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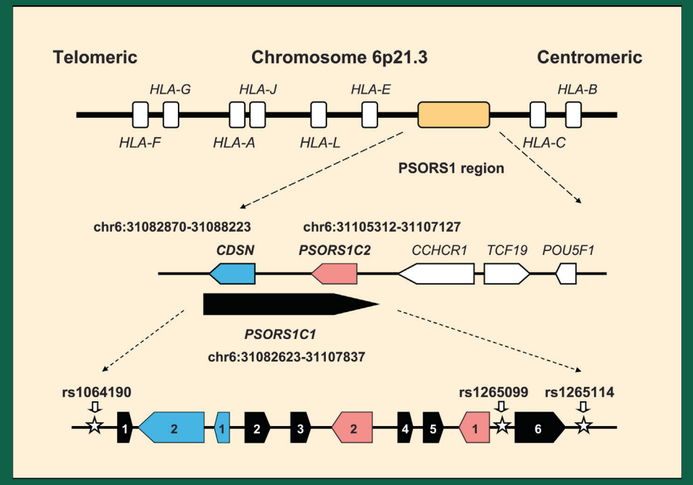

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Editorial

Rare diseases: A mysterious puzzle

Although often affecting only a small population, most rare diseases are genetic and hence afflict the patient throughout life. Personalized medicine is based on a principle that each individual is born with unique biological characteristics. Genomics lays the foundation of personalized medicine, the success of which depends on accurate diagnostic tests capable of identifying patients who can benefit from targeted therapy.

Kawasaki disease (KD) is an acute, self-limited, and systemic vasculitis that is a prime cause of acquired heart disease in children. To date, human leukocyte antigen (HLA) genes within the major histocompatibility complex region on chromosome 6p remain the best documented association for KD. Other non-HLA candidate genes in this region, such as those genes located in the psoriasis susceptibility 1 (PSORS1) region, play potential roles in developing KD. In a study in this issue [1], a single nucleotide polymorphism was identified in the PSORS1 region that contributes to KD susceptibility in Taiwanese children of Han Chinese ethnicity. A strong correlation between PSORS1C1 gene polymorphism and cardiac artery aneurysm in KD patients was observed.

Pediatric obesity looms ever more prevalent and has a major impact on public health. A complication of childhood obesity, acanthosis nigricans (AN), is associated with obesity as a manifestation of cutaneous insulin resistance. Clinical observation plus a pioneer genetic approach revealed association of the insulin/insulin-like growth factor receptor pathway with pre-obese and co-obese AN. Insulin resistance caused by AN might result in failure of suppression of excessive energy intake with ensuing obesity. This study forges a link in complex pathogenesis of obesity under scrutiny of patients' phenotype and genotype association.

Gilles de la Tourette syndrome (TS) is a neuropsychiatric disorder characterized by both motor and vocal tics. Pathogenesis remains obscure; current evidence points to a defective dopamine system. Single nucleotide polymorphisms serve as a tool to study complex gene-associated diseases like TS. In this genetic study, dopamine transporter and dopamine β-hydroxylase genes may not be useful as markers to predict susceptibility to TS, whose etiology is therefore unknown. Childhood TS may involve complex interaction between environmental influences. Further studies must confirm these assertions.

Parkinson's disease (PD) is characterized by progressive neuronal cell loss and decline in movement. β-Glucosidase (GBA), an enzyme deficient in Gaucher's disease, has been linked with PD. GBA mutation was shown in a study in this issue to be associated with PD patients in Central Taiwan. The connection between mutant GBA and parkinsonism remains unclear, therefore, a future study must identify the pathological mechanism. The aforementioned study implicates the mutation as a genetic risk factor in sporadic PD, accounting for higher prevalence of the disease in allele frequencies.

An example of the role of genetic and environmental factors in etiology is birth defects. This issue probes seasonal variation in respiratory defects and Down syndrome in Norway: namely, in March and February, respectively. Further studies must explain such variations, which likely represent environmental causes.

Pathogenesis of rare diseases is too complex to ferret out a common mechanism; etiology entails a gamut of genetic and environmental factors. Fundamental data to profile risk factors and discover novel therapeutic targets are vital. Tailoring therapy based on pharmacogenomic tests may save lives and bolster patient care. A challenge to healthcare teams is to consider how new genomic information affects management decisions and ensure personalized medical care.

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Review article

Personalized medicine: A paradigm shift in healthcare

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ABSTRACT

Personalized medicine is based on the established principle that each individual is born with unique biological characteristics. Genomics, the science of studying the genes in a genome and their interactions with each other, forms the foundation of personalized medicine. Several genomic methods are currently used to identify susceptibility loci for diseases or phenotypic traits, namely, linkage analysis, candidate gene association studies, and genome-wide association studies. The success of personalized medicine depends on having accurate diagnostic tests capable of identifying patients who can benefit from targeted therapy. Larger cohort studies plus the application of genome-wide association studies offer great potential for identifying the genetic factors that influence the pharmacology of specific drugs. By combining these approaches, physicians can predict health risks, determine and quantify the dynamics of disease development, and tailor therapeutic protocols to the needs of the individual. In this review, we focus on the effect of genetic profiling on disease outcomes as well as the potential of genomic methods to predict disease and drug response.

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1. Introduction

Traditional clinical diagnosis and management focuses on the patient's clinical symptoms and signs, medical and family history, and data from laboratory and imaging studies to diagnose and treat illnesses. Personalized medicine is a relatively new paradigm of evidence-based medicine that is based on the established principle that each individual is born with unique biological and genetic characteristics. Also known as P4 medicine, personalized medicine takes into account the patient's genetic profile (personalized medicine), anticipates health-related problems and focuses on wellness, not disease (preventive medicine), directs appropriate treatment using predictive models (predictive medicine), and encourages patients to take more responsibility for their health and healthcare (participatory medicine) [1,2]. In this article, we

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review the personalization aspect of the four-part paradigm by focusing on the effect of genetic profiling on disease outcomes as well as the potential of genomic methods to predict disease and drug response.

There is considerable variation between patients with the same disease. For example, some patients show no response to treatment, whereas others rapidly respond to therapy. Underlying this variation are alterations in the coding sequence or expression of hundreds of genes that confer disease susceptibility. A number of these genes are associated either with disease etiology or with clinical response to treatment. Therefore, it is believed that analysis of the genomic, proteomic, and metabolomic profiles of patients for the presence of drug targets and biomarkers will lead to improvements in diagnostic accuracy, prevention measures, and targeted therapies.

Genomics, the science of studying the genes in a genome and their interactions with each other [3], forms the foundation of personalized medicine [1,2,4,5]. The sequence of the 3 billion base pairs in the human genome has been publicly available since the completion of the International Human Genome Project in 2003. Recent advancements in technology, such as next-generation sequencing and improved computational methods to handle the huge amount of data generated by the new sequencing platforms, have changed the way we perceive medicine. Advances in genomic and high-throughput technologies will soon have a profound impact on the management of diseases. Such platforms will enable presymptomatic diagnosis, stratification of disease, assessment of disease progression, evaluation of patient response to therapy, and identification of relapses [6,7].

2. Human disease and genes

Genetic disorders are classified into several major groups. The first group comprises chromosomal disorders such as Down syndrome, which is caused by an extra copy of chromosome 21. The second group consists of single gene disorders, such as cystic fibrosis and sickle cell anemia. The majority of genetic diseases, however, are multifactorial in nature. Indeed, rather than being associated with changes in only one or a few genes or proteins, many diseases are likely a manifestation of multiple interconnected aberrant pathways and numerous molecular abnormalities. Many birth defects such as cleft lip and neural tube defects as well as many adult disorders, including heart disease, diabetes, and cancer, result from a combination of multiple genetic and environmental causes [6].

Several methods are currently used to identify phenotypic features of diseases and disease-susceptibility loci, including linkage analysis, candidate gene association studies, and genome-wide association studies (GWASs). Linkage analysis is useful for identifying familial genetic variants that have large effects and has been successfully used to discover several mutations responsible for monogenic forms of disease, such as maturity-onset diabetes of the young (MODY). In this disease, heterozygous mutations in the Glucokinase (*GCK*) gene were shown to cause MODY2 [8], whereas mutations in the hepatocyte nuclear factor-1β (*HNF-1β*) gene were shown to be related to the development of MODY5 [9]. Furthermore, linkage studies of type 2 diabetes mellitus (T2DM)-linked chromosomal regions have identified potential causative genetic variants in genes, including calpain-10 (CAPN10) [10], ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1) [11], hepatocyte nuclear factor 4 alpha (HNF4A) [12,13], and adiponectin (ADIPOQ) [14]. Disease-related genes can also be identified on the basis of association testing in populations rather than in families. The methods include candidate gene association studies and GWASs. Candidate gene association is based on measurements of selected biomarkers from relevant pathophysiological pathways. For example, of the scores of candidate genes related to T2DM that have been investigated using this approach, the *PPARG* and *KCNJ11* genes were found to be directly linked to the development of the disease. The *PPARG* gene encodes the peroxisome proliferator-activated receptor γ, a type II nuclear receptor that plays a fundamental role in adipogenesis and insulin sensitivity by regulating the transcriptional activity of various genes. The *KCNJ11* gene, located on the short arm of chromosome 11, encodes the pore-forming subunit of the ATP-sensitive potassium channel Kir6.2 in pancreatic β cells. Gain-of-function mutations in *KCNJ11* open the potassium channel and inhibit the depolarization of β cells, leading to a defect in insulin secretion.

However, significant interethnic differences occur in the risk allele frequency at discrete loci. Variants of the *KCNQ1* gene were first identified in Asians, and it was found that the frequency of the minor allele in that population (30–40%) was much higher than the frequency in Europeans (<10%). In addition, linkage analysis has demonstrated that the presence of the *TCF7L2* gene increases the risk of developing T2DM in almost all ethnic groups. However, risk allele frequencies of single-nucleotide polymorphisms (SNPs) in TCF7L2 in European populations were shown to be higher than those in Japanese (40% vs. 5%), indicating that TCF7L2 variants have little effect on T2DM susceptibility in the Japanese population.

The HapMap project demonstrated that genotyping of approximately 500,000 SNPs is sufficient to cover about 75% of the common variants (MAF of >5%) in the genome. Furthermore, improvements in high-throughput technology for SNP genotyping, which allows for the simultaneous genotyping of hundreds of thousands of SNPs and the development of biostatistical methods to handle the large volumes of data being produced, have opened up new possibilities for GWASs. GWASs are used to compare, in an unbiased manner, the genomes of individuals with or without a disorder of interest (such as T2DM) and to identify differences among a large number of common SNPs. Through such studies, many genetic variants have been identified and placed in pathways that were not previously associated with a particular disease. In addition, disease-associated SNPs have also been ascribed to genes with currently unknown functions [15]. For example, the results of a genome-wide linkage analysis conducted in Japanese sibling pairs [16,17] and GWASs in individuals of European ancestry and in Korean and Taiwanese populations [18–21] have identified the candidate loci for Kawasaki disease. However, these loci do not fully explain the genetic risk

for Kawasaki disease, suggesting that additional genetic factors remain to be discovered.

Recently, two new loci, one at BLK (encoding B-lymphoid tyrosine kinase) and one at CD40, have been found to be associated with Kawasaki disease in Han Chinese [22] and Japanese populations [23]. In another example, a two-stage GWAS was conducted in Han Chinese in Taiwan [24]. The study comprised 2798 patients with T2DM and 2367 healthy controls. The researchers not only confirmed that the *KCNQ1* gene was associated with T2DM but also identified two novel genetic susceptibility loci: *PTPRD* and *SRR*. Interestingly, these two newly identified genes are in pathways that were not previously associated with T2DM. In a recent GWAS comprising 6952 patients with T2DM and 11,865 healthy controls conducted by the Asian Genetic Epidemiology Network consortium, eight additional genetic loci were found to be associated with T2DM, namely, variations in or near the *GLIS3*, *PEPD*, *FITM2-R3HDML-HNF4A*, *KCNK16*, MAEA, *GCC1-PAX4*, *PSMD6*, and *ZFAND3* genes [25].

Most GWASs have been designed to find relatively common variants, typically focusing on those with allele frequencies of >5%. This is because studies require very high statistical power (and therefore, a very large sample size) to detect associations with relatively rare alleles. Therefore, it is possible that some rare genetic variants that play critical roles in disease onset or therapeutic response remain undiscovered. A substantial part of the missing heritability could be attributable to variants with large or intermediate effect sizes and relatively low frequencies. Such variants are likely to have escaped detection by current methods, as their low penetration would preclude linkage analysis, and their frequency would be too low for detection in GWASs. The strategies for identifying such variants largely depend on the frequency of said variants in test populations. Some variants with allele frequencies in the range of 1–5% might be identified by increasing the genotype density and cohort sizes. In this regard, the 1000 Genomes Project has extended the catalog of known human variants to include those with frequencies close to or <1%. However, detection of many of these rare or intermediate variants will require next-generation sequencing rather than traditional GWAS or genotyping [26].

2.1. *Prediction of disease*

In traditional medicine, physicians use different parameters, including patient characteristics and data from laboratory tests and imaging studies to identify an individual's health risk, to predict a patient's response to drugs and to monitor disease status during and following therapy. For example, there is compelling evidence that increasing age, higher body mass index/waist circumference, impaired fasting glucose, impaired glucose tolerance, higher glycated hemoglobin (HbA1c) level, and metabolic syndrome are important risk factors for T2DM. Several scores have been created on the basis of the combination of the clinical features that could predict the risk of diabetes. For example, the Diabetes Risk Calculator includes parameters of age, waist circumference, gestational diabetes, height, race/ethnicity, hypertension, family history of diabetes, and exercise. The value of the area under the curve (AUC) of the Diabetes Risk Calculator [27] was 0.70 for detecting impaired fasting glucose, impaired glucose tolerance, or undiagnosed diabetes. The Framingham Risk Score includes parameters of age, sex, obesity, hypertension, parental history of diabetes, low levels of HDL cholesterol, elevated triglyceride levels, and impaired fasting glucose. The AUC of this risk model was 0.85 for predicting T2DM in middle-aged adults [28].

In recent years, researchers have discovered numerous genes with variations in sequence or expression that contribute to disease susceptibility, and some of those variations provide the basis for targeting the molecular causes of some diseases [22]. The results from other studies have also indicated that genetic variability may be responsible for heterogeneous patient responses to treatment [4,29]. The number of SNPs included in genetic models has increased from three in 2005 to 40 in 2011, and the combinations of genes in each model have differed [30]. Recent studies have compared the predictive ability of risk models that include genetic variants only to those that combine genetic variants with clinical risk factors and found that genetic risk models have lower AUC values (0.55–0.68) than clinical models (AUC, 0.61–0.92) [30]. Incorporation of genetic factors into clinical risk models only marginally improved and in some cases did not improve the AUC value (Table 1) [31–42]. The contribution to disease risk by any one of these genetic factors is small (1.1–1.5-fold increased risk). However, research shows that the discriminative power of genetic risk factors improved as the duration of follow-up increased, whereas that of clinical risk factors decreased [33].

3. Pharmacogenomic studies

Pharmacogenomics is a science that examines the relationships among genetic variations and individual responses to pharmaceutical agents [43]. It has been applied in the field of personalized medicine to develop ways of optimizing drug therapy by stratifying patients into responders, individuals who demonstrate a therapeutic or an adverse response, and nonresponders. Pharmacogenomics technically differs from the science of pharmacogenetics, with the former referring to the general study of all different genes that determine drug behavior, and the latter referring to the study of inherited differences (variation) in drug metabolism and response; however, the distinction between pharmacogenomics and pharmacogenetics is considered arbitrary, and the two terms tend to be used interchangeably. The following are some examples related to the effect of gene variants on drug treatment.

3.1. *Warfarin treatment, blood clotting, and the CYP2C9 gene*

Warfarin, an anticoagulant drug, is widely used to prevent thrombosis, but because of interindividual variations in dose requirements, hemorrhagic complications caused by warfarin therapy are common. Two genes, cytochrome P450 2C9 (*CYP2C9*) and vitamin K epoxide reductase (*VKORC1*), are associated with the pharmacokinetics and pharmacodynamics of warfarin, respectively [44]. The metabolism of (*S*)-

Table 1 – Predict models combined with clinical risk and genetic markers for type 2 diabetes.

