets were derived from the Avon Longitudinal Study of Parents and Children (ALSPAC, UK) and from the Cooperative Health Research in the Region of Augsburg (KORA, Germany).

Discovery set

Sample I: PsyCoLaus

A total of 645 cases (relapsers) and 338 controls (abstainers) were selected from a population-based collection (PsyCoLaus), which included 3713 individuals, between 35 and 66 years of age, who were residents of the City of Lausanne (Switzerland). A detailed description of recruitment procedures and assessments has been provided by Firmann *et al.* (2008) and Preisig *et al.* (2009). In brief, a community survey (CoLaus) was conducted of 6738 individuals randomly selected from the list of residents in the city of Lausanne (Switzerland) between 2003 and 2006. DNA and plasma samples were collected for the study of genetic variants and biomarkers. Between 2004 and 2008, all 35-year-old to 66-year-old individuals of the CoLaus sample were invited to participate in a psychiatric sub-study (PsyCoLaus). Sixty-seven per cent of them accepted the comprehensive psychiatric evaluation. The psychiatric evaluation was carried out through a semistructured interview, which allows for the establishment of the lifetime and 12-month prevalence of DSM-IV (American Psychiatric Association, 1994) mental disorders, the Diagnostic Interview for Genetic Studies (DIGS) (Nurnberger *et al.*, 1994). A specific chapter of the DIGS assessed nicotine addiction. The mean age of the participants was 50.94 (SD 8.8 years). Fifty-three per cent of them were women. Only individuals of European origin were considered for genetic analysis. The Institutional Ethics Committee of the University of Lausanne approved both the CoLaus and the PsyCoLaus studies. All participants provided written informed consent after having received a detailed description of the goal and funding of the study.

Sample II: SHIP

A total of 190 cases (relapsers) and 652 abstainers were identified from the SHIP. SHIP was a cross-sectional survey in West Pomerania, the north-east area of Germany (John *et al.*, 2001; Volzke *et al.*, 2010). A sample from the population aged 20–79 years was drawn from population registries. First, the three cities of the region (with 17 076 to 65 977 inhabitants) and the 12 towns (with 1516 to 3044 inhabitants) were selected, and then 17 out of 97 smaller towns (with <1500 inhabitants) were drawn at random. Second, from each of the selected communities, individuals were drawn at random, proportional to the population size of each community and stratified by age and sex. Only individuals with German citizenship and main residency in the study area were included. Finally, 7008 individuals were sampled, with 292 individuals of each sex in each of the twelve 5-year age strata. To minimize dropouts by migration or death, participants were selected in two waves. The net sample (without migrated or deceased individuals) included 6267 eligible participants. Selected participants received a maximum of three written invitations. In case of nonresponse, letters were followed by a telephone call or by home visits if contact by telephone was not possible. The SHIP population finally comprised 4308 participants (corresponding to a final response of 68.8%). All participants

signed a written informed consent after having received a detailed description of the goal and funding of the study. SHIP was approved by the local Institutional Review Board and conformed to the principles of the Declaration of Helsinki.

Replication set

Sample III: ALSPAC

A total of 1451 cases (relapsers) and 547 abstainers were identified from the ALSPAC (Golding *et al.*, 2001), a prospective study that recruited pregnant women of European ancestry from Bristol, UK, with expected delivery dates between April 1991 and December 1992. Cigarette smoking behaviour of women before and during pregnancy was determined from questionnaires. A questionnaire was administered in the 18th gestational week, asking about lifetime, prepregnancy and first trimester smoking behaviour (whether or not the woman smoked and, for smokers, the quantity of cigarettes per day). Data on known covariates of smoking behaviour (Lu *et al.*, 2001) were also collected by a questionnaire, including age, age at smoking initiation, socioeconomic status, educational level, parity and partner's smoking status. There were 6227 pregnant women of European ancestry on whom data on genotype and smoking status immediately before pregnancy were available. A total of 1998 women reported smoking immediately before pregnancy, of whom 547 reported not smoking in the first trimester. All women provided written informed consent and ethical approval was obtained from the ALSPAC Law and Ethics Committee and the Local Research Ethics Committee.

Sample IV: KORA

A total of 483 cases (relapsers) and 438 abstainers were identified from the KORA S3 study. The study population for genetic analyses was recruited from the KORA S3 survey. It is an independent population-based sample from the general population living in the region of Augsburg, Southern Germany, and was examined in 1994/1995. All participants were of European descent. The standardized examinations applied have been described in detail elsewhere (Lowel *et al.*, 2005; Wichmann *et al.*, 2005). A total of 3006 individuals participated in a follow-up examination in 2004/05 (KORA F3). From the KORA F3 survey, 2978 individuals between 35 and 79 years of age were selected for genotyping in this study. All participants provided written informed consent after a detailed description and explanation of the study. The study was approved by the local ethical committee.

Phenotype definition

Definitions of 'relapser' and 'abstainer' were based on data available in the literature and on analysis carried out on the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) (data not shown).

A first criterion was applied to exclude occasional and light smokers: both cases and controls had to be past or current smokers and declared to smoke/have smoked at least 10 cigarettes per day, when regular smokers.

Abstainers (controls) were defined as those who smoked daily at least for 2 years and have declared at least 3 years of abstinence. The rationales for these criteria are based on a series of findings:

(1) Analysis of the NESARC data shows that the risk of remission from ND is the highest in the first year of smoking (5%) and constant over time after the first year (Chilcoat and Webb, 2007). Thus, selecting individuals who have smoked for at least 2 years decreases the variability relative to their probability to achieve remission from ND, but they are likely to vary with respect to their ability to achieve sustained abstinence after quitting attempt.
(2) Available data indicate that the wide majority of individuals who quit smoking and abstain for 3 years will remain abstinent (true abstainers). Findings from the US Surgeon General's report (1990) suggest that the rates of relapse level off after about 3 years of abstinence (Hughes *et al.*, 2008). Other studies, and analysis on the NESARC sample (data not shown), show the rate of relapse decreasing to around 5% after more than 2 years of abstinence (Hughes *et al.*, 2004; Segan *et al.*, 2006; Herd *et al.*, 2009).

Relapsers (cases) were defined as those who were daily smokers, who have smoked for at least 5 years and declared that they have attempted to quit smoking unsuccessfully. This means that both cases and controls had at least 5 years since onset of daily smoking.

This phenotype definition was applied to the PsyCoLaus, SHIP and KORA collections to obtain the cases and controls included in the current analysis. It was not possible to apply the exact phenotype definition for the ALSPAC sample.

For the ALSPAC replication sample, all pregnant women who reported smoking immediately before pregnancy ($n = 1998$) were selected. It is standard practice in the UK for health professionals to advise pregnant women to stop smoking during pregnancy. Indeed, 547 women reported to have stopped smoking in the first trimester and were classified as abstainers (controls). However, 1451 pregnant women were still smoking during their first trimester of pregnancy and were defined as 'relapsers' (cases). As no direct information on the true intention to stop smoking was assessed, our definition relies on the assumption that the majority of pregnant women seriously consider stopping smoking during pregnancy, given the considerable social and medical pressures to do so.

Sample preparation, genotyping and quality control

Discovery set

Sample I: PsyCoLaus

Genomewide SNP genotyping was performed on ~6000 samples for the CoLaus study, of which PsyCoLaus is a subset, using the Affymetrix 500K SNP chip (Affymetrix Inc., Santa Clara, California, USA). A total of 336 samples were excluded from the study as they either had a call rate less than 90% or had gender inconsistencies. Marker quality control (QC) performed also resulted in the exclusion of markers that were monomorphic (4052), had a call rate less than 95% (30 873), deviated significantly ($P < 10^{-3}$) from Hardy–Weinberg equilibrium (35 417) or had minor allele frequencies less than 0.01 (61 654), leaving a total number of 370 162 genotyped markers for analysis. Imputation of SNP genotypes was performed using the software IMPUTE v0.5.0 (University of Oxford, Oxford, UK) (Marchini *et al.*, 2007) based on HapMap CEU II genotypes. Approximately 2.5 million markers were imputed and analysed for the association with the smoking relapse genotype.

Sample II: SHIP

The SHIP samples were genotyped using the Affymetrix Human SNP Array 6.0 (Affymetrix Inc.). The SHIP DNA for genotyping was prepared at the Greifswald University and genotyping was performed at Affymetrix Inc. Genotypes were determined using the Birdseed2 clustering algorithm (Korn *et al.*, 2008). For QC purposes, several HapMap CEPH trio families were added as control samples, allowing to calculate experimental reproducibility, trio accuracy and HapMap concordance. At the chip level, only individuals with a genotyping call rate on QC probesets (QC call rate) of at least 86% were included. Finally, all arrays had a sample call rate greater than 92%. The overall genotyping efficiency of the samples was 98.55%. Imputation of genotypes in the SHIP cohort was performed using the software IMPUTE v0.5.0 based on HapMap CEU II genotypes. The genetic data analysis workflow was created using the Software InforSense. Genetic data were stored using the database Caché (InterSystems GmbH, Darmstadt, Germany).

Replication set

Sample III: ALSPAC

For top SNP markers from the discovery phase, genotyping was performed by KBiosciences (Hoddesdon, UK; *http://www.kbioscience.co.uk*), using their own system of fluorescence-based competitive allele-specific PCR (KASPar). The genotyping call rate was greater than 94%. Duplicate samples (7%) were genotyped for QC purposes. Concordance between duplicate samples was greater than 99%. There was no evidence of deviation from Hardy–Weinberg equilibrium ($P \geq 0.01$).

Sample IV: KORA

In total, 21 SNPs, which were identified from regions with the lowest *P*-value in the meta-analysis, were genotyped with the MassARRAY system using the iPLEX technology (Sequenom Inc., San Diego, California, USA) in all 'relapsers' and 'abstainers' in KORA S3 ($n = 2978$). The call rate of all SNPs was greater than 92%.

Statistical methods

Genomewide association analysis

Logistic regression was used to assess the association between each genetic marker and the smoking relapse phenotype, assuming an additive genetic model and adjusting for age and sex as covariates. Before analysis, the degree of identity by descent (IBD) sharing between all pairs of participants was estimated using PLINK analysis software (Purcell *et al.*, 2007), leading to the exclusion of one individual. The analyses were carried out using the PLINK software and QUICKTEST (v 0.94; *http://toby.freeshell.org/software/quicktest.shtml*). The principal component analysis approach was used to assess population stratification (Price *et al.*, 2006). The Genomic Control (GC) method was also used to correct for any presence of population stratification. Linkage disequilibrium analyses were also carried out for the top markers identified. All top markers identified were in Hardy–Weinberg equilibrium in all cohorts.

Meta-analysis: sample I and sample II GWAS

A meta-analysis of association signals obtained from each of the discovery GWAS samples (PsyCoLaus and SHIP) was carried out. The meta-analysis was carried out using a fixed-effect inverse-variance weighted model (Normand, 1999). Both studies were GC-corrected before meta-analysis. As the overall lambdaGC of the meta-results was 0.987, the uncorrected (*P*-value) and the corrected (pvalGC) *P*-values were the same. No significant heterogeneity was observed between the individual cohorts for the top SNPs identified in the discovery sample.

Replication analysis: sample III and IV and selection of SNPs

This study had funding for de-novo genotyping of nine SNPs. For the three loci with *P*-values less than 10^{-6}, two SNPs each were chosen: the SNPs with the smallest *P*-value and the one with the second smallest as backup. The remaining SNPs were selected using the one with the smallest *P*-value from each of the other three loci having association *P*-values less than 5×10^{-6}. For two of the three backup SNPs, genotyping failed, which resulted in seven successfully genotyped SNPs. Thus, the SNP with the smallest association *P*-value of each of the six top loci with *P*-values less than 5×10^{-6} was available for replication analysis.