Author (year published)	Outcome no. of cases/study population	Clinical risk factors + biomarkers		+ Genetic markers		Different[a]
Balkau et al (2008) [31]	203 i.c./3817 (30–64 y)	Men: smoking, WC, FPG, GGT Women: FH, BMI, FPG, TG	AUC: 0.850 (men) 0.917 (women)	2 genes	AUC: 0.851 (men) 0.912 (women)	
Vaxillaire et al (2008) [32]	187 i.c./3442 (30–65 y)	Age, sex, average BMI over 9 y	AUC: 0.82	3 genes	AUC: 0.83	
Lyssenko et al (2008) [33]	2201 i.c./18,831	Age, sex, BMI, BP, FH, FPG HDL, TG, WC	AUC: 0.74	11 genes	AUC: 0.75	Yes
van Hoek et al (2008) [34]	490 i.c.+545 p.c./5297 (>55 y)	Age, sex, BMI	AUC: 0.66	17 genes (18 SNP)	AUC: 0.68	Yes
Meigs et al (2008) [35]	255 i.c. /2377	Age, sex, BMI, FH, FPG, SBP, HDL, and TG	C: 0.900	18 genes	C: 0.901	No
Lango et al (2008) [36]	2309 p.c./2598 controls (35–70 y)	Age, sex, BMI	AUC: 0.78	17 genes (18 SNPs)	AUC: 0.80	Yes
Schulze et al (2009) [37]	801 i.c. /1,962	Age, height, WC, hypertension, intake of red meat, whole gram bread, coffee, alcohol, PA, smoking, glucose, HbA1c, TG, HDL, CHOL, GGT, ALT	AUC: 0.9000	20 genes	AUC: 0.9002	No
Lin et al (2009) [38]	356 p.c./5360 (35–75 y)	Age, BMI, FH, WHR, TG/HDL ratio	AUC: 0.86	15 genes	AUC:0.87	Yes
Sparso et al (2009) [39]	4093 cases/5302 g.t.	Age, sex, BMI	0.92	19 genes	0.93	N/A
Talmud et al (2010) [40]	302 i.c./5535 (35–55 y)	Age, sex, BMI, drug treatment, FH, smoking (Cambridge risk score)	AUC: 0.72	20 genes	AUC: 0.73	Yes
Talmud et al (2010) [40]	302 i.c. /5535 (35–55 y)	Age, sex, BMI, FH, HDL, TG, FPG (FHOS risk score)	AUC: 0.78	20 genes	AUC: 0.78	No
Wang et al (2010) [27,41]	518 i.c./7232 (45–74 y)	Age, BMI,WC, BP medication, history of blood glucose, PA, daily consumption of vegetables, fruits or berries, TG, HDL, adiponectin, ALT	C: 0.772	19 genes	C: 0.772	N/A
De Miguel-Yanes et al (2011) [42]	144 i.c. <50 y; 302 i.c. ≧50 y /3471	Sex, BMI, FH, FPG, SBP, HDL, TG	C: 0.903	40 genes	C: 906	No

ALT = alanine aminotransferase; AUC = area under curve; BMI = body mass index; BP = blood pressure; CHOL = cholesterol; FPG = fasting plasma glucose; FH = family history; FHOS = Framingham Offspring study; GGT = gamma-glutamyltransferase; HbA1c = hemoglobin A1c; HDL = high density lipoprotein; i.c. = incident cases; g.t. = glucose tolerance; N/A = not applicable; PA = physical activity; p.c. = prevalent cases; SBP = systolic blood pressure; pop. = population; TG = triglyceride; WC = waist circumference; WHR = waist/hip ratio.
a Different between model with clinical Risk factors and/or biomarkers and model with genetic markers.

warfarin to its inactive metabolite is mediated by the cytochrome p450 enzyme CYP2C9. Persons with at least one copy of either CYP2C9*2 or CYP2C9*3 require less warfarin for effective anticoagulation compared to the general population [45]. Consistent with this observation, hemorrhagic complications are more common in persons carrying the CYP2C9*2 or CYP2C9*3 allele. The function of VKORC1 is the gamma carboxylation of glutamyl residues, which is required for the activation of multiple vitamin K-dependent clotting factors [23]. A functional variant in the promoter region (.1639G>A) of the *VKORC1* gene was found to alter a transcription factor-binding site [46]. *In vitro* studies confirmed that the A (minor) allele reduces transcription of the gene by almost 50% compared with a promoter bearing the wild-type G allele. Several pharmacogenetic warfarin dose-prediction models have been developed [29,47]. These dose-initiation models indicate that the CYP2C9 and VKORC1 polymorphisms account for 18% and 30% of the observed variability of warfarin, respectively. Specifically, the VKORC1 variant predicts warfarin sensitivity, and the CYP2C9*2/*3 variants affect warfarin clearance. Clinical factors—including age, sex, body size, race/ethnicity, smoking status, and relevant concomitant medications—are responsible for an additional 12% of dose variability, meaning that, overall, VKORC1 and CYP2C9*2/*3 variants as well as clinical factors account for up to 60% of interindividual variability [48].

3.2. *Herceptin treatment of breast cancer and the HER2/neu gene*

About 18–20% of patients with breast cancer show amplification of the *HER2/neu* gene or overexpression of its protein product. Those factors are associated with increased risk of disease recurrence and poor prognosis. The mean relative risk for overall survival is 2.74 for patients who are HER2-positive [49]. The development of HER2-targeted therapies for patients with HER2-positive disease has dramatically reduced the risk of recurrence after the initial therapy and has led to improved prognosis. Various HER2-targeted drugs are approved or in development, such as monoclonal antibodies

that are directed against its external domain (e.g., trastuzumab and pertuzumab), small molecule tyrosine kinase inhibitors (e.g., lapatinib and neratinib), anti-HER2 antibodies conjugated to toxic molecules (e.g., trastuzumab-DM1 or T-DM1), and chaperone antagonists (e.g., geldanamycin) [50]. Herceptin (trastuzumab) is a recombinant humanized IgG1-kappa monoclonal antibody that selectively binds with high affinity to the extracellular domain of HER2. Based on results from randomized clinical trials, trastuzumab-containing regimens are now recommended for women with HER2-positive metastatic breast cancer [51]. Data from trials of first-generation adjuvant regimens combining trastuzumab with various chemotherapeutic drugs showed significant improvements in disease-free and overall survival rates [49]. Studies on second-generation adjuvant regimens comprising other HER2-targeted agents such as lapatinib and pertuzumab are underway, and newer drugs such as T-DM1 and neratinib are being actively tested in the metastatic setting.

3.3. *Carbamazepine therapy and the HLA-B*1502 allele*

Carbamazepine is an important treatment for seizure disorders, bipolar disorder, trigeminal neuralgia, and chronic pain. However, carbamazepine is also associated with hypersensitivity reactions that range from benign urticaria to life-threatening cutaneous disorders, including Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). Both conditions (SJS–TEN) are associated with significant morbidity and mortality. The incidence of SJS in Han Chinese is higher than that in Caucasians (8 cases per million person-years in the Han Chinese population compared with 2–3 cases in Caucasians) [52]. Recently, pharmacogenomic studies have found a strong association between carbamazepine-induced SJS–TEN and the HLA-B*1502 and HLA-B*5801 alleles in the Han Chinese populations in Taiwan [52,53] and in other Asian countries [54,55]. Furthermore, the incidence of SJS–TEN among people treated with the drug is substantially reduced when individuals carrying HLA-B*1502 are excluded from carbamazepine therapy [53]. Patients of Han Chinese descent with molecular evidence of that allele should, therefore, be treated with other classes of antiepileptic drugs. However, the allele frequency of HLA-B*1502 is markedly lower in Caucasians (1–2%) than in Han Chinese (8%). In addition, no association between HLA-B and carbamazepine-induced SJS–TEN has been found in Caucasian patients. Therefore, the United States Food and Drug Administration recommends genetic screening for patients of Asian ancestry before initiation of carbamazepine therapy.

3.4. *Antidiabetic drugs*

Blood glucose control is a priority in the treatment of T2DM. Pharmacogenetic studies have been performed on three classes of drugs commonly used in the treatment of diabetes: metformin, sulfonylureas, and thiazolidinediones [56]. Metformin suppresses hepatic gluconeogenesis by activating AMP-activated protein kinase, which inhibits the expression of hepatic gluconeogenic genes PEPCK and Glc-6-pase by increasing the expression of the small heterodimer partner. It was recently found that variations in the organic cation transporter 1 and 2 (OCT1 and OCT2), proteins responsible for the hepatic transport of metformin, might affect metformin response [57]. Sulfonylureas bind to ATP-sensitive potassium channels in pancreatic β cells, thereby stimulating insulin release in a glucose-independent manner [58]. Recently, polymorphisms in drug target genes (*ABCC8*, *KCNJ11*) and diabetes risk genes (*TCF7L2* and *IRS-1*) have been shown to be associated with variability in the response to sulfonylurea drugs in patients with T2DM [59]. Pearson et al [60] found that patients with hepatocyte nuclear factor-1α (*HNF-1α*) gene mutations were supersensitive to treatment with sulfonylureas but responded poorly to treatment with metformin. In addition, research has shown that individuals with the TCF7L2 risk genotype respond poorly to treatment with sulfonylureas [61]. Variations in CYP2C9, an enzyme involved in sulfonylurea drug metabolism, KCNJ11, K+ inward rectifier Kir6.2, and the sulfonylurea receptors SUR1and ABCC8 are all genetic modifiers of the response to treatment with sulfonylurea. For thiazolidinediones, the focus has been on PPAR-γ variants, although the results are currently inconclusive. Despite these associations, the genetic data currently available are insufficient to support management decisions for the common forms of T2DM [62].

4. Conclusion

Results from large cohort studies will provide fundamental data that can be used to profile risk factors and discover novel therapeutic targets for patients. Tailoring therapy based on pharmacogenomic testing results may save lives and improve patient care. Finally, the decreasing price of new technologies that gather personal genomic information will facilitate their transition from basic research settings to the clinical setting, thereby reshaping clinical diagnostic paradigms. The challenge to healthcare teams is to consider how the new genomic information may be leveraged to influence management decisions and to fulfill the promise of personalizing medical care.

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Original article

Genetic variations within the PSORS1 region affect Kawasaki disease development and coronary artery aneurysm formation

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ABSTRACT

Background: Kawasaki disease (KD) is a pediatric systemic vasculitis, for which both genetic and environmental factors are suspected. HLA gene clusters within the major histocompatibility complex (MHC) region at chromosome 6p21.3 have been recently linked to KD. However, little was known about the roles of non-MHC genes during KD development. This study examines the association of psoriasis susceptibility 1 (PSORS1) genetic variants at the MHC region with KD development.

Methods: A total of 93 KD patients and 680 healthy children were enrolled. Three unique single nucleotide polymorphisms (SNPs) that cover *PSORS1C1*, *PSORS1C2*, and *CDSN* genes were genotyped by *Taqman* genotyping assay. The frequency of genetic variants was assessed by χ^2 analysis.

Results: Polymorphism rs1064190 located at the promoter region of *PSORS1C1* is found to be associated with KD development ($p = 0.026$) with higher T/T genotype frequency ($p = 0.006$) the T-A-T haplotype is more frequent in KD patients than normal controls ($p = 0.033$). In addition, T allele at rs1064190 shows a protective allele for coronary artery aneurysm (CAA) formation in KD patients ($p = 0.015$). The plasma activity of GOT/GPT, the indicators for KD heart damage, are significantly lower in patients with T/T genotype than in those with non-T/T genotype.

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Conclusion: Results suggest pivotal roles of polymorphisms within the PSORS1 region in KD pathogenesis. Since this region has been linked to psoriasis, this finding provides molecular evidence to suggest the possible linkage between KD and psoriasis.
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1. Introduction

Kawasaki disease (KD) is an acute, self-limited, and systemic vasculitis, a leading cause of acquired heart disease in children [1,2]. Clinical symptoms include skin rash, inflammation of mucous membranes in the mouth, nose, or throat, plus swollen lymph nodes with persistent high fever. During the acute stage, the activation of vascular endothelial cells and increased serum levels of proinflammatory cytokines are involved in the occurrence of inflamed and injured vessels. The inflammation of blood vessels can affect functions of coronary arteries and lead to worse complications. Cardiac sequelae, such as coronary artery aneurysm (CAA), are among the key aspects of this disease [3,4]. Patients with these cardiovascular complications are at increased risk of developing ischemic heart disease, which may lead to myocardial infarction and sudden death.

Currently, there is no specific test for KD because its etiology remains uncertain, although many clinical observations support the involvement of microorganism infection [5,6]. Molecular mimicry or superantigens from *Staphylococci* are suspected as the causes to trigger the activation of common immune pathways, resulting in inflammation [7]. Nevertheless, the epidemiological study indicates KD affecting all ethnic groups, yet it is more prevalent among the children of Asian and Pacific Island descent. Therefore, both host immune dysregulation and genetic susceptibility were suggested as key factors to determine KD development [8,9]. Genome-wide studies further provided cogent evidence that functional polymorphisms play important roles in KD development [10–12]. Variations in the genes involved in regulating immune functions and inflammation have been found related to KD susceptibility [13–15]. Some genes playing roles in cardiovascular pathogenesis, e.g., formation of CAAs, likewise also proved elemental to KD development [12,16].

However, the variations in the gene clusters within the major histocompatibility complex (MHC) region at chromosome 6p21.3 have been linked to dysregulated inflammation disorders such as immune-mediated vascular disease [17]. To date, human leukocyte antigen (HLA) genes within this region remain the best documented association for KD, e.g., variations in HLA-Bw22 (now designated Bw54), HLA-Bw51, and HLA-G [9,18]. Still, potential roles of other non-HLA candidate genes in the MHC region were not well studied in KD patients. The psoriasis susceptibility 1 (PSORS1) region is known as one major susceptibility loci on chromosome 6 located telomeric to HLA-C locus for psoriasis and psoriatic arthritis by a linkage disequilibrium mapping [19–21]. With the completion of the human genome project, at least 10 genes were defined in this region, while only certain genes can express detectable ribonucleic acid (RNA) and protein levels in skin cells, e.g., *PSORS1C1*, *PSORS1C1*, and *CDSN* [22,23]. Although the biological functions of these genes are largely unknown, sequence analysis revealed a highly polymorphic nature of the gene structure with more than 10 SNPs/kb, a common trait shared by HLA genes [24,25]. Therefore, it is widely believed that these genes may still perform critical roles in regulating T-cell responses to self-antigens and local immunological activation [22]. Interestingly, clinical investigations indicated that certain KD cases may develop psoriatic lesions, suggesting common pathogenic mechanisms shared by both these immune-mediated diseases [26–28]. Potential functions in immune regulation arouse interest in genetic variations in the PSORS1 region determining KD susceptibility, as proposed here. The linkage of genetic variation with CAA formation was also studied.

2. Materials and methods

2.1. Study population

A total of 93 patients who fulfilled diagnostic criteria for KD were identified at China Medical University Hospital from 1998 to 2005 and enrolled in this study. Clinical observation indicated that all the patients in this study underwent regular echocardiography examinations, beginning at the acute stage of KD, at 2 and 6 months after onset, and once a year thereafter. For the control group, DNA samples of 680 healthy children were randomly selected from the Han Chinese Cell and Genome Bank. Controls were matched for sex and age with patients. Since the estimated prevalence of KD in Taiwan is less than 1/1000 children, we assume that there are no KD cases in the control group. This study was approved by the Institutional Review Board at China Medical University, and informed consent was obtained from parents.

2.2. Genomic DNA extraction and genotyping of SNPs in the PSORS1 region

Genomic DNA was extracted from peripheral blood leukocytes via a standard protocol (Genomic DNA kit; Qiagen, Valencia, CA). DNA fragments containing rs1064190, rs1265099, and rs1265114 SNP sites were amplified by polymerase chain reaction (PCR) using *Taqman* SNP genotyping assay system from Applied Biosystems, Inc. (Carlsbad, CA). Probe search and design are available on their website (https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=ABGTKeywordSearch). Probe IDs for rs1064190, rs1265099, and rs1265114 were C-2436655-20, C-

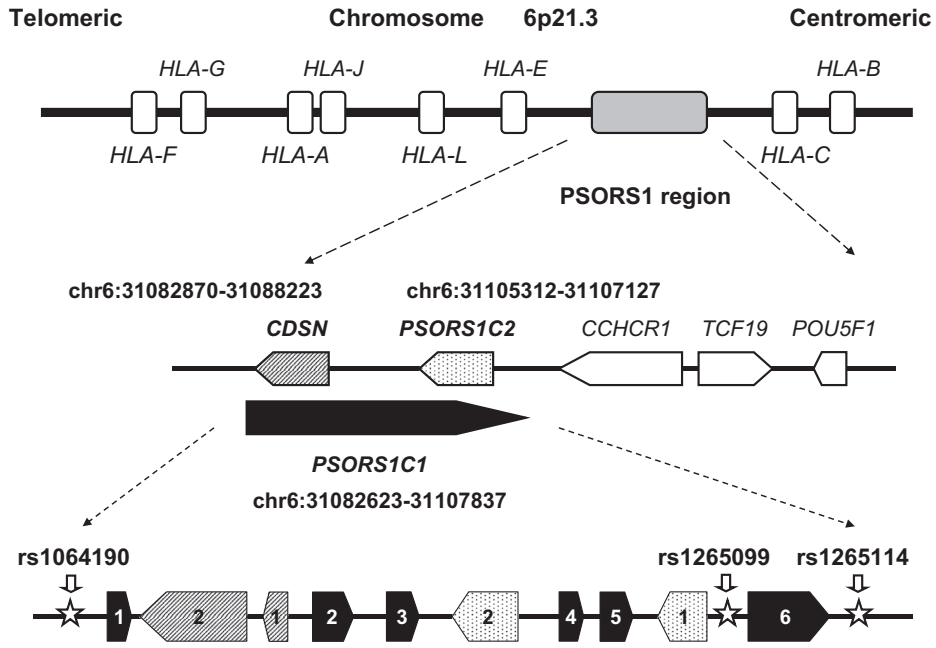

Fig. 1 – Gene map of a subsection of the PSORS1 region located at the MHC gene cluster region on chromosome 6p. *PSORS1C1* gene (black boxes) overlaps with *PSORS1C2* (spotted boxes) and *CDSN* (striped boxes) genes on 6p21.3. Unique SNP sites, rs1064190, rs1265099, and rs2076311, which cover these three genes were studied for association with KD development. Exons for each gene were numbered, albeit not to scale. KD = Kawasaki disease; MHC = major histocompatibility complex; PSORS1 = psoriasis susceptibility 1.

2438331-1, and Sheu-SNP-002, respectively. PCR amplification comprised initial denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 10 seconds, 56°C for 10 seconds, and 72°C for 20 seconds, with one additional cycle at 72°C for 5 minutes. Genetic variation was detected by reading fluorescence signals of PCR products. Positive signal indicates a perfect match between the probe and tested DNA, thus identifying wild-type alleles.

Table 1 – Polymorphisms within the PSORS1 region in KD and control.

SNP	Genotype/allele	Control	KD	KD vs. Control		
				p	OR	95% CI
rs1064190		(*n* = 659)	(*n* = 93)			
	TT	138 (20.9)	33 (35.5)	***0.006****	1.87	1.07–3.26
	GT	318 (48.3)	34 (36.6)		0.83	0.49–1.43
	GG	203 (30.8)	26 (28.0)		1.00	
	allele T	594 (45.1)	100 (53.8)	***0.026****	1.42	1.04–1.93
	allele G	724 (54.9)	86 (46.2)		1.00	
rs1265099		(*n* = 656)	(*n* = 92)			
	GG	135 (20.6)	21 (22.8)	0.849	1.20	0.65–2.21
	AG	321 (48.9)	45 (48.9)		1.08	0.64–1.80
	AA	200 (30.5)	26 (28.3)		1.00	
	allele A	591 (45.0)	87 (47.3)	0.568	1.09	0.80–1.49
	allele G	721 (55.0)	97 (52.7)		1.00	
rs1265114		(*n* = 664)	(*n* = 93)			
	TT	6 (0.9)	0 (0.0)	0.443	0.00	—
	CT	78 (11.7)	14 (15.1)		1.32	0.71–2.44
	CC	580 (87.3)	79 (84.9)		1.00	
	allele T	90 (6.8)	14 (7.5)	0.705	1.12	0.62–2.01
	allele C	1238 (93.2)	172 (92.5)		1.00	

Numbers in parentheses indicate the percentage of the genotypic or allelic frequency.
CI = confidence interval; KD = Kawasaki disease; OR = odds ratio; PSORS1 = psoriasis susceptibility 1; SNP = single nucleotide polymorphism.
*Numbers in bold italics indicate significant differences.

Table 2 – Polymorphisms within the PSORS1 region in KD patients and associations with CAA formation.

SNP	Genotype/allele	Control	KD-CAA (+)	KD-CAA (–)	KD-CAA (+) vs. Control			KD-CAA (–) vs. Control			KD-CAA (+) vs. KD-CAA (–)		
					p	OR	95% CI	*p*	OR	95% CI	*p*	OR	95% CI
rs1064190		(*n* = 659)	(*n* = 30)	(*n* = 63)									
	TT	138 (20.9)	11 (36.7)	22 (34.9)	0.623	1.35	0.58–3.14	***0.033****	2.31	1.14–4.67	0.109	0.58	0.2–1.68
	GT	318 (48.3)	7 (23.3)	27 (42.9)		0.37	0.14–0.96		1.23	0.63–2.40		0.30	0.1–0.94
	GG	203 (30.8)	12 (40)	14 (22.2)		1.00			1.00			1.00	
	allele T	594 (45.1)	29 (48.3)	71 (56.3)	0.619	1.14	0.68–1.91	***0.015****	1.57	1.09–2.27	0.305	0.72	0.39–1.34
	allele G	724 (54.9)	31 (51.7)	55 (43.7)		1.00			1.00			1.00	
rs1265099		(*n* = 656)	(*n* = 30)	(*n* = 62)									
	GG	135 (20.6)	8 (26.7)	13 (21)	0.712	1.48	0.54–4.04	0.972	1.07	0.51–2.26	0.830	1.38	0.41–4.65
	AG	321 (48.9)	14 (46.7)	31 (50)		1.09	0.45–2.65		1.07	0.58–1.97		1.02	0.36–2.89
	AA	200 (30.5)	8 (26.7)	18 (29)		1.00			1.00			1.00	
	allele A	591 (45.0)	30 (50)	57 (46)	0.451	1.22	0.73–2.05	0.844	1.04	0.72–1.50	0.608	1.18	0.63–2.18
	allele G	721 (55.0)	30 (50)	67 (54)		1.00			1.00			1.00	
rs2076311		(*n* = 664)	(*n* = 30)	(*n* = 63)									
	TT	6 (0.9)	0 (0)	0 (0)	0.150	0.00	—	0.739	0.00	—		—	—
	CT	78 (11.7)	7 (23.3)	7 (11.1)		2.26	0.94–5.45		0.93	0.41–2.11	0.123	2.43	0.77–7.73
	CC	580 (87.3)	23 (76.7)	56 (88.9)		1.00			1.00			1.00	
	allele T	90 (6.8)	7 (11.7)	7 (5.6)	0.146	1.82	0.8–4.11	0.599	0.81	0.37–1.79	0.140	2.25	0.75–6.72
	allele C	1238 (93.2)	53 (88.3)	119 (94.4)		1.00			1.00			1.00	

Numbers in parentheses indicate percentage of the genotypic or allelic frequency.
CAA = coronary artery aneurysm; CI = confidence interval; KD = Kawasaki disease; OR = odds ratio; PSORS1 = psoriasis susceptibility 1; SNP = single nucleotide polymorphism.
*Numbers in bold italics indicate significant differences.

Table 3 – Haplotype analysis within the PSORS1 region in KD patients and healthy control subjects.

Haplotype	Estimated haplotype frequency				*p*			
	Control ($n = 680$)	KD total ($n = 93$)	KD-CAA (+) ($n = 30$)	KD-CAA (−) ($n = 63$)	KD total vs. Control	KD-CAA (+) vs. Control	KD-CAA (−) vs. Control	KD-CAA (+) vs. KD-CAA (−)
G-A-C	29.1%	23.8%	18.5%	25.8%	0.132	0.077	0.440	0.274
G-A-T	1.0%	0.6%	0.6%	0.7%	0.584	0.798	0.747	0.976
G-G-C	21.6%	19.9%	16.6%	21.9%	0.587	0.350	0.949	0.399
G-G-T	3.3%	2.0%	0.9%	2.4%	0.347	0.305	0.591	0.488
T-A-C	24.9%	27.5%	31.5%	25.4%	0.455	0.247	0.903	0.380
T-A-T	0.1%	1.0%	1.1%	1.3%	***0.033****	0.082	***0.012****	0.923
T-G-C	17.6%	21.4%	26.7%	19.0%	0.212	0.074	0.704	0.231
T-G-T	2.3%	4.0%	4.0%	3.5%	0.183	0.418	0.404	0.882

Numbers in parentheses indicate the percentage of haplotype frequency.
CAA = coronary artery aneurysm; KD = Kawasaki disease; PSORS1 = psoriasis susceptibility 1.
*Numbers in bold italics indicate significant differences.

2.3. *Clinical symptoms and association study*

Clinical information of KD patients in this study was gleaned from clinical notes, e.g., blood tests, muscle function of heart, and fever duration. All the patients in this study were treated with intravenous immunoglobulin (IVIG; 2 g/kg infused over 8-12 hours) and oral aspirin (80-100 mg/kg/day); echocardiographs were obtained by the pediatric cardiologist before or within 14 days of IVIG administration. CAAs were diagnosed according to criteria proposed by the Japanese Kawasaki Disease Research Committee (Research Committee on KD). Coronary arteries were classified as abnormal if the internal lumen diameter was >3 mm in children younger than 5 years or >4 mm in children older than 5 years, the internal diameter of a segment measured ≥1.5 times that of an adjacent segment, or the coronary lumen was clearly irregular.

2.4. *Plasma activity assay of glutamic oxaloacetic transaminase and glutamic pyruvic transaminase*

Blood samples were collected from KD patients and subjected to plasma separation. Determination of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activity in plasma was conducted as per Reitman and Frankel [29] using kits purchased from RANDOX Laboratories (Antrim, UK). After adding the substrate, the developed color was measured using a spectrophotometer at a wavelength of 505 nm; the international activity unit of the sample was calculated by normalizing the data with the standard curve using aspartate as substrate for GOT and alanine as substrate for GPT.

2.5. *Statistic analysis*

Allelic and genotype frequency distributions for three polymorphisms of KD patients and controls were performed by χ^2 analysis with SPSS software (version 10.0, SPSS Inc., Chicago, IL), with $p < 0.05$ considered statistically significant. Allelic and genotype frequencies were expressed as percentages of total alleles and genotypes, The odds ratio (OR) derived from the allelic or genotype frequency with 95% confidence interval (95% CI). Haplotypes were determined by the Bayesian statistical method available in program Phase 2.1; adherence to Hardy-Weinberg equilibrium constant was tested via χ^2 test with one degree of freedom.

3. Results

The gene locus for the PSORS1 region has been mapped to the MHC region on chromosome 6p, a region linked to a range of autoimmune diseases. Within the MHC region, 201 reliable, polymorphic, and evenly spaced SNPs were previously genotyped [24]. Among these defined SNPs, rs1064190, rs1265099, and rs1265114 proved unique to the PSORS1 region covering *PSORS1C1*, *PSORS1C2*, and *CDSN* genes (Fig. 1). Notably, *PSORS1C1* genomic sequence overlaps with genes *CDSN* and *PSORS1C2* such that our study evaluated the association of these three genes with KD development. Genotyping PCR via *Taqman* system analyzed the genetic variants of these three SNPs in study subjects and controls. No significant differences in allele and genotype frequencies for rs1265099 and rs1265114 emerged, yet a significant difference was found in frequency T/T at rs1064190 ($p = 0.006$; OR = 1.87, 95% CI = 1.07-3.26). In addition, KD patients have a higher frequency of T allele at rs1064190 as compared to controls ($p = 0.026$, OR = 1.42, 95% CI = 1.04–1.93) (Table 1).

The formation of CAAs is a prime cause of heart attack and a significant risk of death or disability for KD patients. We aimed to understand the impact of polymorphisms in the PSORS1 region on CAA formation. As shown in Table 2, we analyzed genotype frequency as well as allele frequency at rs1064190, rs1265099, and rs1265114 with respect to the symptoms of CAA formation in KD patients. Data showed no links of rs1265099 or rs1265114 with CAA formation in KD patients, neither in genotype frequency (rs1265099: $p = 0.849$; rs1265114: $p = 0.443$) nor in allele frequency (rs1265099: $p = 0.568$; rs1265114: $p = 0.705$). However, polymorphisms at rs1064190 were found to determine CAA formation in patients. As compared with controls, KD patients not suffering from CAAs showed a greater tendency to carry the T/T genotype ($p = 0.033$; OR = 2.31, 95% CI = 1.14–4.67), as shown in Table 2. T allele at this SNP site was found to be protective against CAA formation in KD patients ($p = 0.015$; OR = 1.57,

Table 4 – Association between rs1064190 alleles and clinical parameters in children with KD CAA (+) and KD CAA (−).

Clinical parameters[a]	KD CAA (+)			KD CAA (−)			p/p_{adj}[b] KD CAA (+) vs. KD CAA (−)		
	TT ($n = 11$)	non-TT ($n = 19$)	Total ($n = 30$)	TT ($n = 22$)	non-TT ($n = 41$)	Total (n = 63)	TT	non-TT	Total
Age, y	1.45 ± 0.95	2.26 ± 2.11	1.88 ± 1.78	1.44 ± 0.97	1.9 ± 1.55	1.80 ± 1.43	0.989/1.0	0.489/1.0	0.825/1.0
WBC, $\times 10^3/mm^3$	16.63 ± 5.8	17.01 ± 6.37	15.94 ± 6.15	13.66 ± 6.11	13.35 ± 4.21	13.97 ± 5.09	0.227/1.0	0.021/0.231	0.159/1.0
Hemoglobin, g/dL	10.82 ± 1.32	11.11 ± 1.29	11.08 ± 1.31	11.29 ± 1.11	11.36 ± 0.94	11.31 ± 0.98	0.404/1.0	0.558/1.0	0.476/1.0
Platelet, $\times 10^3/mm^3$	436.67 ± 193.74	530.36 ± 266.89	475.79 ± 219.75	421.13 ± 149.65	370.67 ± 99.95	393.85 ± 131.80	0.843/1.0	0.018/0.198	0.098/1.0
ESR, mm/h	90 ± 34.95	92.42 ± 30.41	94.21 ± 27.53	81.06 ± 34.32	75.13 ± 33.13	76.33 ± 34.07	0.556/1.0	0.123/1.0	0.045/0.495
CRP, mg/dL	15.75 ± 4.97	13.66 ± 7.84	13.71 ± 7.15	6.55 ± 5.22	7.29 ± 4.88	7.80 ± 5.55	***<0.001/0.009****	***0.001/0.011****	***<0.001/0.009****
GOT, IU/L	48.63 ± 48.26	99.85 ± 123.51	88.00 ± 150.20	53.69 ± 55.63	89.89 ± 127.41	66.36 ± 81.36	0.829/1.0	0.815/1.0	0.155/1.0
GPT, IU/L	47.13 ± 36.36	79.5 ± 93.1	72.83 ± 145.59	38.31 ± 43.95	92.46 ± 117.08	56.74 ± 57.02	0.630/1.0	0.736/1.0	0.050/0.55
Fever duration (before IVIG)	6.67 ± 2.06	7 ± 2.61	7.30 ± 2.15	5.27 ± 0.96	5.94 ± 1.46	5.61 ± 1.37	0.033/0.363	0.084/0.924	***<0.001/0.008****
Fever duration (after IVIG)	1.22 ± 1.09	2.23 ± 3	1.95 ± 2.57	1.33 ± 2.13	1.28 ± 1.59	1.31 ± 1.72	0.886/1.0	0.198/1.0	0.237/1.0
Total fever duration	7.89 ± 1.83	9.23 ± 3.77	8.85 ± 3.45	6.6 ± 1.99	7.19 ± 2.07	6.71 ± 2.27	0.129/1.0	0.024/0.264	***0.003/0.033****

*Numbers in bold italics indicate significant differences.