For the ALSPAC replication sample, the association between smoking relapse and the seven top risk-associated

polymorphisms identified in the discovery phase was assessed. Logistic regression analysis, adjusting for age and sex as covariates, was carried out to assess the association between each dichotomized variable and the risk polymorphisms.

For the KORA sample, analyses were carried out using SNPTEST software (*https://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html*).

Meta-analysis of the discovery and replicated samples

The results obtained from the discovery and replicated sets were combined together in a meta-analysis using the fixed-effect inverse-variance weighted analysis approach. Heterogeneity tests between the discovery and the replicated sets were also assessed.

Results

The demographic and main clinical characteristics of the four samples ($n = 4744$) are shown in Table 1.

For the discovery phase, we carried out association analyses separately within each cohort (PsyCoLaus and SHIP) under an additive genetic model adjusting for age and sex as covariates. The results from the two samples were then combined in a meta-analysis using the fixed-effect inverse-variance weighted model. A Manhattan plot showing genomewide association for the discovery phase is shown in Fig. 1.

We followed up our most promising association findings (lead SNPs from the top seven most associated genomic regions from the discovery phase meta-analysis) in the replications sets. No between-sample heterogeneity with respect to the effect sizes was observed in the discovery set for the seven top SNPs ($I^2 = 0$, heterogeneity $P > 0.05$), although the allele frequencies of some of these loci varied considerably between the GWAS samples. The allele frequencies in the two replication samples were quite similar. Therefore, we cannot exclude the possibility that at least some of the observed heterogeneity in the allele frequencies could be related to the imputation of the genotypes in the discovery samples. Association results for these seven top SNPs from the two discovery samples, two replication sets and the combined samples are reported in Table 2. The Q–Qplot obtained for the GC-corrected *P*-values from the meta-analysis of the discovery samples is shown in Fig. 2.

None of the seven top findings from the discovery phase could be replicated in both replication samples, and none reached the threshold for genomewide significance in the combined sample ($n = 4744$), which is based on a fixed-effect inverse-variance weighted model (Normand, 1999). Achieving a nominally significant association with a consistent direction of effect in both discovery samples ($\beta = 0.504$; P combined = 5.64E−06) and the ALSPAC replication sample ($\beta = -0.27$; $P = 0.004$; $n = 1932$), one SNP, rs1008509, within the *Xylosyltransferase II* (*XYLT2*) gene was not associated with smoking relapse in the second replication sample (KORA) ($\beta = 0.19$; $P = 0.138$; $n = 912$). We also queried our GWAS meta-analysis results from the discovery samples for a panel of SNPs within candidate genes that were associated with smoking phenotypes in three recently published GWAS (Liu *et al.*, 2010; Thorgeirsson *et al.*, 2010; Tobacco and Genetics Consortium, 2010). Except for rs1028936 near *PPP1R3C* ($P = 0.02$), none of these candidate SNPs reached nominal significance in our results (Table 3).

Discussion

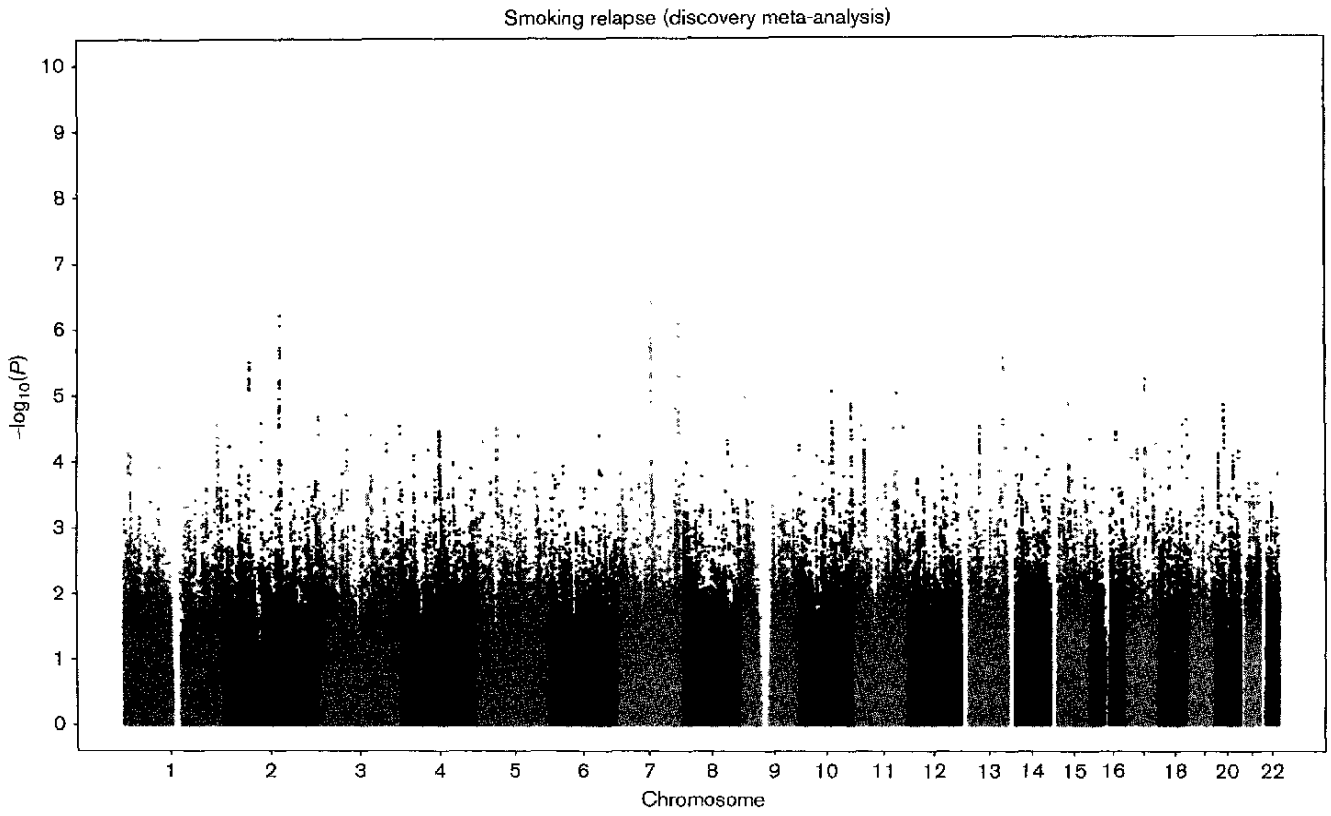
Using an unbiased genomewide approach involving meta-analysis of findings across four independent European samples, we have failed to identify any convincing genetic associations with the clinically relevant phenotype of smoking relapse. Further, we did not find SNPs associated with other smoking phenotypes to be associated with smoking relapse, which may suggest alternative biological mechanisms of pathogenesis.

Table 1 Demographics and main clinical features of the four samples, totalling 2769 cases (relapsers) and 1975 controls (abstainers)

	Sample size	Sex: women (%)	Age at interview	Age of smoking onset (mean±SD) (years)	CPD (mean±SD)
Sample I – PsyCoLaus (Affymetrix 500K)					
Cases	645	326 (51)	49.59±8.52	18.3±4	22.15±10.4
Controls	338	149 (44)	52.06± 8.58	18.7±4	20.65±10.7
Sample II – SHIP (Affymetrix 6.0)					
Cases	190	114 (60)	40.4±13	18.0±4	16.7±6.8
Controls	652	528 (81)	57.3±14	18.5±5	19.1±9.5
Sample III – ALSPAC					
Cases	1451	1451 (100)	26.6±5	16.0±3	14.6±7
Controls	547	547 (100)	27.2±5	16.9±3	8.0±7
Sample IV – KORA (Affymetrix 500K)					
Cases	483	205 (42.4)	41.5±11	17.7±4	20.4±9
Controls	438	121 (27.6)	50.0±12	17.7±4	25.4±15

ALSPAC, Avon Longitudinal Study of Parents and Children; CPD, cigarettes per day; KORA, Cooperative Health Research in the Region of Augsburg; PsyCoLaus, population-based study from Lausanne; SHIP, Study of Health in Pomerania.

Fig. 1



Summary of the meta-analysis of the GWAS in smoking relapse versus abstinence of the two discovery samples (PsyCoLaus and SHIP). GWAS, genomewide association studies; PsyCoLaus, population-based study from Lausanne; SHIP, Study of Health in Pomerania.

ND remains a major public health problem worldwide, being associated with an increased risk of developing several medical illnesses (including lung cancer, cardiovascular and lung diseases), which is reduced by cessation of smoking (Teo *et al.*, 2006; White, 2007; Godtfredsen *et al.*, 2008). The majority of smokers express the intention to quit, and a wide percentage make a serious attempt (Centers for Disease Control and Prevention, 2005). However, success rates after attempts to quit smoking remain modest, with abstinence rates varying between 5%, at 1 year after unaided attempt, and 25%, for long-term abstinence with a pharmacologic aid (Zhu *et al.*, 2000; Moore *et al.*, 2009). It could therefore be important to identify the genetic contribution to a smoker's ability/inability to maintain long-term abstinence after a quitting attempt. Understanding of the biological underpinning of this important smoking-related phenotype may help identify new strategies to prevent individuals from relapsing to drug-seeking and drug-taking behaviours during protracted abstinence.

Few GWAS and candidate-gene studies have focused on phenotypes that are related to the ability to quit smoking and more importantly to remain abstinent, despite this being the unresolved issue in the treatment of ND, and thus of greatest clinical relevance. Although 'genomewide significance' is rarely attained for individual markers for nicotine cessation, more sophisticated methods of analyses have provided evidence for the contribution of genetic markers in smoking cessation. On the basis of GWAS data, Uhl *et al.* (2010) could show 'more clustering of nominally positive results within small genomic regions, more overlap between these genomic regions and those identified in six prior successful smoking cessation GWA studies and sets of genes that fall into gene ontology categories that appear to be biologically relevant. The 1000 SNPs with the strongest associations form a plausible Bayesian network; no such network is formed by randomly selected sets of SNPs'.

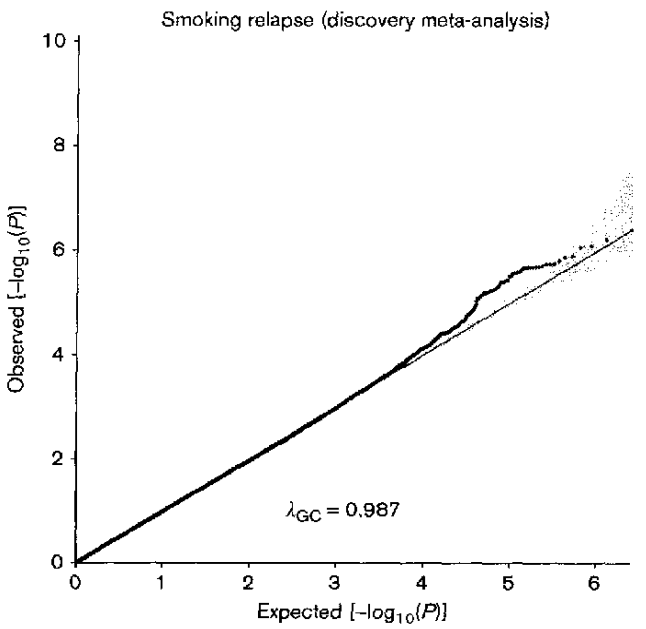
Here, we report the results from two GWAS and two independent replication samples of a smoking-related phenotype that has not been tested previously in GWAS studies: the smoking relapse phenotype. We identified cases and controls within four community-based studies applying a rather strict, empirically derived definition of smoking relapse versus smoking abstinence. Given the rather small sample size of 835 cases (relapsers) and 990