CAA = coronary artery aneurysm; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; GOT = glutamate oxaloacetate transaminase; GPT = glutamic pyruvic transaminase; IVIG = intravenous immunoglobulin; KD = Kawasaki disease; WBC = white blood cell.

a Data for each group are expressed as mean ± SD.

b p_{adj} is adjusted p after Bonferroni correction, i.e., $p_{adj} = p \times 11$.

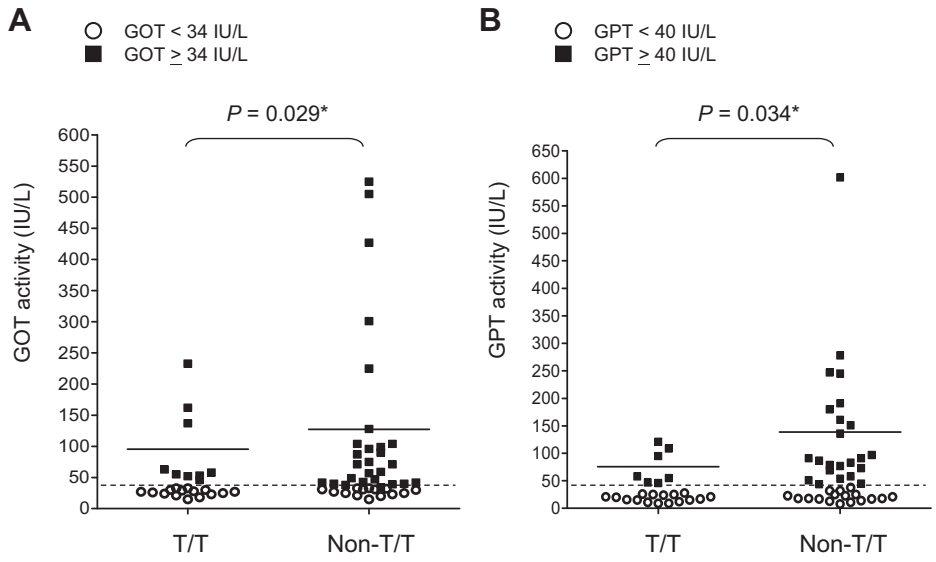

Fig. 2 – Analyses of plasma activity of (A) GOT and (B) GPT in KD patients subgrouped by genotype at SNP site rs1064190. Patients with normal plasma activity of GOT (< 34 IU/L) and GPT (< 40 IU/L) are represented using open circles; those with elevated activity of GOT and GPT, which are indicators for heart damage in KD patients, are represented using filled squares. *$p = 0.029$. **$p = 0.034$. GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase; KD = Kawasaki disease.

95% CI = 1.09–2.27). However, none of these can serve as a predictive genetic marker to differentiate patients with or without CAAs.

As genetic variations in the PSORS1 region were found to control KD development and CAA formation, it is interesting to rate the impact of diverse genetic combinations on KD patients. Table 3 plots haplotype frequencies using the three polymorphisms studied. Among eight haplotypes, the most common one in KD patients was T-A-C and that in normal controls was G-A-C. In particular, the T-A-T frequency was found to be higher in KD patients (1.0%) than that in normal controls (0.1%) ($p = 0.033$) and highly frequent in CAA-free KD patients (1.3%) ($p = 0.012$). The G-A-C haplotype appeared to be less in KD patients with CAA (18.5%) than that in normal controls (29.1%), although data did not show statistical significance ($p = 0.077$). In contrast, the frequency of the T-G-C haplotype was found to be higher in KD patients with CAA (26.7%) than that in controls (17.6%), but data were not statistically significant ($p = 0.074$).

Our data indicated rs1064190 SNP plays critical roles in KD development and CAA formation. We, therefore, would like to know whether genetic variations at this SNP site could also associate with clinical parameters critical for KD diagnosis. Table 4 shows KD patients with CAAs averaging higher C-reactive protein (CRP) level and longer fever duration time than those without CAAs, hinting at acute inflammation associated with CAA formation. Yet, the association of increased CRP and fever with CAA did not significantly differ between T/T and non-T/T (G/T or G/G genotype) subsets, maybe owing to relatively small case numbers (Table 4). Hence, we tried another approach to subgroup patients using cutoffs of 34 IU/L for GOT (Fig. 2A) and 40 IU/L for GTP (Fig. 2B), setting standards to define normal ranges of GOT and GPT activity in humans. Our data indicated the association of non-T/T genotype with the abnormal elevated serum activity of GOT ($p = 0.029$) and GPT ($p = 0.034$), which are key indicators for abnormal heart functions in KD patients. Data point to the likelihood that the T/T genotype may possess protective functions to reduce heart damage in KD patients, leading to lower GOP or GTP level.

4. Discussion

To the best of our knowledge, this is the first research to study the association of SNPs in the PSORS1 region and KD development. Among the three SNP sites studied, rs106419 located at the promoter region of the *PSORS1C1* gene (psoriasis susceptibility 1 candidate 1, also known as *SEEK1*) was found to play crucial roles in KD development. Patients with T/T versus G/T or G/G genotypes have a higher probability of developing KD. Interestingly, the T/T genotype is a protective factor associated with a low frequency of CAA formation and heart damage. Considering the haplotype frequencies between the case and control groups, KD patients with the T-A-T haplotype appeared to be significantly "at-risk" compared with other haplotypes. Such data suggest functional roles of genes within the PSORS1 region in KD, also revealing that genetic variations in non-MHC genes at chromosome 6p21.3 could contribute to KD susceptibility. Thus, other non-MHC genes in this susceptible region might also play roles in KD development. With limited sample size in this study, further research must ascertain how these non-MHC genes work with MHC genes in regulating immune functions and KD development.

Besides genetic variations, recent studies indicated that microorganism infection triggered KD [5–7]. Certain subsets

of T-lymphocytes, e.g., V-beta 2+ and V-beta 8+, were constitutively activated in KD patients, suggesting a systemic immunoactivation caused by superantigens [30]. With KD more prevalent among children of Asian and Pacific Island descent, interactions/combinations between genetic and environmental factors may play important roles to control the susceptibility to KD. The genetic variations in the PSORS1 region have been associated with psoriasis and psoriatic arthritis on the basis of a genome-wide association study [19–21]. Our findings provide molecular evidence for the linkage between these kinds of autoimmune diseases. In fact, KD is described in association with the development of several psoriasiform eruptions [26–28]. A number of studies discovered bacterial antigens from *Staphylococcus aureus* and *Streptococcus pyogenes* as common causes for the activation of autoimmune response in both KD and psoriasis patients [28,31,32]. It is, therefore, not surprising that genetic variations in the PSORS1 region can also determine the etiology of KD. Our findings and prior studies support a hypothesis that KD and certain types of psoriasis share common pathogenic mechanisms in disease development [28,33–35]. Currently, it is not clear why genetic variations in the *PSORS1C1* gene contribute to KD development. Previous studies have demonstrated *PSORS1C1* gene as the key gene associated with psoriasis or psoriatic arthritis in the PSORS1 region [23,36], indicating novel functions of PSORS1C1 in inducing autoimmunity. Since the PSORS1C1 protein is extremely variable owing to the highly polymorphic gene structure and splicing variants [23], probing additional SNPs in this gene may clarify susceptibility to KD. Further functional studies may reveal details of mechanisms controlled by PSORS1C1 variants during KD development.

In summary, our study identified an SNP in the PSORS1 region that contributes to KD susceptibility in Taiwanese children of Han Chinese ethnic background. We observed significant association between the *PSORS1C1* gene polymorphism and the occurrence of cardiac artery aneurysm in KD patients. Our data showed that the plasma activity of GOT and GPT was significantly lower in KD patients with the T/T genotype at this SNP site than in those with the G/T or G/G genotype. Results suggest the polymorphism of *PSORS1C1* gene playing roles in KD pathogenesis.

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Original article

Appearance of acanthosis nigricans may precede obesity: An involvement of the insulin/IGF receptor signaling pathway

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ABSTRACT

Background: Obesity is one of the main causes of preventable death. Complications of childhood obesity include cardiovascular risk, impaired glucose tolerance, type 2 diabetes mellitus, and acanthosis nigricans (AN; associated with obesity as a manifestation of cutaneous insulin resistance). An interaction between AN and obesity as well as a detailed mechanism for the pre- and co-obesity appearance of AN in children are still to be revealed.

Purposes: This research tries to assess involvement of the insulin/insulin-like growth factor (IGF) receptor pathway in childhood pre- and co-obesity AN via a study of the association of polymorphisms of the INSR, IRS1, and IGF1R genes with pre- and co-obesity AN.

Methods: In total, 99 children with pre- and co-obesity AN and 100 healthy controls were genotyped and analyzed by the polymerase chain reaction-restriction fragment length polymorphism method.

Results: Intergroup frequency differed starkly for INSR His1085His and IGF1R IVS7-20, but not in the IRS1 Ala804Ala or IGF1R Thr766Thr genotypes. The T allele of INSR His1085His and C allele of IGF1R IVS7-20 both conferred a starkly(p=0.04 and 2.84E-6 = 2.84×10^{-6}, respectively)higher risk for AN.

Conclusion: The above findings suggest that certain genetic variants in insulin/insulin-like growth factor (IGF) receptor pathway might be correlated with the appearance of AN prior to or concurrent with obesity, and also reveal the insulin/IGF receptor pathway as crucial in pre- and co-obesity AN.

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1. Introduction

Obesity is one of the leading causes of preventable death. With the increasing intake of high-calorie food and a sedentary lifestyle, pediatric obesity looms ever more prevalent and is exerting a major impact on public health in the 21st century. This universal trend of pediatric obesity is reported not only in Occidental countries (up to 25% of children in the USA), but also in Oriental nations like Taiwan [1]. There is no denying that obese children tend to become obese adults, especially those whose obesity lasts to their adolescence. Complications of obesity include cardiovascular risk, hypertension, dyslipidemia, endothelial dysfunction, type 2 diabetes mellitus and impaired glucose tolerance, acanthosis nigricans (AN), hepatic steatosis, precocious puberty, hypogonadism and polycystic ovary syndrome, obstructive sleep disorder, orthopedic complications, cholelithiasis, and pseudotumor cerebri [2–4].

AN is a hyperpigmented, velvety cutaneous thickening easily observed on certain parts of the body, including the axillae, sides of the neck, groin, antecubital and popliteal surfaces, umbilical area, and, in more severe cases, even spread over the whole body and mucosal surface. In the literature, AN is reported to be closely associated with obesity as a manifestation of cutaneous insulin resistance [5]. In addition, endocrinopathies, malignancy (most frequently gastric carcinoma in adults), genetic syndromes, and the use of drugs may also lead to the development of AN [6–10].

From a clinical viewpoint, it is taken for granted that obesity often comes prior to the appearance of AN of cutaneous insulin resistance. Interestingly, from questionnaires given to child patients with AN that we met in the China Medical University Hospital, a large proportion (about 50%) of their AN syndromes appeared prior to or together with obesity, which we will describe as pre- and co-obesity AN (PCOAN).

This clinical observation may challenge the traditional rationale that elevated insulin concentration owing to excessive weight gain and subsequent insulin resistance in obese people results in both direct and indirect activation of insulin-like growth factor (IGF-1) receptors on keratinocytes and fibroblasts, leading to epidermal proliferation and the appearance of AN [11]. Insulin and leptin resistance have been proved to be responsible for a failure of appetite and suppression of excessive energy intake [12]. Also, defective insulin binding and post-insulin receptor function, plus genetic defects within the insulin receptor gene, have been documented in patients with AN [13–16].

From our clinical experience and limited previous reports, we hypothesized that differences in the genetic background of the insulin/IGF receptor and its associated signaling pathway in these children with PCOAN should play a key role in excessive weight gain and AN, no matter which occurred first. Insulin resistance itself may precipitate excessive weight gain via a failure to prevent redundant energy intake, facilitating the concurrent emergence of AN arising from epidermal proliferation. Fig. 1 plots an overall flowchart of our hypothesis.

To understand the genomic role of insulin/IGF receptor pathway-related genes in PCOAN, we chose four single nucleotide polymorphisms (SNPs) from three genes—*INSR* His1085His (rs1799817), *IRS1* Ala804Ala (rs1801123), *IGF1R* IVS7-20 (rs2272037), and *IGF1R* Thr766Thr (rs3743262)—and probed their genotypic distribution in a Taiwanese child population with PCOAN.

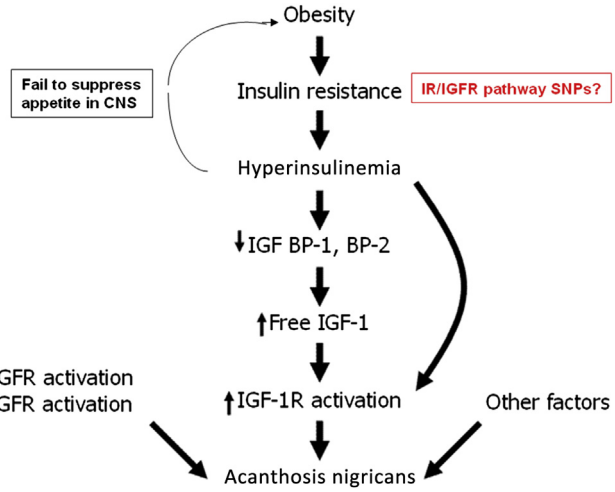

Fig. 1 – Postulated mechanisms for the pathogenesis of pre- and co-obesity acanthosis nigricans. CNS = central nervous system; EGFR = epidermal growth factor receptor; FGFR = fibroblast growth factor receptor; IGF = insulin-like growth factor; IGFR = insulin-like growth factor receptor; IGF-1R = insulin-like growth factor receptor 1; IR = insulin receptor; SNP = single nucleotide polymorphism. *Note.* From Acanthosis nigricans: A practical approach to evaluation and management, by S.P. Higgins, M. Freemark, and N.S. Prose, 2008, *Dermatol Online J* 14(9), p.2. Credit to S.P. Higgins, Copyright 2008, Name of Copyright Holder: The Regents of the University of California, Davis campus, UCDHS Department of Dermatology.

2. Materials and methods

2.1. Study population and sample collection

The children and adolescents (5–18 years old) were recruited to the study group from the Department of Pediatrics at the China Medical University Hospital in central Taiwan. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared. All participants with a BMI above the 95th percentile for age and gender were classified as obese on the basis of BMI category by the Department of Health of Taiwan. A morning serum after an 8-hour fast was obtained for glucose and insulin measurement. Insulin resistance was determined using the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) index in the fasting state. Detailed medical and family histories were obtained for all participants, and physical examinations were performed. All the participants showed normal thyroid and adrenal function. Controls, enrolled from the Department of Family Medicine, were free of obesity, AN, and other obesity-related diseases. Each person donated samples with informed consent. This study was approved by the Institutional Review Board Committee of China Medical University Hospital.

2.2. Genotyping assays

Genomic DNA was prepared from peripheral blood leukocytes by a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan), and then processed as reported in previous studies [17–25]. Briefly, the following primers were used: *INSR* C3255T rs1799817, 5′-TTGGGTGAGGGCTTGGGTGGAAG-3′ and 5′-CCTGTGTCCTCT GTCGCTCTGTG-3′; *IRS1* C2412T rs1801123, 5′-CTCCTACTACT CATTGCCAAG-3′ and 5′-CAGACAAGTAGCCAGACTGAT-3′; *IGF1R* IVS7-20(C/T) rs2272037, 5′-GACCTCCCATTATAGAAA GTG-3′ and 5′-CCAGTGAGCTTGCGAAGAAG-3′; and *IGF1R* C2298T rs3743262, 5′-TCCACGGTTA AGATTCTTCTG-3′ and 5′-TCCACTAGGTTGTGAGGAAG-3′.