Table 2 Summary of meta-analysis results for the top seven SNPs identified in the discovery phase

SNP	Gene	Chromosome	Allele	Sample I		Sample II		Discovery Set		Sample III		Sample IV		Combined Set		Allele frequency			
				Effect	*P*-value	Effect	*P*-value	Effect	*P*-value	Effect	*P*-value	Effect	*P*-value	Effect	*P*-value	Total (*n*)[a]	I	II	III	IV
rs1008509	*XYLT2*	17	t	−0.454	2.31E−03	−0.567	5.65E−04	−0.5044	5.64E−06	−0.268	4.00E−03	0.190	0.138	−0.234	1.58E−04	4669	0.36	0.81	0.78	0.80
rs6464419	*DPP6*	7	a	−0.536	8.30E−05	−0.661	2.22E−03	−0.5707	8.11E−07	0.164	0.104	0.049	0.721	−0.273	4.36E−05	4660	0.76	0.15	0.15	0.17
rs11900784	*ANTXR1*	2	a	0.539	7.43E−05	0.563	1.22E−02	0.5453	3.09E−06	−0.053	0.617	−0.320	0.037	0.286	4.47E−05	4649	0.97	0.87	0.87	0.87
rs787176	*GTDC1*[b]	2	t	−0.416	7.53E−04	−0.563	1.40E−04	−0.4754	6.05E−07	−0.057	0.471	0.077	0.397	−0.181	3.49E−04	4585	0.27	0.67	0.68	0.58
rs13018437	*ARHGAP15*[b]	2	a	0.669	1.86E−05	0.475	9.24E−03	0.5876	8.53E−07	−0.082	0.354	−0.086	0.456	0.214	4.30E−04	4649	0.86	0.18	0.23	0.24
rs2769920	*FAM155A*	13	a	1.163	7.20E−03	1.398	8.63E−05	1.3021	2.63E−06	0.007	0.930	−0.256	0.039	−0.003	9.60E−01	4645	0.45	0.07	0.25	0.22
rs6465083	*GRM3*	7	t	0.530	6.57E−06	0.379	1.26E−02	0.4743	3.97E−07	−0.170	0.038	0.030	0.788	0.091	9.19E−02	4619	0.10	0.28	0.27	0.28

Sample I – PsyCoLaus; Sample II – SHIP; Discovery Set- PsyCoLaus and SHIP; Sample III – ALSPAC; Sample IV – KORA.
ALSPAC, Avon Longitudinal Study of Parents and Children; KORA, Cooperative Health Research in the Region of Augsburg; PsyCoLaus, population-based study from Lausanne; SNP, single-nucleotide polymorphism; SHIP, Study of Health in Pomerania.
[a]Because of some missing values or genotype errors, the number of cases varies between the different SNPs.
[b]These SNPs represent the same locus.

Fig. 2



Q–Qplot obtained for the GC-adjusted *P*-values from the meta-analysis of the discovery samples. GC, Genomic Control.

controls (abstainers) analysed in our two discovery samples, it seemed acceptable to aim for replication of the top markers, even though they did not reach genomewide significance in the discovery samples. However, even in the total sample of 4744 individuals, none of the top SNPs analysed reached the association at the genomewide level. These results suggest that – as for other smoking phenotypes – no individual variant with a substantial effect exists for the likelihood of achieving long-term abstinence from smoking. However, even the total sample size of 4744 individuals is rather small in the field of GWAS, and provides modest power to detect SNPs with a small effect. Among the top findings obtained from the discovery set, some have a biological rationale, in particular, the *glutamate metabotropic receptor 3* (*GRM3*) gene. Like other addictions, ND has to be considered as a chronic brain disorder with a high relapse rate in individuals aiming for abstinence. It is likely that neuroadaptative processes occur in the brain during chronic nicotine exposure and lead to the high rates of relapse after attempts to quit smoking. Genes involved in relapse may specifically alter the brain's capacity to return to its normal 'preaddictive' state and thereby maintain a long-term susceptibility for nicotine relapse.

Glutamate is the major excitatory neurotransmitter in the central nervous system, and it has been shown to be directly modulated by nicotine administration. Experimental animal studies showed that the development of ND produces neuroadaptive changes in glutamate receptors, and that

Table 3 Association with smoking relapse of SNPs in candidate genes from recently published GWAS on smoking phenotypes (Liu *et al.*, 2010; Thorgeirsson *et al.*, 2010; Tobacco and Genetics Consortium, 2010)

Gene name	Chromosome	SNPs	Consortium sample	Meta-analysis (*P*-value in this study)
CHRNA3/CHRNA5				
	15q25	rs1051730	TAG, ENAGE, OX-GSK	0.094
		rs16969968	TAG, OX-GSK	0.133
		rs55853698	OX-GSK	–
		rs6495308	OX-GSK	0.852
CHRNB3				
	8p11	rs6474412	ENGAGE	0.372
		rs13280604	ENGAGE	0.368
Near *PPP1R3C*				
		rs1329650	TAG	0.066
		rs1028936	TAG	0.020
EGLN2, near *CYP2A6*				
		rs3733829	TAG	0.533
CYP2A6, CYP2B6				
	19q13	rs7937	ENGAGE	0.858
		rs1801272	ENGAGE	0.803
		rs4105144	ENGAGE	0.810
		rs7260329	ENGAGE	0.937
BDNF				
		rs6265	TAG	0.815
		rs1013442	TAG	0.962
		rs4923457	TAG	0.527
		rs4923460	TAG	0.735
		rs4074134	TAG	0.528
		rs1304100	TAG	0.948
		rs6484320	TAG	0.684
		rs879048	TAG	0.453
Near *DBH*				
		rs3025343	TAG	0.836

GWAS, genomewide association studies; SNP, single-nucleotide polymorphism.

these receptors may be involved in early nicotine withdrawal symptoms and in relapse to nicotine-seeking and nicotine-taking behaviours after a period of extended abstinence (Dravolina *et al.*, 2007; Liechti and Markou, 2008).