The following cycling conditions were performed: one cycle at 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; and final extension at 72°C for 10 minute. Polymerase chain reaction (PCR) products, except *IGF1R* IVS7-20(C/T) rs2272037, were studied after digestion with MspA1I, HphI, and DraIII, restriction enzymes for *IRS1* C2412T rs1801123 (cut from 189+257 bp A type into 125+132+189 bp G type), *IGF1R* C2298T rs3743262 (cut from 432 bp T type into 181+251 bp C type), and *INSR* C3255T rs1799817 (cut from 418 bp T type into 92+326 bp C type), respectively. The PCR product of *IGF1R* IVS7-20(C/T) rs2272037 was purified using QIAEX II (Qiagen, Hilden, Germany) and applied to direct sequencing for SNP type detection. Direct sequencing used a BigDye 3.1 Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with an ABI 3100 Genetic Analyzer (Applied Biosystems).

2.3. Statistical analyses

Our study selected only those matches with all SNP data (case/control = 99/100) for final analysis. Pearson's two-sided Chi-square or Fisher's exact test was used to compare the genotype distribution between cases and controls. Data were recognized as statistically significant at $p < 0.05$.

3. Results

Table 1 outlines the clinical characteristics and analysis of the 99 child patients with PCOAN and the 100 healthy controls, groups similar in gender at enrollment. The control group were of a greater age, but even so had a lower BMI, serum fasting glucose, insulin level and HOMA score ($p < 0.005$), i.e., had higher insulin sensitivity. Defining insulin resistance as a fasting insulin level above 15 μIU/ml, none of the controls but 61 of the 99 patients in the PCOAN group were insulin-resistant. Fourteen patients had impaired fasting glucose, and four fulfilled the criteria for diabetes (Table 1). These differences are detailed in the Discussion section.

Table 2 shows the genotype frequencies of *INSR* His1085His (rs1799817), *IRS1* Ala804Ala (rs1801123), *IGF1R* IVS7-20 (rs2272037), and *IGF1R* Thr766Thr (rs3743262) in the controls and patients with PCOAN. The genotype distributions of various genetic polymorphisms of *INSR* His1085His and *IGF1R* IVS7-20 differed significantly in PCOAN patients versus controls ($p = 0.046$ and 8.77E-6 = 8.77×10^{-6}, respectively), whereas those for *IRS1* Ala804Ala or *IGF1R* Thr766Thr did not ($p > 0.05$) (Table 2). The distributions of *INSR* His1085His *C homozygote/heterozygote/T homozygote in the controls and patients with PCOAN were 36.0%/53.0%/11.0% and 18.2%/61.6%/20.2%, respectively (Table 2). The proportions of IRS1 Ala804Ala *C homozygote/heterozygote/T homozygote in controls and patients with PCOAN were 53.0%/30.0%/9.0% and 39.4%/47.5%/13.1%, respectively (Table 2). The proportions of IGF1R IVS7-20 *A homozygote/heterozygote/G homozygote in controls and patients with PCOAN were 36.0%/51.0%/13.0% and 80.8%/18.2%/1.0% respectively (Table 2). The *IGF1R* Thr766Thr *C homozygote/heterozygote/T homozygote proportions in controls and patients with PCOAN were 43.0%/40.0%/17.0% and 32.6%/46.5%/20.9%, respectively (Table 2). Polymorphisms of *IGF1R* IVS7-20 and *INSR* His1085His thus correlate significantly with PCOAN.

Table 3 shows the frequencies of *IRS1* Ala804Ala (rs1801123), *IGF1R* IVS7-20 (rs2272037), *IGF1R* Thr766Thr (rs3743262), and *INSR* His1085His alleles (rs1799817) for controls and patients with PCOAN. Allele frequency distributions of *INSR* His1085His and *IGF1R* IVS7-20 *C are associated with higher susceptibility to PCOAN. Distributions of the *INSR* His1085His C/T allele in controls and patients with PCOAN

Table 1 – Clinical and biochemical features of the pre-obesity and co-obesity AN patients (PCOAN) and control groups.

	PCOAN group	Control group	p
Age	10.8 (4–18)	24 (19–40)	<0.0001
Sex (male/female)	55/44	50/50	0.435
BMI (kg/m^2)	30.7 (22.4–44.2)	20.1 (18–22)	<0.0001
Fasting glucose (mg/dL)	101.1 (77–327)	81.5 (64–96)	<0.0001
Fasting insulin (μIU/ml)	21.1 (3.9–77.9)	10.3 (6.3–12.4)	<0.0001
HOMA-IR	5.4 (3.7–21.3)	1.8 (0.8–2.6)	<0.0001

Data are presented as mean (range).
HOMA-IR = Homeostasis Model Assessment for Insulin Resistance.

Table 2 – Distributions of *IRS1*, *INSR*, and *IGF1R* genotypes among pre-obesity and co-obesity AN patients and control groups.

Genotype	Controls	Patients	p[a]
INSR His1085His (rs1799817)			0.0462
CC	36 (36.0)	18 (18.2)	
CT	53 (53.0)	61 (61.6)	
TT	11 (11.0)	20 (20.2)	
IRS1 Ala804Ala (rs1801123)			0.2516
AA	53 (53.0)	39 (39.4)	
AG	38 (38.0)	47 (47.5)	
GG	9 (9.0)	13 (13.1)	
IGF1R IVS7-20 (rs2272037)			8.77E-6 = 8.77×10^{-6}
CC	36 (36.0)	80 (80.8)	
CT	51 (51.0)	18 (18.2)	
TT	13 (13.0)	1 (1.0)	
IGF1R Thr766Thr (rs2272037)			0.4253
CC	43 (43.0)	28 (32.6)	
CT	40 (40.0)	40 (46.5)	
TT	17 (17.0)	18 (20.9)	

Data are presented as n (%).

Table 3 – Distributions of *IRS1*, *INSR*, and *IGF1R* alleles among pre-obesity and co-obesity AN patients and control groups.

Allele	Controls	Patients	*p*
INSR His1085His (rs1799817)			0.0255
Allele C	125 (62.5)	97 (49.0)	
Allele T	75 (37.5)	101 (51.0)	
IRS1 Ala804Ala (rs1801123)			0.1025
Allele A	144 (72.0)	125 (63.1)	
Allele G	56 (28.0)	73 (36.9)	
IGF1R IVS7-20 (rs2272037)			2.84E-6 $= 2.84 \times 10^{-6}$
Allele C	123 (61.5)	178 (89.9)	
Allele T	77 (38.5)	20 (10.1)	
IGF1R Thr766Thr (rs2272037)			0.2043
Allele C	126 (63.0)	96 (55.8)	
Allele T	74 (37.0)	76 (44.2)	

Data are presented as *n* (%).

were 62.5%/37.5% and 49.0%/51.0%, respectively (Table 3). Distributions of the *IRS1* Ala804Ala A/G allele in controls and patients with PCOAN were 72.0%/28.0% and 63.1%/36.9%, respectively. The proportions of the *IGF1R* IVS7-20 C/T allele in controls and patients with PCOAN were 61.5%/38.5% and 89.9%/10.1%, respectively. Distributions of the *IGF1R* Thr766Thr C/T allele in controls and patients with PCOAN were 63.0%/37.0% and 55.8%/44.2%, respectively.

4. Discussion

AN reportedly shows a strong ethnic influence and is common in children and adolescents, especially among populations with high rates of adult diabetes [26,27]. However, two recent reports have challenged those who describe AN as a reliable predictor of hyperinsulinemia [28,29]. Hirschler et al reported the BMI of patients with AN as being significantly greater than that of participants without AN, whereas neither fasting immunoreactive insulin nor the HOMA-IR index differed in Hispanic individuals [28]. A Japanese study found a significant difference between AN-positive and AN-negative groups in the duration of obesity, as well as the age and percent overweight [30]. We proposed a clinical observation in a large proportion of PCOAN patients that AN may occur prior to or concurrent with the appearance of obesity, and then investigated its possible mechanism via a pioneering genetic approach.

According to the results shown in Table 1, we can provide some explanations and annotations for the data presented and the stories behind them. Both groups had a similar gender distribution, which prevents gender bias: females were reported to have a higher peripheral insulin sensitivity than males [31]. The PCOAN group had a younger age and higher serum fasting glucose and insulin levels than the control group. Blunted insulin sensitivity in the PCOAN group supports our hypothesis that PCOAN may stem from an indigenous genetic difference in the insulin/IGF receptor pathway, since insulin sensitivity tends to decline with age [32]. In our cohort, 30% of patients with PCOAN showed no insulin resistance, implying that other factors (such as epidermal growth factor receptor, fibroblast growth factor receptor, and leptin) play a key role in such non-insulin-resistant patients [33]. By contrast, the use of fasting serum insulin level or HOMA-IR as an indicator of insulin resistance may be inappropriate if the glucose clamp test is viewed as a gold standard. However, it is nigh on impossible to perform a clamp test in obese children, owing to the parents' wishes and technical aspects [34]. The PCOAN group manifested a higher HOMA-IR index than the controls, i.e., higher insulin resistance. No consensus was reached on a cut-off value for HOMA-IR in obese children, but a value above 3 was generally considered to denote insulin resistance [35,36].

Previous genetic studies of AN have focused largely on the β-adrenergic receptor and discuss the association of AN with obesity, cardiovascular disease, and type 2 diabetes [37–47]. Our paper first evaluated another pathway closely related to obesity—the insulin/IGF receptor—and its genetic association with PCOAN. Of the four SNPs probed in this study, we can report the variant *INSR* His1085His and *IGF1R* IVS7-20 genotypes as being positively correlated with susceptibility to PCOAN (see Tables 2 and 3). This suggests that both insulin and IGF receptor subpathways are involved in PCOAN, in addition to involvement of their downstream genes and crosstalk between them, meriting further investigation.

At the cellular molecular level, insulin interacts with not only the insulin receptor, but also the IGF receptor, activating downstream effectors and even crosstalking with each other [48]. Along with the aforementioned insulin resistance that might result in failure to suppress excessive energy intake, with ensuing obesity, this concept fits our results showing a strong correlation of the insulin/IGF receptor pathway with PCOAN. It is well known that most obesity may originate from a disturbed interaction between genetics and the environment that cannot be explained by a single factor; our study specified one genetic factor, related to the insulin/IGF receptor pathway, as playing a particular role in the complex pathogenesis of obesity, under scrutiny of the patients' phenotype and genotype association.

Future molecular studies are needed to elucidate the complex relationship between polymorphisms and PCOAN. A genetic population study of AN, like an association study, can derive correlations among AN and other diseases, such as obesity and diabetes mellitus. Our preliminary data not only provide evidence that the T allele of *INSR* His1085His and C allele of *IGF1R* IVS7-20 are correlated with the appearance of AN proceeding or concurrent with obesity, but also reveal that the insulin/IGF receptor pathway may play a leading role in PCOAN in Taiwan.

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Original article

Association analysis between Tourette's syndrome and two dopamine genes (DAT1, DBH) in Taiwanese children

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ABSTRACT

Background: Recent research suggests that Tourette's syndrome (TS) may result from a defect in the dopamine system. Several candidate gene polymorphisms have been implicated in attention deficit hyperactivity disorder, including the dopamine transporter (*DAT1*) and dopamine β-hydroxylase (*DBH*) genes. A high rate of comorbidity between attention deficit hyperactivity disorder and TS indicates that they may share the same pathophysiology.
Purpose: We aimed to test the hypothesis that the dopamine gene might play a role in TS.
Methods: An association study, using an independent sample of patients from the midland region of Taiwan, was performed to investigate whether *DAT1* and *DBH* gene polymorphisms can be used as markers of susceptibility to TS. A total of 160 children with TS and 83 normal control participants were included in the study. Polymerase chain reaction was used to identify polymorphisms in the *DAT1* (40 bp VNTR) and *DBH* (TaqI A2) genes. Genotypes and allelic frequencies for the *DAT1* and *DBH* gene polymorphisms in both groups were compared.
Results: The results showed that genotypes and allelic frequencies in both groups were not significantly different. The most common genotype for *DAT1* (40 bp VNTR) was the 10,10 homozygote in both groups. The most common genotype for *DBH* (TaqI A2) was the T homozygote in both groups.
Conclusion: These data suggest that the *DAT1* and *DBH* genes may not be useful markers to predict susceptibility to TS.
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1. Introduction

Gilles de la Tourette syndrome (TS) is a neuropsychiatric disorder characterized by both motor and vocal tics. In addition, affected individuals frequently display symptoms such as attention deficit hyperactivity disorder (ADHD) and/or obsessive–compulsive disorder. In the 1970s, investigators first demonstrated that TS has a familial concentration [1]. TS was

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then shown to be transmitted vertically from generation to generation, and studies of twin pairs confirmed a genetic influence [2,3]. To date, the gene search in TS has been unsuccessful [4], which is illustrative of the many factors that can complicate genetic analysis of complex human traits [5].

The pathogenesis of TS remains obscure. Current evidence suggests that TS may result from a defect in the dopamine system [6–10]. Studies have focused mainly on the dopamine transporter gene [*DAT1* 40 bp variable tandem nucleotide repeat (VNTR)], and the dopamine beta hydroxylase gene (*DBH TaqI* A2) in ADHD [11–13]. ADHD is common in TS probands and is reported to affect about 50–70% of referred TS cases [14–16]. These observations led us to test the polygenic hypothesis by examining the potential effect of *DAT1* and *DBH* in TS. We previously used single nucleotide polymorphisms (SNPs) as a tool in genetic studies of polygenic disorders [17–21]. SNPs are markers that may provide a new way to identify complex gene-associated diseases such as TS. In this study, we tested the hypothesis that genetic variation in the *DAT1* (40 bp VNTR) and *DBH* (*TaqI* A2) genes confers susceptibility to TS. Two SNP markers have been identified in these genes, allowing researchers to detect disease-causing gene associations [22].

2. Materials and methods

The study included Taiwanese children with TS ($n = 100$ in the *DAT1* group and $n = 160$ in the *DBH* group, respectively) and normal control participants ($n = 83$). This study was approved by the Ethics Committee of the China Medical University Hospital, Taichung, Taiwan. All parents signed informed consent before blood tests were performed. TS patients and the controls were both recruited from the midland regions of Taiwan. Diagnosis of TS followed the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) [23]. The criteria for TS are as follows: the presence of multiple motor and at least one vocal tic (not necessarily concurrently); a waxing and waning course with tics evolving in a progressive manner; the presence of tic symptoms for at least 1 year; the onset of symptoms before 21 years of age; the absence of a precipitating illness (e.g., encephalitis, stroke, or degenerative disease) or medication; the observation of tics by a knowledgeable neurologist; and marked distress or significant impairment in social, occupational, or other important areas of functioning. A pediatric neurologist (I-C.C.) examined the children and made sure that all cases were unrelated. The 83 controls were healthy volunteers with no history of psychiatric treatment.

All children underwent peripheral blood sampling for genotype analyses. Genomic DNA was isolated from peripheral blood by mean of a DNA extractor kit (Genomaker DNA extraction kit; Blossom, Taipei, Taiwan). A total of 50 ng of genomic DNA was mixed with 20 pmol of each polymerase chain reaction (PCR) primer in a total volume of 25 μL containing 10 mM Tris-hydrochloride, pH 8.3; 50 mM potassium chloride; 2.0 mM magnesium chloride; 0.2 mM each deoxyribonucleotide triphosphate; and 1 U of DNA polymerase (Amplitaq; Perkin Elmer, Foster City, CA, USA). Four PCR primers were used to amplify the correlated gene. The sequences of these primers were as following (from the 5′ to 3′ end): *DBH* (444 g/a): upstream, CCTGGAGCCCAGTGCTTGTC; downstream, ACGCCCTCCTGGGTACTCGC; and *DAT1*: upstream, TGTGGTGTAGGGAACGGCCTGAGA; downstream, AAATTCCAGTGGGGTCCCTTCCTG. The PCR conditions were as follows: 35 cycles of: 95°C for 30 seconds, 60°C for *DBH* (444 g/a) or 66.5°C for *DAT1* for 30 seconds, and 72°C for 45 seconds, followed by 72°C for 7 minutes, and then held at 4°C. The polymorphisms were analyzed by PCR amplification followed by restriction analysis with *Eco*NI for *DBH* (444 g/a). The PCR products were directly analyzed on 3% agarose gel by electrophoresis, and each allele was identified according to its size.

Allelic frequencies were expressed as a percentage of the total number of alleles. The genotypes and allelic frequencies for *DAT1* and *DBH* polymorphisms in both groups were compared. Using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) with the χ^2 test was used for statistical analyses. A p value of <0.05 was considered statistically significant.

3. Results

Genotype proportions and allele frequencies for *DAT1* and *DBH* were not significantly different between the groups (Tables 1 and 2). The most common genotype for *DAT1* was the 10,10 homozygote in both groups. The allele 10 frequency for *DAT1* in TS patients was 87% and in controls it was 88.6% (Table 1).

The most common genotype for *DBH* was the T homozygote in both groups. The proportions of T homozygotes, T/C heterozygotes, and C homozygotes for *DBH* were: in TS patients, 75.6%, 22.5%, and 1.9%, respectively; and in controls,

Table 1 – Genotypes for DAT1 (40 bp VNTR) polymorphisms in children with Tourette's syndrome and in normal individuals.

	Tourette patients, n (%) ($n = 100$)	Controls, n (%) ($n = 83$)	p
Genotype			
11,11	1 (1)	0	0.795
10,13	0	1 (1.2)	
10,11	2 (2)	2 (2.4)	
10,10	75 (75)	66 (79.5)	
10,9	19 (19)	11 (13.3)	
10,8	1 (1)	0	
10,7	1 (1)	1 (1.2)	
10,6	1 (1)	0	
9,9	0	1 (1.2)	
9,7	0	1 (1.2)	
Allelic frequency			
Allele 13	0	1 (0.6)	0.858
Allele 11	4 (2)	2 (1.2)	
Allele 10	174 (87)	147 (88.6)	
Allele 9	19 (9.5)	14 (8.4)	
Allele 8	1 (0.5)	0	
Allele 7	1 (0.5)	2 (1.2)	
Allele 6	1 (0.5)	0	

The p-values were calculated using the χ^2 test.

Table 2 – Genotypes and allele frequencies for DBH (TaqI A2) polymorphisms in children with Tourette's syndrome and in normal individuals.

	Tourette patients, *n* (%) (*n* = 160)	Controls, *n* (%) (*n* = 83)	*p*
Genotype			
T/T	121 (75.6)	65 (78.3)	0.900
C/T	36 (22.5)	17 (20.5)	
C/C	3 (1.9)	1 (1.2)	
Allelic frequency			
Allele T	278 (86.9)	147 (88.6)	0.700
Allele C	42 (13.1)	19 (11.4)	

The *p*-values were calculated using the χ^2 test.

78.3%, 20.5%, and 1.2%, respectively. The allele T and C frequencies for DBH in TS patients were 86.9% and 13.1%, respectively; and in controls, 88.6% and 11.4%, respectively (Table 2).

4. Discussion

Dopamine transport was first described 40 years ago [24]. *DAT* was itself identified and its molecular structure described more than 20 years later [25]. The human *DAT* gene is localized on chromosome 5p15.3 [26]. A genetic polymorphism of a 40 bp VNTR polymorphic sequence in the 3′ untranslated region of exon 15 of the gene has been described [27]. This VNTR of exon 15 is repeated 3–11 times, most typically 10 times. The 10-repeat shows ethnic heterogeneity with a frequency of 0.7 among Caucasians and Hispanics in the USA, 0.54 in African Americans, and 0.9 in Asians [28–30]. *DATs* are expressed in a small number of neurons in the brain, mainly in the striatum and nucleus accumbens, but also in the globus pallidus, cingulate cortex, olfactory tubercle, amygdala, and midbrain [31]. *DAT*, like the transporters for norepinephrine and serotonin, is a Na^+/Cl^- dependent transmembrane transport protein [32] which regulates the concentration of dopamine in the synaptic cleft.

DBH appears to be a strong candidate for investigation in TS, because it catalyzes the conversion of dopamine to norepinephrine and therefore influences both the dopaminergic and adrenergic systems. Serum *DBH* levels are under strong genetic control and show large interindividual variation [30]. Alleles of several polymorphisms at the DBH locus have been found to be associated with serum *DBH* levels.

In the present study we did not find significant evidence for association in our TS samples. The role of the dopaminergic system in the pathogenesis of TS is still not known. Preliminary studies have suggested that the pathogenesis of tics involves neuronal activity within subcortical neuronal circuits [33]. Therefore, this raises the possibility that classic neurotransmitters, dopamine and serotonin, may be involved in the pathobiology of TS. However, other investigators have emphasized that abnormalities of dopamine fail to explain many clinical and laboratory observations, including the description of unchanged tics in four adults who developed parkinsonism and received treatment with L-dopa [34].

Our review of the literature found that recent linkage studies have not provided any positive results regarding: dopamine D1-5 receptors [35–37], glycine α-1 subunit, GABAA receptor α-1, α-6, and γ-2 subunits (GABRA1, GABRA6, GABRG2), GABAA receptor β-1 and α-2 subunits (GABRB1, GABRA2), glutamate receptor GLUR1, the α-adrenergic receptor ADRA1, the β-adrenergic receptor ADRB1, and the glucocorticoid receptor GRL [38]; norepinephrine transporter gene [39]; or catechol-o-methyltransferase [40]. Other investigators have sought to identify associations between TS and other movement disorders [41]. Further studies will be required to confirm these assertions.

The etiology of TS is therefore unknown. In fact, TS in children may involve a complex interaction between environmental influences, especially infection, autoimmune contributions, epigenetic factors, and genetic factors. Our study suggests that the *DBH* and *DAT1* genes may not contribute to the etiology of TS. Further studies could focus on the analysis of other dopaminergic genes in TS patients. Our results could provide the database for a further survey of *DBH* and *DAT1* gene polymorphisms.

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Original article

Increased incidence of Parkinsonism among Chinese with β-glucosidase mutation in central Taiwan

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ABSTRACT

Parkinson's disease (PD) is characterized by progressive neuronal cell loss and decline in movement. Recently, much attention has been focused on dopaminergic neurotoxicity, which causes neurogeneration in the nervous system, thus implicating dysfunction in predisposition to PD pathogenesis. The enzyme deficient in Gaucher's disease, β-glucosidase (GBA), has been cited as being linked with PD. This study investigated whether a mutation in GBA is associated with PD patients in central Taiwan; a GBA mutation was detected by polymerase chain reaction and sequencing in 148 patients with PD and 120 normal controls. The results revealed a significant difference between PD patients and normal controls in GBA mutation and a statistical correlation between GBA mutation L444P and PD formation. It could be concluded that patients who carry the T/C of GBA mutation, L444P, have a higher risk of developing PD in Chinese patients in middle Taiwan.

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1. Introduction

Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic neurons and the presence of intracellular Lewy body inclusions within the *substantia nigra*. The presence of age-associated cytopathogenic process is required for a definite diagnosis. Clinically, PD patients exhibit at least one of resting tremor, muscle rigidity, bradykinesia, and postural instability, which are features of neurodegenerative disorder [1,2]. Patients with familial PD provide a good insight for examination of candidate genes; however, the incidence of PD pathogenesis is still too complex to unify the common mechanisms of etiologies. In particular, the major causes of the disease occur in a sporadic manner, possibly suggesting

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that the susceptibility of PD is involved in a wide spectrum of genetic and environmental factors.

Current theories of PD pathogenesis are based on available data from familiar histories of the disease. Mutations in five causal genes—α-synuclein, parkin, DJ-1, PINK1, and leucine-rich repeat kinase 2 (LRRK2) [3–9]—have been identified as molecular events increasing risk of disease. Evidence for the association of the PD candidate gene *LRRK2*, which encodes the protein kinase dardarin and is located within *PARK8* locus on chromosome 12p11.2-q13.1 [4,5], is growing. Genetic evidence of its linkage to hereditary late-onset PD was originally detected in a Japanese family [4,10]. Then, as currently identified from a rare familial pedigree of PD, finding several *LRRK2* gene mutations revealed it as causing autosomal-dominant familiar Parkinsonism [4,5].

The gene encoding β-glucosidase (also known as glucocerebrosidase; GBA1) has been localized to chromosome 1q21 [11]; a highly homologous pseudogene sequence exists at 16 kb downstream [11,12]. More than 200 mutations, point mutations, deletions, insertions, splicing aberrations, and various rearrangements have been identified within the *GBA* gene [13–15]. Environmental factors are known to play a role in PD pathogenesis [16,17]. This case–control association study explored a hypothesis of common coding variation within *GBA* gene also tending toward susceptibility of sporadic PD among Chinese in central Taiwan. In the search for variants, we undertook the potentially functional mutation for frequent analysis in sporadic forms of PD cases and ethnically-matched controls among the Taiwanese population.

2. Patients and methods

2.1. Patients

A standard clinical examination was performed on every participant, and PD diagnosis was confirmed according to published neurological criteria [1,2,18]. The cohort enrolled 148 PD cases and 120 healthy controls from the same population in central Taiwan. Patients were of Taiwanese ethnicity, with sporadic PD based on pedigree assay, as each member in the absence of family history was observed.

2.2. PCR amplification

PCR amplification of the DNA fragments covering mutation L444P of GBA was conducted from genomic DNA of patients and controls; PCR amplified the DNA fragments of GBA according to Tsai et al [9]. Briefly, PCR reaction was performed under the conditions: PCR carried out in total volume of 50 μL, containing 50 ng genomic DNA, 1× Taq polymerase buffer, 5 pmol of each primer, and 0.25 U of‘ AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA, USA). Amplification conditions were: (1) denaturation at 95°C for 4 minutes; (2) 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds; and (3) final elongation at 72°C for 10 minutes. Preventive contamination entailed PCR reaction mixture without DNA (negative control) in each run of amplification.

2.3. DNA sequencing

All PCR fragments were purified by QIAEX II (Qiaqgen, Hilden, Germany) and directly sequenced to identify mutations. The direct sequencing was achieved by an ABI Prism 3100 DNA sequencer (PE Applied Biosystems) with the BigDye Dideoxy Terminator Sequencing Kit (PE Applied Biosystems).

2.4. Statistical analysis

The parameters were compared using Fisher's exact test, with $p < 0.05$ statistically significant. SPSS software (version 10.0; SPSS Inc., Chicago, IL, USA) was used to performed calculations.

3. Results

The L444P mutation was screened by PCR and sequencing. The screening of L444P mutation showed all patients carrying PD hetero- or homozygote genotype in their *GBA* gene. Table 1 plots genotype distribution and allelic frequencies for L444P mutation in patients and controls. Significant differences arose between patients and controls ($p < 0.001$) in L444P genotype distribution, with T/C homozygote distribution in patients (4.51%) higher than in controls (0%). Also, allelic frequency of L444P mutation showed statistical significance, distinguishing PD patients from controls ($p = 0.048$; Fisher's exact test).

4. Discussion

This study analyzes the *GBA* gene in PD among the Chinese population of central Taiwan. Data indicate GBA mutation L444P is associated with PD. Since significance is dominant, this association is not likely to stem from a chance event. Earlier study showed L444P mutation as second most common Gaucher's disease (GD) mutation among non-Jewish patients, accounting for 37% of total mutations surveyed [19,20]. In our research, L444P accounts for 4.51% of PD cases. A cause for the linkage of mutant *GBA* with Parkinsonism remains unclear; further studies must identify the pathological mechanism. Recently, the mutation in *GBA* was noted in relation to

Table 1 – Distribution of genotypes among Parkinson's disease (PD) patients and healthy controls.

	PD patients	Normal controls	*p*
	n (%)	*n* (%)	
Genotype			
T/T	148 (95.48)	120 (100)	<0.001
T/C	7 (4.51)	0 (0)	
C/C	0 (0)	0 (0)	
Allelic frequency			
Allele T	303 (97.74)	240 (100)	0.048
Allele C	7 (2.26)	0 (0)	

p values calculated by Fisher's exact test.

synucleinopathies. Some studies pay attention to this association [21,22]; others find a correlation between Parkinsonism and the *GBA* mutation, the gene encoding the enzyme deficient in autosomal, recessively-inherited lysosomal storage, also known as GD. Rare GD cases developing Parkinsonism have been identified [23,24]. Neuropathological evaluation of several such patients showed intraneuronal inclusions with α-synuclein in brain regions related to GD [24,25]. Additional studies focusing on diverse cohorts with Parkinsonism indicate higher frequency of specific *GBA* mutations. Increasing *GBA* mutations in PD, identification of mutations in the spectrum of synucleinopathy [26], and higher frequency of Parkinsonism among GD patients [25] led us to research this correlation further, via the genetic approach. Our analysis lends compelling evidence of the *GBA* gene L444P mutation contributing significant association, thereby implicating it as one possible genetic risk factor for sporadic PD and accounting for higher prevalence of disease in allele frequencies. These data may extend the survey of GBA to other populations for sporadic form of PD. Detailed study of molecular mechanisms must explore the role of GBA and related drug development in PD.

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Original article

Seasonal variation of birth defects in Norway

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ABSTRACT

Background: Seasonal variation in the occurrence of birth defects provides indirect evidence of the causal role of environmental factors, because genetic factors do not exhibit seasonality.
Aim: This study was undertaken to assess the seasonal variation of birth defects in Norway.
Methods: We conducted a nationwide cross-sectional study of 326,560 births in years 1993–1998, using information from the Medical Birth Registry in Norway. We applied the Lorenz curve and associated Gini index and its 95th percentiles from 10,000 Monte Carlo simulations to identify specific birth defects and birth defect groups with statistically significant seasonal variation. For identified outcomes we applied logistic regression analysis to quantify deviations of risk in high and low peak months.
Results: The Gini index indicated statistically significant seasonal variation ($\alpha = 0.05$) for any birth defect, 0.040 (95th percentile = 0.024), respiratory defects, 0.140 (95th percentile = 0.141), and for Down syndrome, 0.148 (95th percentile = 0.126). Based on logistic regression adjusting for maternal age, parity, centrality, population density, and industrial profile, highest risk for respiratory defect was among infants born in March (adjusted odds ratio [OR] 1.82, 95% confidence interval [CI] 1.33–2.50), and for Down syndrome in February (adjusted OR 1.64, 95% CI 1.21–2.22) compared to risks of infants born in other months.
Conclusion: Findings suggest that environmental factors with seasonal variation play a role in the etiology of respiratory defects and Down syndrome.
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1. Introduction

Accumulating evidence indicates both genetic and environmental factors playing roles in etiology of birth defects [1]. It is very likely that multilevel interaction exists between genetic and environmental factors [2]. Seasonal variation in the occurrence of birth defects yields indirect evidence of a causal role of environmental factors such as prenatal exposure to disinfection by-products, which has been reported to exhibit seasonality [3].

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A series of epidemiologic studies have assessed seasonal variation of birth defects [4–30]. Seasonal variation in occurrence of neural tube defects has received the most attention, but studies conducted in diverse regions provide conflicting results. Studies in the United Kingdom [8], Newfoundland [9], and South Africa [14] reported significant seasonal variation in occurrence of neural tube defects; research in Canada [6], Utah [7], South America [11], Italy [12], Japan [20], and Northern Germany [21] found none. Others report seasonal variation in occurrence of oesophagial atresia [12], diaphragmatic hernia [12], cleft lip [22,27,28], anomaly of pulmonary valule [29], ventricular septal defects [25,30], and Down syndrome [17].

We previously reported relations between exposure to disinfection by-products and risk of birth defects, in particular neural tube, cardiac, respiratory system, and urinary tract defects [31,32]. Among specific birth defects, only risk of ventricular septal defect was significantly elevated with an exposure response pattern [32]. Elaboration of seasonal variation of these and other birth defects would provide additional insight into the role of environmental factors. We thus evaluated seasonal variation in occurrence of birth defects in Norway, using population-based information on all births registered by the nationwide Medical Birth Registry for the years 1993–1998.

2. Methods

2.1. Study population

The source population comprised all 361,767 newborns registered by the Norwegian Birth Registry from 1993 to 1998. We excluded 35,207 (9.7%) due to incomplete information on gestational age. The study population included 326,560 (90.3%) term births, with study protocol approved by the Institutional Review Board of Bloomberg School of Public Health at Johns Hopkins University, in compliance with principles outlined in the Helsinki Declaration.

2.2. Birth defects

We focused on the most common specific birth defects and five groups of defects: neural tube, cardiac, respiratory, oral cleft, and urinary tract defects. These were used in the previous study of Norwegian births [14].