The rs1008509 SNP within the *XYLT2* gene was among the top findings in the discovery set that was replicated in the ALSPAC cohort ($P = 0.004$), but could not be replicated in KORA. The protein encoded by this gene is an isoform of xylosyltransferase, which belongs to a family of glycosyltransferases. This enzyme transfers xylose from UDP-xylose to specific serine residues of the core protein and initiates the biosynthesis of glycosaminoglycan chains in proteoglycans including chondroitin sulphate, heparan sulphate, heparin and dermatan sulphate. *XYLT2* is expressed ubiquitously in humans, but it is also strongly expressed in various parts of the brain that could therefore provide a link to smoking relapse.

The *DPP6* gene encodes a dipeptidylpeptidase-like protein that can regulate the biological activity of neuropeptides, it binds special potassium channels altering their expression and biophysical properties and may be involved in neural plasticity (Kaulin *et al.*, 2009; McNicholas *et al.*, 2009; Nadin and Pfaffinger, 2010). Interestingly, *DPP6* was also identified by Uhl *et al.* (2008) as one of the genes harbouring allelic variants that distinguished successful versus unsuccessful quitters.

Previous GWAS have consistently identified a locus on chromosome 15 for ND. Other markers have been less consistently associated with smoking phenotypes, such as smoking cessation (Uhl *et al.*, 2008; Liu *et al.*, 2010; Thorgeirsson *et al.*, 2010; Tobacco and Genetics Consortium, 2010). We queried our meta-analysis for the top findings from these GWAS. As we hypothesized, when tested in the individuals selected on the basis of our relapse/abstinence criteria, none of the selected genes reached adjusted nominal significance. This seems to indicate that the neurobiology of smoking relapse and long-term abstinence is distinct from the other smoking phenotypes (i.e. smoking quantity, ND, smoking cessation). Another interpretation might be a considerable genetic heterogeneity between populations, even closely related ones, which would have implications for any meta-analysis that was not highly powered to overcome such heterogeneity (Li *et al.*, 2003; Conti *et al.*, 2008; Uhl *et al.*, 2008). Moreover, no overlap was found between our top markers and the results from Uhl *et al.* (2010). Several neurotransmitter systems seem to be involved and may have variable relevance in the different phases of smoking (i.e. development of dependence, withdrawal upon cessation, relapse after sustained abstinence).

These results call for further collaborative efforts, because larger, prospectively assessed, phenotypically homogeneous samples will increase the power to detect a genetic association for this clinically relevant smoking

phenotype. The definition of relapse and long-term abstinence requires a comprehensive assessment of smoking history and habits in large community-based samples to have a more phenotypically homogeneous and well-characterized sample of large size.

Study limitations

Although we used two discovery samples and two independent replication samples, the overall sample size of the study was rather small. We used three population-based samples and one sample of pregnant women. The latter sample (ALSPAC) was clearly different from the other sample as the participants were female, younger and smoked few cigarettes per day. We still assumed that the genetic architecture of abstinence versus relapse would be present in this group while being under psychosocial pressure of stopping their smoking behaviour during pregnancy. However, the lack of replication of the top findings may be partially attributed to the phenotypic heterogeneity between the discovery and the replication samples. Further, we did not account statistically for the putative impact of smoking quantity on the ability to quit. However, we analysed all genetic markers that have been implicated in smoking quantity in the recent GWAS (Liu *et al.*, 2010; Thorgeirsson *et al.*, 2010; Tobacco and Genetics Consortium, 2010). None of those markers showed any association with relapse at all, making it less likely that smoking quantity is a mediator of the genetic architecture of relapse. Further, we follow the pathophysiological model that smoking quantity acts directly on the ability to quit but not on the allele distribution of a risk gene for relapse. Thus, it is unlikely that smoking quantity itself acts as a confounder between a risk gene and smoking relapse. In all, we applied a rather strict phenotype definition for the selection of cases and controls; however, study design, recruiting strategy, assessment instruments and cultural differences among the four samples may have introduced phenotypic heterogeneity.

Acknowledgements

The authors would like to express their gratitude to the Lausanne inhabitants who volunteered to participate in the PsyCoLaus study. They also thank all the investigators of the CoLaus study, who made the psychiatric study possible, as well as many GSK employees who contributed to the execution of this study, and especially Emiliangelo Ratti for supporting this work within GSK Drug Discovery.

The authors are very grateful to all the families who took part in this study, the midwives for their help in recruiting them and the whole ALSPAC team, which includes interviewers, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, receptionists and nurses.

The PsyCoLaus study was supported by grants from the Swiss National Science Foundation (#3200B0-105993, #3200B0-118308, 33CSC0-122661) and from GlaxoSmith Kline (Psychiatry Center of Excellence for Drug Discovery and Genetics Division, Drug Discovery, Verona, R&D).

SHIP is part of the Community Medicine Research net of the University of Greifswald, Germany, which is funded by the Federal Ministry of Education and Research (Grants No. 01ZZ9603, 01ZZ0103, and 01ZZ0403), the Ministry of Cultural Affairs as well as the Social Ministry of the Federal State of Mecklenburg, West Pomerania. Genomewide data have been supported by the Federal Ministry of Education and Research (Grant No. 03ZIK012) and a joint grant from Siemens Healthcare, Erlangen, Germany and the Federal State of Mecklenburg, West Pomerania. The University of Greifswald is a member of the 'Center of Knowledge Interchange' program of the Siemens AG.