All births after the 16th week of gestational age are compulsorily reported to the Medical Birth Registry. During the child's 1st week of life, a physician (usually a pediatrician) makes diagnoses of birth defects, which are recorded in the registry. Hence, birth defects diagnosed later in life are excluded from the registry. According to the International Classification of Diseases, Eighth Revision (ICD-8), up to three birth defects are coded for each child.

2.3. Covariates

We used routine birth registry data to construct covariates: maternal age (younger than 20 years; 20–34 years; age 35 years or older), and parity (0; 1; 2; and $\geq$3 previous deliveries). We received municipal-level data from the Norwegian Social Science Data services, to construct three municipal level indicators of socioeconomic status: centrality, population density, and industrial profile. Centrality means urbanity and geographical placement in relation to a regional center. In the current analyses, we divided data into three levels, low (municipalities with urban centers up to 15,000 residents), medium (urban areas up to 50,000 residents), and high (includes a regional center). Population density is the proportion of urban population in a municipality. We categorized the data as: (1) <20%; (2) 20–39.9%; (3) 40–59.9%; (4) 60–79.9%; and (5) 80% or more. Industrial profile indicates relative distribution of trade in a municipality, given by three levels: mainly agriculture/fisheries (low), mainly industry (medium), and mainly services (high).

2.4. Statistical methods

We applied the Lorenz curve and associated Gini index described by Lee [33] to assess seasonal variation of birth defects. This method is more sensitive to minute temporal changes, its power relatively higher than that of other commonly used seasonality tests, such as χ^2 goodness-of-fit, Edwards, Roger, and Kuiper. Analyses proceeded in three phases: (1) construction of the Lorenz curve, (2) calculation of the Gini index, and (3) iteration of phases one and two using smoothing techniques.

The main parameter in analyses was the monthly birth defect ratio (R_i), calculated for each of 12 months by dividing number of cases (C_i) occurring in a given month i during 6 years by number of days (D_i) in the corresponding month in the same time period (Table 1). First, we ranked the months according to the monthly birth defect ratio from lowest to highest. We constructed the Lorenz curve by plotting cumulative percentage of cases in rank order (y-axis) against cumulative percentage of days (x-axis). The area between a diagonal line and curve (A_s) quantifies seasonality; i.e., deviation from homogeneous monthly birth defect ratio (Fig. 1).

The Gini index was defined as two times A_s, varying from 0 representing no seasonal variation, to 1 with maximal seasonal variation. We used 10,000 Monte Carlo simulations for each sample size to derive approximate Gini index distribution describing chance variation of the Gini index and to define statistical significance of observed seasonal variation. We used the 95th percentile Gini index value to assess statistical significance at the $\alpha = 0.05$ level.

Monthly birth defect ratios are subject to substantial chance variation due to the relatively small numbers of cases. We used a smoothing technique to reduce chance variation. We first calculated the 3-month moving average R_i for each month, with two weighting schemes (1/3, 1/3, 1/3 and 1/4, 2/4, 1/4), then used the smoothed R_i to derive expected cases for each month. Gini indices were defined for both weighting schemes, as described previously (Gini-1 and Gini-2).

When seasonal variation was identifed by Gini indices, we used the prevalence odds ratio to quantify timing of the peak and amplitude in seasonal variation. We compared the risk of birth defects in each month to the rest of the months, applying logistic regression to estimated odds ratios adjusted for possible confounding factors such as maternal age, parity, centrality, population density, and industrial profile of the municipality where the mother lived during pregnancy.

Table 1 – Monthly ratio of respiratory defects and corresponding 3-month moving average in Norway 1993–1998.

Month	No. of cases (C_i)	Number of days (D_i)	Monthly birth defect ratio (R_i)	Three-month moving average			
				with weights: 1/3,1/3,1/3		with weights: 1/4, 2/4, 1/4	
				Monthly birth defect ratio (R_{i1})	No. of cases (C_{i1})	Monthly birth defect ratio (R_{i2})	No. of cases (C_{i2})
Jan	28	186	0.15054	0.14806	27.54	0.14868	27.65
Feb	26	169	0.15385	0.18390	31.08	0.17639	29.81
Mar	46	186	0.24731	0.17261	32.11	0.19128	35.58
Apr	21	180	0.11667	0.16613	29.90	0.15376	27.68
May	25	186	0.13441	0.13184	24.52	0.13248	24.64
Jun	26	180	0.14444	0.13596	24.47	0.13808	24.85
Jul	24	186	0.12903	0.14134	26.29	0.13826	25.72
Aug	28	186	0.15054	0.13208	24.57	0.13669	25.43
Sep	21	180	0.11667	0.13029	23.45	0.12688	22.84
Oct	23	186	0.12366	0.10048	18.69	0.10627	19.77
Nov	11	180	0.06111	0.10818	19.47	0.09642	17.35
Dec	26	186	0.13978	0.11714	21.79	0.12280	22.84
Total	305	2191			303.9		304.2

3. Results

3.1. *Any birth defect*

Among 326,560 births in the study population during 1993–1998, we identified 10,207 births (3.13%) with a birth defect of interest. Table 2 plots the number and prevalence (%) of birth defects, empirical Gini indices and 95th percentile Gini index values from Monte Carlo simulations. The Gini index for any birth defect was 0.039 (95th percentile = 0.024). The Gini index was larger than the 95th value from the Monte Carlo simulation indicating a significant seasonal variation, at the 0.05 level. To reduce sampling variation of R, we used the smoothing technique to Gini-1 and Gini-2. Values for Gini-1 and Gini-2 are also given in Table 2. In general, test statistics for Gini, Gini-1, and Gini-2 were similar. Based on the month of birth, Table 3 shows statistically significant increases in risk for any birth defect in February (adjusted odds ratio [OR] 1.13, 95% confidence interval [CI] 1.06–1.22) and October (adjusted OR 1.09, 95% CI 1.02-1.17). Fig. 2 graphs seasonal variation of birth defect by month of birth.

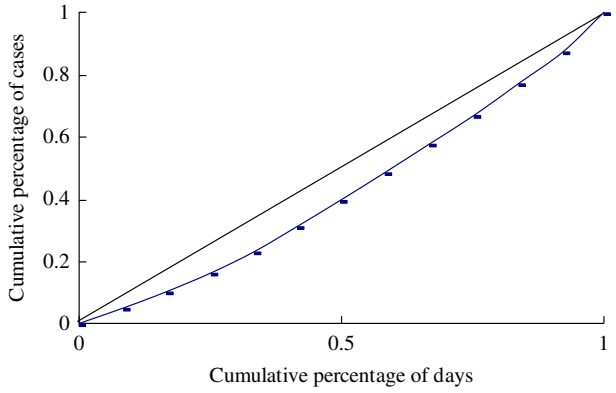

Fig. 1 – Lorenz curve of respiratory defects in Norway during 1993–1998.

3.2. *Neural tube defects*

We identified 250 births (0.08%) with neural tube defects: 87 (0.03%) anecephalus, 21 (0.01%) encephalocele, and 142 (0.04%) spina bifida cases. Gini index of neural tube defects (Table 2) was estimated as 0.112 (95th percentile = 0.156), lower than 95th percentile of 10,000 Monte Carlo simulations with sample size 246. Gini index for anencephalus was 0.144 (95th percentile = 0.261), for encephalus 0.316 (95th percentile = 0.507), and for spina bifida 0.110 (95th percentile = 0.205). Thus there was no significant seasonal variation of these defects (Table 2).

3.3. *Cardiac defects*

A total of 931 cardiac defects (0.29%) were identified; Table 2 shows no substantial seasonal variation (Gini = 0.056, 95th percentile = 0.081). Nor did seasonal variation appear in specific cardiac defects: transposition of great vessels (Gini = 0.232, 95th percentile = 0.318), left heart ventricular hyposplasia (Gini = 0.196, 95th percentile = 0.305), tetralogy of Fallot (Gini = 0.332, 95th percentile = 0.400), ventricular septal defet (Gini = 0.093, 95th percentile = 0.113), and atrial septal defect (Gini = 0.143, 95th percentile = 0.217).

3.4. *Respiratory defects*

In all, 305 infants (0.09%) were identified with respiratory defects. The Gini-2 index for respiratory defects was 0.101 (95th percentile = 0.088), showing that a null hypothesis of no seasonal variation was rejected at $\alpha = 0.05$. The test statistic for Gini-1 was 0.098 (95th percentile = 0.082), consistent with Gini-2, but for Gini borderline seasonal variation was shown (Gini = 0.140, 95th percentile = 0.141). Thus, respiratory defects exhibited significant seasonal variation, with the highest risk in March (adjusted OR 1.82, 95% CI 1.33–2.50) and lowest risk in November (adjusted OR 0.46, 95% CI 0.25–0.83), as shown in Fig. 3.

Table 2 – Seasonal variation of birth defects in Norway 1993-1998. Gini index magnitude indicates probability of observed seasonal variation; 95% percentiles represent limits of statistical significance on $\alpha = 0.05$.

Outcomes			Gini	Gini-1	Gini-2
Any birth defect					
N, Prevalence (%)	10,207	3.13			
Index value			0.040*	0.024*	0.027*
95th percentile			0.024	0.014	0.015
Neural tube defects					
N, Prevalence (%)	250	0.08			
Index value			0.112	0.034	0.039
95th percentile			0.156	0.093	0.099
Anencephalus					
N, Prevalence (%)	87	0.03			
Index value			0.144	0.057	0.067
95th percentile			0.261	0.155	0.164
Encephalocele					
N, Prevalence (%)	21	0.01			
Index value			0.316	0.183	0.192
95th percentile			0.507	0.310	0.329
Spina bifida					
N, Prevalence (%)	142	0.04			
Index value			0.110	0.043	0.050
95th percentile			0.205	0.121	0.129
Cardiac defects					
N, Prevalence (%)	931	0.29			
Index value			0.056	0.030	0.035
95th percentile			0.081	0.048	0.051
Transposition of great vessels					
N, Prevalence (%)	58	0.02			
Index value			0.232	0.110	0.123
95th percentile			0.318	0.189	0.201
Tetralogy of Fallot					
N, Prevalence (%)	36	0.01			
Index value			0.332	0.134	0.164
95th percentile			0.400	0.241	0.257
Ventricular septal defect					
N, Prevalence (%)	472	0.14			
Index value			0.093	0.038	0.041
95th percentile			0.113	0.066	0.071
Atrial septal defect					
N, Prevalence (%)	126	0.04			
Index value			0.143	0.067	0.078
95th percentile			0.217	0.128	0.137
Respiratory defects					
N, Prevalence (%)	305	0.09			
Index value			0.140	0.098*	0.101*
95th percentile			0.141	0.082	0.088
Oral cleft defects					
N, Prevalence (%)	631	0.19			
Index value			0.067	0.043	0.044
95th percentile			0.098	0.057	0.061
Cleft palate					
N, Prevalence (%)	183	0.06			
Index value			0.136	0.090	0.094
95th percentile			0.181	0.106	0.114
Cleft lip					
N, Prevalence (%)	133	0.04			
Index value			0.182	0.112	0.120
95th percentile			0.211	0.124	0.132
Cleft palate with cleft lip					
N, Prevalence (%)	315	0.10			
Index value			0.126	0.040	0.037
95th percentile			0.138	0.081	0.086
Urinary tract defects					
N, Prevalence (%)	399	0.12			
Index value			0.119	0.068	0.071
95th percentile			0.122	0.072	0.076
Renal agenesis					
N, Prevalence (%)	55	0.02			
Index value			0.227	0.105	0.123
95th percentile			0.326	0.196	0.208
Cystic kidney disease					
N, Prevalence (%)	72	0.02			
Index value			0.244	0.144	0.152
95th percentile			0.286	0.169	0.180
Obstructive defects of urinary tract					
N, Prevalence (%)	183	0.06			
Index value			0.153	0.086	0.092
95th percentile			0.181	0.107	0.114
Esophageal atresia					
N, Prevalence (%)	54	0.02			
Index value			0.267	0.128	0.140
95th percentile			0.327	0.194	0.207
Diaphragmtica hernia					
N, Prevalence (%)	82	0.03			
Index value			0.188	0.077	0.088
95th percentile			0.269	0.159	0.169
Down syndrome					
N, Prevalence (%)	381	0.12			
Index value			0.148**	0.094**	0.103**
95th percentile			0.126	0.074	0.079

*$p < 0.05$; **$p < 0.01$.

3.5. *Oral cleft defects*

Overall, 631 births (0.19%) with oral cleft defects were identified, including 183 cleft palate (0.06%) and 133 cleft lip cases (0.04%), and 315 cases (0.10%) with both cleft palate and cleft lip. The Gini index of oral cleft defects (Table 2) was estimated as 0.067 (95th percentile = 0.098), for cleft palate, 0.136 (95th percentile = 0.181), for cleft lip, 0.182 (95th percentile = 0.211), and for cleft palate with cleft lip, 0.126 (95th percentile = 0.138). There was no significant seasonal pattern.

3.6. *Urinary tract defects*

We found 399 urinary tract defects (0.12%) in the study population, with no significant seasonal variation (Gini = 0.119, 95th percentile = 0.122) (Table 2). Consistently, the effect on specific urinary tract defects, renal agenesis (Gini = 0.227, 95th percentile = 0.326), cystic kidney disease (Gini = 0.244, 95th percentile = 0.286), and obstructive defects of the urinary tract (Gini = 0.153, 95th percentile = 0.181) was lower in estimation of Lorenz curve and associated Gini index compared with 95th percentile of 10,000 Monte Carlo simulations with each sample size of 55, 72, and 183, respectively.

3.7. *Down syndrome*

A total of 381 newborns (0.12%) were identified with Down syndrome; Gini index (Gini = 0.148; 95th percentile = 0.126) showed the null hypothesis of no seasonal variation rejected at $\alpha = 0.05$. Test statistics for Gini-1 (Gini-1 = 0.094; 95th percentile = 0.074), and Gini-2 (Gini-2 = 0.103; 95th

Table 3 – Prevalence odds ratio of birth defects by month of birth in Norway, 1993–1998.

Birth defects	N	P (%)	cOR (95% CI)	aOR (95% CI)
Any birth defect	10,207	3.13		
January	883	3.27	1.05 (0.98-1.13)	1.05 (0.98-1.12)
February	898	3.48	1.13 (1.05-1.21)	1.13 (1.06-1.22)
March	926	3.18	1.02 (0.95-1.09)	1.02 (0.95-1.09)
April	833	2.85	0.90 (0.84-0.97)	0.90 (0.83-0.96)
May	917	3.18	1.02 (0.95-1.09)	1.02 (0.95-1.10)
June	830	2.98	0.95 (0.88-1.02)	0.95 (0.88-1.02)
July	848	2.97	0.94 (0.88-1.01)	0.94 (0.88-1.01)
August	826	3.02	0.96 (0.90-1.03)	0.96 (0.90-1.04)
September	842	3.09	0.99 (0.92-1.06)	0.98 (0.91-1.06)
October	899	3.41	1.10 (1.03-1.18)	1.09 (1.02-1.17)
November	793	3.24	1.04 (0.97-1.12)	1.04 (0.97-1.12)
December	712	2.90	0.92 (0.85-0.99)	0.92 (0.85-1.00)
Respiratory defects	305	0.09		
January	28	0.10	1.12 (0.76-1.65)	1.12 (0.76-1.66)
February	26	0.10	1.09 (0.73-1.62)	1.09 (0.73-1.64)
March	46	0.16	1.81 (1.32-2.48)	1.82 (1.33-2.50)
April	21	0.07	0.75 (0.48-1.17)	0.76 (0.49-1.18)
May	25	0.09	0.92 (0.61-1.39)	0.92 (0.61-1.39)
June	26	0.09	1.00 (0.67-1.49)	1.00 (1.67-1.49)
July	24	0.08	0.89 (0.59-1.35)	0.89 (0.59-1.35)
August	28	0.10	1.11 (0.75-1.63)	1.10 (0.75-1.63)
September	21	0.08	0.81 (0.52-1.27)	0.81 (0.52-1.26)
October	23	0.09	0.93 (0.61-1.42)	0.92 (0.60-1.41)
November	11	0.04	0.46 (0.25-0.84)	0.46 (0.25-0.83)
December	26	0.11	1.15 (0.77-1.71)	1.14 (0.77-1.71)
Down syndrome	381	0.12		
January	35	0.13	1.12 (0.79-1.59)	1.08 (0.76-1.53)
February	48	0.19	1.68 (1.24-2.28)	1.64 (1.21-2.22)
March	39	0.13	1.16 (0.83-1.62)	1.15 (0.83-1.61)
April	32	0.11	0.93 (0.65-1.34)	0.93 (0.65-1.33)
May	35	0.12	1.04 (0.74-1.48)	1.03 (0.73-1.46)
June	31	0.11	0.95 (0.66-1.37)	0.76 (0.66-1.38)
July	34	0.12	1.02 (0.72-1.45)	1.04 (0.73-1.48)
August	19	0.07	0.57 (0.36-0.91)	0.58 (0.37-0.92)
September	20	0.07	0.61 (0.39-0.95)	0.59 (0.37-0.94)
October	39	0.15	1.30 (0.93-1.81)	1.29 (0.92-1.81)
November	26	0.11	0.90 (0.61-1.35)	0.93 (0.62-1.38)
December	23	0.09	0.79 (0.52-1.21)	0.83 (0.54-1.26)

Logistic regression analysis adjusted for maternal age, parity, centrality, population density, and industrial profile of municipality where the mother lived during pregnancy.
aOR = adjusted odds ratio; cOR = crude odds ratio.

percentile = 0.079) were consistent with Gini. Down syndrome occurred most often in February (adjusted OR 1.64, 95% CI 1.21–2.22), and least frequently in August (adjusted OR 0.58, 95% CI 0.37-0.92) and September (adjusted OR 0.59, 95% CI 0.37–0.94), as shown in Fig. 4.