The UK Medical Research Council (74882), the Wellcome Trust (076467) and the University of Bristol provide core support for ALSPAC. This research was specifically funded by the Wellcome Trust (086684).

The KORA research platform was initiated and financed by the Helmholtz Center Munich, German Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Research (BMBF) and by the State of Bavaria. Part of this work was financed by the German National Genome Research Network (NGFN-2). KORA research was also supported within the Munich Center of Health Sciences (MC Health) as part of LMUinnovativ. N.D. and M.G. were supported by the German Research Foundation (DFG) national priority program SPP1226 and the German Federal Ministry of Education and Research (BMBF): NGFN-Plus FKZ 01GS08158.

Conflicts of interest

The PsyCoLaus study was partially funded by GlaxoSmithKline (GSK). Federica Tozzi, Howard Chilcoat, Justin P. Rubio and Vincent Mooser are, or were at the time of the study, full time employees of GSK. Peter Vollenweider and Gérard Waeber received unrestricted grant from GSK to build the CoLaus study. For the remaining authors there are no conflicts of interest.

References

American Psychiatric Association (1994). *Diagnostic and Statistical Manual of mental disorders (DSM-IV)*. Washington, DC: American Psychiatric Association.

Broms U, Silventoinen K, Madden PA, Heath AC, Kaprio J (2006). Genetic architecture of smoking behavior: a study of Finnish adult twins. *Twin Res Hum Genet* **9**:64–72.

Centers for Disease Control and Prevention (2005). Cigarette smoking among adults – United States, 2004. *MMWR Morb Mortal Wkly Rep* **54**: 1121–1124.

Chilcoat HD, Webb DJ (2007). Time to remission for alcohol, nicotine, and other illegal drug dependence in the United States. Presented at the 69th Annual Meeting of the College of Problems of Drug Dependence, 21 June 2007; Quebec City, Quebec.

Conti DV, Lee W, Li D, Liu J, Van Den BD, Thomas PD, *et al.* (2008). Nicotinic acetylcholine receptor beta2 subunit gene implicated in a systems-based candidate gene study of smoking cessation. *Hum Mol Genet* **17**: 2834–2848.

Dravolina OA, Zakharova ES, Shekunova EV, Zvartau EE, Danysz W, Bespalov AY (2007). mGlu1 receptor blockade attenuates cue- and nicotine-induced reinstatement of extinguished nicotine self-administration behavior in rats. *Neuropharmacology* **52**:263–269.

Firmann M, Mayor V, Vidal PM, Bochud M, Pecoud A, Hayoz D, *et al.* (2008). The CoLaus study: a population-based study to investigate the epidemiology and genetic determinants of cardiovascular risk factors and metabolic syndrome. *BMC Cardiovasc Disord* **8**:6.

Garvey AJ, Bliss RE, Hitchcock JL, Heinold JW, Rosner B (1992). Predictors of smoking relapse among self-quitters: a report from the Normative Aging Study. *Addict Behav* **17**:367–377.

Godtfredsen NS, Lam TH, Hansel TT, Leon ME, Gray N, Dresler C, *et al.* (2008). COPD-related morbidity and mortality after smoking cessation: status of the evidence. *Eur Respir J* **32**:844–853.

Golding J, Pembrey M, Jones R (2001). ALSPAC-the Avon Longitudinal Study of Parents and Children. I. Study methodology. *Paediatr Perinat Epidemiol* **15**:74–87.

Herd N, Borland R, Hyland A (2009). Predictors of smoking relapse by duration of abstinence: findings from the International Tobacco Control (ITC) Four Country Survey. *Addiction* **104**:2088–2099.

Hughes JR, Keely J, Naud S (2004). Shape of the relapse curve and long-term abstinence among untreated smokers. *Addiction* **99**:29–38.

Hughes JR, Peters EN, Naud S (2008). Relapse to smoking after 1 year of abstinence: a meta-analysis. *Addict Behav* **33**:1516–1520.

John U, Greiner B, Hensel E, Ludemann J, Piek M, Sauer S, *et al.* (2001). Study of Health in Pomerania (SHIP): a health examination survey in an east German region: objectives and design. *Soz Praventivmed* **46**:186–194.

Kaulin YA, De Santiago-Castillo JA, Rocha CA, Nadal MS, Rudy B, Covarrubias M (2009). The dipeptidyl-peptidase-like protein DPP6 determines the unitary conductance of neuronal Kv4.2 channels. *J Neurosci* **29**:3242–3251.

Korn JM, Kuruvilla FG, McCarroll SA, Wysoker A, Nemesh J, Cawley S, *et al.* (2008). Integrated genotype calling and association analysis of SNPs, common copy number polymorphisms and rare CNVs. *Nat Genet* **40**:1253–1260.

Lessov CN, Martin NG, Statham DJ, Todorov AA, Slutske WS, Bucholz KK, *et al.* (2004). Defining nicotine dependence for genetic research: evidence from Australian twins. *Psychol Med* **34**:865–879.

Lessov-Schlaggar CN, Pang Z, Swan GE, Guo Q, Wang S, Cao W, *et al.* (2006). Heritability of cigarette smoking and alcohol use in Chinese male twins: the Qingdao twin registry. *Int J Epidemiol* **35**:1278–1285.

Li MD, Cheng R, Ma JZ, Swan GE (2003). A meta-analysis of estimated genetic and environmental effects on smoking behavior in male and female adult twins. *Addiction* **98**:23–31.

Liechti ME, Markou A (2008). Role of the glutamatergic system in nicotine dependence: implications for the discovery and development of new pharmacological smoking cessation therapies. *CNS Drugs* **22**:705–724.

Liu JZ, Tozzi F, Waterworth DM, Pillai SG, Muglia P, Middleton L, *et al.* (2010). Meta-analysis and imputation refines the association of 15q25 with smoking quantity. *Nat Genet* **42**:436–440.

Lowel H, Doring A, Schneider A, Heier M, Thorand B, Meisinger C (2005). The MONICA Augsburg surveys-basis for prospective cohort studies. *Gesundheitswesen* **67 (Suppl 1)**:S13–S18.

Lu Y, Tong S, Oldenburg B (2001). Determinants of smoking and cessation during and after pregnancy. *Health Promot Int* **16**:355–365.

Maes HH, Sullivan PF, Bulik CM, Neale MC, Prescott CA, Eaves LJ, Kendler KS (2004). A twin study of genetic and environmental influences on tobacco initiation, regular tobacco use and nicotine dependence. *Psychol Med* **34**:1251–1261.

Marchini J, Howie B, Myers S, McVean G, Donnelly P (2007). A new multipoint method for genome-wide association studies by imputation of genotypes. *Nat Genet* **39**:906–913.

Marques-Vidal P, Kutalik Z, Paccaud F, Bergman S, Waeber G, Vollenweider P, Cornuz J (2011). Variant within the promoter region of the CHRNA3 gene associated with FTN dependence is not related to self-reported willingness to quit smoking. *Nicotine Tob Res* **13**:833–839.

McNicholas K, Chen T, Abbott CA (2009). Dipeptidyl peptidase (DP) 6 and DP10: novel brain proteins implicated in human health and disease. *Clin Chem Lab Med* **47**:262–267.

Moore D, Aveyard P, Connock M, Wang D, Fry-Smith A, Barton P (2009). Effectiveness and safety of nicotine replacement therapy assisted reduction to stop smoking: systematic review and meta-analysis. *BMJ* **338**:b1024.

Murray S (2006). A smouldering epidemic. *CMAJ* **174**:309–310.

Nadin BM, Pfaffinger PJ (2010). Dipeptidyl peptidase-like protein 6 is required for normal electrophysiological properties of cerebellar granule cells. *J Neurosci* **30**:8551–8565.

Normand SL (1999). Meta-analysis: formulating, evaluating, combining, and reporting. *Stat Med* **18**:321–359.

Numberger JI Jr, Blehar MC, Kaufmann CA, York-Cooler C, Simpson SG, Harkavy-Friedman J, *et al.* (1994). Diagnostic interview for genetic studies. Rationale, unique features, and training. NIMH Genetics Initiative. *Arch Gen Psychiatry* **51**:849–859.

Preisig M, Waeber G, Vollenweider P, Bovet P, Rothen S, Vandeleur C, *et al.* (2009). The PsyCoLaus study: methodology and characteristics of the sample of a population-based survey on psychiatric disorders and their association with genetic and cardiovascular risk factors. *BMC Psychiatry* **9**:9.

Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D (2006). Principal components analysis corrects for stratification in genome-wide association studies. *Nat Genet* **38**:904–909.

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, *et al.* (2007). PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* **81**:559–575.

Saccone SF, Hinrichs AL, Saccone NL, Chase GA, Konvicka K, Madden PA, *et al.* (2007). Cholinergic nicotinic receptor genes implicated in a nicotine dependence association study targeting 348 candidate genes with 3713 SNPs. *Hum Mol Genet* **16**:36–49.

Segan CJ, Borland R, Greenwood KM (2006). Can transtheoretical model measures predict relapse from the action stage of change among ex-smokers who quit after calling a quitline? *Addict Behav* **31**:414–428.

Sullivan PF, Kendler KS (1999). The genetic epidemiology of smoking. *Nicotine Tob Res* 1 **(Suppl 2)**:S51–S57.

Teo KK, Ounpuu S, Hawken S, Pandey MR, Valentin V, Hunt D, *et al.* (2006). Tobacco use and risk of myocardial infarction in 52 countries in the INTERHEART study: a case–control study. *Lancet* **368**:647–658.

Thorgeirsson TE, Geller F, Sulem P, Rafnar T, Wiste A, Magnusson KP, *et al.* (2008). A variant associated with nicotine dependence, lung cancer and peripheral arterial disease. *Nature* **452**:638–642.

Thorgeirsson TE, Gudbjartsson DF, Surakka I, Vink JM, Amin N, Geller F, *et al.* (2010). Sequence variants at CHRNB3-CHRNA6 and CYP2A6 affect smoking behavior. *Nat Genet* **42**:448–453.

Tobacco and Genetics Consortium (2010). Genome-wide meta-analyses identify multiple loci associated with smoking behavior. *Nat Genet* **42**:441–447.

Uhl GR, Liu QR, Drgon T, Johnson C, Walther D, Rose JE, *et al.* (2008). Molecular genetics of successful smoking cessation: convergent genome-wide association study results. *Arch Gen Psychiatry* **65**:683–693.

Uhl GR, Drgon T, Johnson C, Ramoni MF, Behm FM, Rose JE (2010). Genome-wide association for smoking cessation success in a trial of precessation nicotine replacement. *Mol Med* **16**:513–526.

Volzke H, Alte D, Schmidt CO, Radke D, Lorbeer R, Friedrich N, *et al.* (2011). Cohort profile: the Study of Health in Pomerania. *Int J Epidemiol* **40**:294–307.

White WB (2007). Smoking-related morbidity and mortality in the cardiovascular setting. *Prev Cardiol* **10**:1–4.

Wichmann HE, Gieger C, Illig T (2005). KORA-gen – resource for population genetics, controls and a broad spectrum of disease phenotypes. *Gesundheitswesen* **67 (Suppl 1)**:S26–S30.

Zhu S, Melcer T, Sun J, Rosbrook B, Pierce JP (2000). Smoking cessation with and without assistance: a population-based analysis. *Am J Prev Med* **18**:305–311.