4. Discussion

Based on Monte Carlo simulation, there was a statistically significant seasonal variation in the occurrence of any birth defect, respiratory defects, and Down syndrome. As a rule, birth defects occurred more often in February and October.

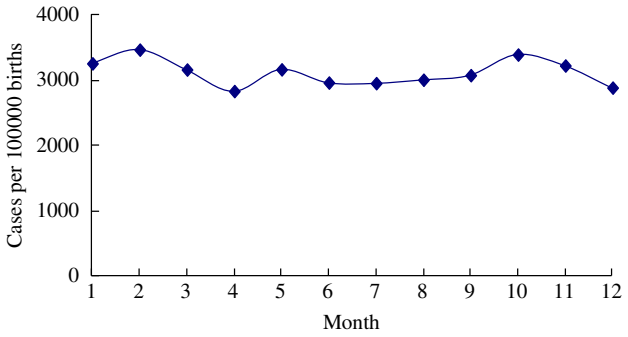

Fig. 2 – Seasonal variation of any birth defect in Norway, 1993–1998.

The highest occurrence of respiratory defects was in March, and Down syndrome in February. This seasonal variation of birth defects may imply an effect of environmental factors such as prenatal exposure to disinfection by-products [3] or viral infections [17,24,25], which are potential determinants of birth defects and known to exhibit seasonality. Taking into account length of gestation, the highest peak of conception of respiratory defects for all births over 28 weeks' gestation was in June and during the summer months. Interestingly, disinfection by-products might vary seasonally and increase with temperature [3]. The etiology of Down syndrome is still controversial and diffcult to understand. There are two possible explanations related to seasonal variation of Down syndrome. One can be expected as a consequence of seasonal variation in hormone production by the hypothalamus–pituitary–ovarian axis [19,34]; another is that the fetal brain is much more sensitive to viral infection during the first few months of gestation [17]. Further research should elaborate on whether a pregnant woman's exposure to disinfection by-products or virus infections is responsible for this seasonality.

4.1. *Validity of results*

The Medical Birth Registry supplied health information on large numbers of newborns, making it possible to assess seasonal variation of relatively rare birth defects. We excluded approximately one-tenth of these births due to insufficient gestational age data. This exclusion was not likely to introduce selection bias; characteristics of excluded individuals did

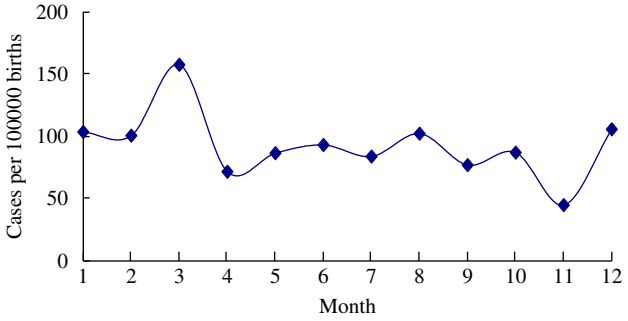

Fig. 3 – Seasonal variation of respiratory defects in Norway, 1993–1998.

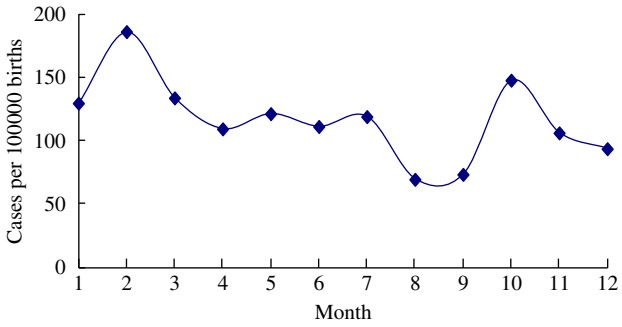

Fig. 4 – Seasonal variation of Down syndrome in Norway, 1993–1998.

not differ substantially from those included (data not shown). Because date of birth does not reflect the actual time period when the defect was induced and some defects also cause reduced length of gestation, we also estimated date of conception to evaluate the seasonal variation.

The issue of multiple comparisons should be considered when interpreting results of specific birth defects. In the current study of 32 comparisons (32 types of diagnostic defect and 1 seasonal pattern), two to three statistically significant associations at the 0.05 level would be found by chance alone [35,36]. Weak associations are more likely due to chance than strong associations. Thus, weak seasonal variation in occurrence of hydrocephalus could arise from multiple comparisons. Each reported association must be considered in light of previous epidemiologic and toxicologic evidence. This study had limited power to detect some of the rarer defects: e.g., only 21 of 326,560 newborns developed encephalocele.

Misclassification of birth defects is a potential source of random error, because diagnosis of birth defects is difficult due to the rarity of each condition. In general, these birth defects may be underreported, because we only included those diagnosed within the first week of life. However, we have no reason to believe that underreporting would be substantially related to month of birth. Therefore, in the presence of a true seasonal variation, underreporting would dilute the observed association rather than lead to erroneous inferences.

4.2. *Synthesis with previous knowledge*

Results indicate overall seasonal variation of birth defects in Norway, with peaks in February and October suggesting environmental factors playing a causal role. We evaluated consistency of seasonality over time by stratifying the study population into two strata according to year of birth and found no significant period effect. No previous study assessed the overall seasonal variation.

Newborns with neural tube defects was one group in Norway with risk related to exposure to disinfection by-products. Consistent with previous studies in Poland [5], Canada [6], Utah [7], South America [11], Italy [12], Japan [20], and northern Germany [21], we found no seasonal variation in the occurrence of neural tube defects in Norway. We noted seasonal variation in Down syndrome and respiratory tract defects. Stolwijk et al. reviewed 13 studies of Down syndrome published as of 1997 and concluded that there was no systematic seasonal pattern [19], pointing out that studies from the extreme end of the northern hemisphere suggested a seasonal pattern. Two such studies were from Sweden [37] and northern Finland [38]. Of the more recent studies, seasonal variation appeared in Hertfordshire, England [17] but not in a large population-based study of 7994 newborns with Down syndrome in England and Wales [23]. We did not identify previous observations of seasonal variation in respiratory defects. We found no seasonal variation in occurrence of several birth defects, which in previous studies have shown seasonality: esophageal atresia [12], diaphragmatic hernia [12], cleft lip [22,27,28], and ventricular septal defects [25,30].

5. Conclusion

In summary, this study indicates seasonal variation in occurrence of respiratory defects and Down syndrome in Norway. The peak occurrence of respiratory defects was in March and of Down syndrome in February. Further studies are needed to explain reasons for seasonal variation, which are likely to represent environmental causes of these birth defects.

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Case report

Möbius syndrome in a male with XX/XY mosaicism

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ABSTRACT

We report the case of a 2-year-old male with congenital symmetric facial diplegia, and bilateral paralysis of abduction of the eyes. Findings were compatible with a diagnosis of Möbius syndrome. Routine G-banded chromosome analysis revealed a mosaic karyotype with 40 cells showing normal 46,XX and 10 cells showing normal 46,XY. An XX male attributed to XX/XY mosaicism was diagnosed. The phenotype of our patient did not coincide with any described form of XX reversal syndrome, but and was a unique combination of both syndromes. The disorder of this patient is likely to represent a genetic condition with pleiotropic effects on brain development and sex determination, providing adding further evidence for the heterogeneity of Möbius syndrome and sex reversal syndromes.

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1. Introduction

Möbius syndrome has been defined as congenital paresis or paralysis of the facial nerve that can be accompanied by paralysis or dysfunction of other cranial nerves, either unilaterally or bilaterally. The abducens nerves are most frequently involved, with concomitant paralysis of the hypoglossal nerve and hemiatrophy of the tongue present in one-third of cases. Most have congenital dysphagia, drooling, malocclusion, velopharyngeal incompetence, dysarthria, and delayed speech. Trigeminal nerve involvement with trismus is less frequent. Talipes equinovarus, malformations of the hands and fingers, and Poland anomaly may be associated.

Multiple factors are probably involved in pathogenesis, which is understandable in view of the many sites of pathology. Although often caused by environmental effects during pregnancy [1], a few cases have been familial with autosomal dominant and perhaps autosomal recessive inheritance. A pedigree has been described with seven affected members and a reciprocal translocation between chromosomes 1 and 13, demonstrable by banding techniques, which suggests that cytogenetic investigation is appropriate in the evaluation of

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affected patients [2]. We report the case of a Möbius syndrome patient with 46,XX/46,XY mosaicism.

2. Case report

The patient, the first son born to a 34-year-old mother and 35-year-old father, was conceived by *in vitro* fertilization because of his mother's fallopian tube obstruction. There was no family history of cranial nerve palsy. Pregnancy was uncomplicated, delivery was normal at 39 weeks, birth weight was 2550 g, and Apgar scores were unknown. He had feeding problems owing to inefficient sucking and swallowing due to paresis of the facial muscles as a newborn.

On physical examination at age 18 months, the child had a height of 84 cm (50–75 percentile), weight of 11 kg (25–50 percentile), and head circumference of 47.2 cm (25–50 percentile). A characteristic craniofacial appearance included epicanthic folds, a flattened nasal bridge, micrognathia, a high arched palate, hypertelorism, a small mouth with down-turned corners, and mild ptosis. Neurologic examination noted bilateral facial diplegia and abducens nerve palsy with conjugated horizontal gaze palsies. There was a palsy of the upper face with a relative sparing of the lower half of the face, including the perioral muscles and platysma. Sucking remained slow, but no aspiration or respiratory distress occurred. The eyes could not be totally closed. No other abnormal physical signs were noted, including talipes equinovarus or hypoplasia of the pectoralis muscle. The genitalia were normal for age, with both testes descended. At age 24 months, the Bayley Scales of Infant Development (BSID-II) showed a mental developmental age of 20 months, motor developmental age of 24 months, and language developmental age of 19 months. Brainstem auditory evoked response showed no sensorineural hearing loss. Echocardiogram was normal. Magnetic resonance imaging of the head including the brainstem was normal. He received speech therapy for his dysarthria. At age 3.5 years, electromyography revealed bilateral facial neuropathy. Routine G-banded chromosome analysis revealed a mosaic karyotype with 40 cells showing normal 46,XX and 10 cells showing normal 46,XY. An XX male attributed to XX/XY mosaicism was diagnosed. Abdominal CT scan revealed no female genital organ. At age 7 years, no significant abnormality was found in psychomotor development and the child had good performance at school. The Wechsler Intelligence Scale for Children, Third Edition (WISC-III) showed a verbal IQ of 83 and a performance IQ of 106. The patient showed the typical facial picture of Möbius syndrome.

3. Discussion

The exact etiology and pathogenesis of Möbius syndrome remain unknown, with two causes proposed: primary genetic [3] and primary ischemic [4]. Teratogenicity is suggested as a pivotal etiologic factor in both [5], but postulated etiologic mechanisms are based on limited pathologic observation. While the essential features of the syndrome are somewhat limited, it can be accompanied by neuromuscular and other abnormalities [6–8]. Various craniofacial, musculoskeletal, and cardiac malformations, as well as mental retardation, may be associated, giving rise to the term Möbius-like syndrome [9]. The disorder is usually sporadic, although a few cases have been familial. Ziter et al [2] observed congenital facial diplegia in seven members of three generations of a family with reciprocal translocation between chromosomes 1 and 13. Slee et al [10] observed deletion of 13q12.2 in a female patient. Kremer et al [11], by linkage analysis in a Möbius syndrome family, excluded chromosome 13q as a candidate region and found linkage markers at 3q21–q22. Localization of the present gene argues for genetic heterogeneity. Genetic heterogeneity has been suggested before, based on the clinical variability of the syndrome and segregation of the disorder in families [12].

Human males with a 46,XX karyotype were first described in 1964 by three different groups of investigators [13–15]. The frequency of this syndrome is estimated at 1 in 20,000 newborn males, although there are considerable geographic variations [16]. Most patients (85%) have a normal male phenotype at birth and are usually diagnosed after puberty when consulting a physician due to hypogonadism, gynecomastia, and/or infertility [17]. Although the clinical and endocrinologic features of XX males resemble those of 47, XXY Klinefelter's syndrome, XX males present normal or even low height and do not differ from the general population with regard to intelligence [18]. Our case is a XX male with XX/XY mosaicism. Mosaicism including a second cell line with a Y chromosome has been claimed as the origin of some cases of XX males.

The unique feature in this patient is a XX male with Möbius syndrome, and although numerous cases have been reported, no previous cases with sex reversal have been described. Möbius syndrome is a rare disorder, with incidence in the population not determined. The incidence of XX maleness is 1/20,000. Certainly a rare occurrence of both disorders in one patient raises the possibility of two phenotypes etiologically related. While sex-reversal syndromes are not usually associated with any neurologic abnormality, several multiple malformation syndromes can cause genital ambiguity and also result in neurologic involvement. In our reviews, sex-reversal patients with chromosomal deletion, such as deletion of 9p, 10q, or 18p, have development delay and neurologic signs [19–21]. This case of Möbius and XX sex-reversal syndrome is likely to represent a genetic condition with pleiotropic effects on brain development and sex determination, providing evidence of heterogeneity in Möbius and sex-reversal syndromes.

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Submitted manuscripts are considered with the understanding that they have not been published previously in print or electronic format (except in abstract or poster form) and are not under consideration in totality or in part by another publication or electronic medium.

7. Basic Criteria

Articles should be written in English (using American English spelling) and meet the following basic criteria: the material is original, the information is important, the writing is clear and concise, the study methods are appropriate, the data are valid, and the conclusions are reasonable and supported by the data.

8. Article Categories

8.1. *Review Articles*

These should aim to provide the reader with a balanced overview of an important and topical subject in the field, and should be systematic and critical assessments of literature and data sources. They should cover aspects of a topic in which scientific consensus exists as well as aspects that remain controversial and are the subject of ongoing scientific research. All articles and data sources reviewed should include information about the specific type of study or analysis, population, intervention, exposure, and tests or outcomes. All articles or data sources should be selected systematically for inclusion in the review and critically evaluated.

By invitation only. The format for review articles will be jointly decided by the Editors and the contributing author. Typical length: no more than 4000 words, 50–100 references.

8.2. *Original Articles*

These may be randomized trials, intervention studies, studies of screening and diagnostic tests, laboratory and animal studies, cohort studies, cost-effectiveness analyses, case-control studies, and surveys with high response rates, which represent new and significant contributions to the field.

Section headings should be: Abstract, Introduction, Methods, Results, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

The Introduction should provide a brief background to the subject of the paper, explain the importance of the study, and state a precise study question or purpose.

The Methods section should describe the study design and methods (including the study setting and dates, patients/participants with inclusion and exclusion criteria, or data sources and how these were selected for the study, patient samples or animal specimens used, explain the laboratory methods followed), and state the statistical procedures employed in the research.

The Results section should comprise the study results presented in a logical sequence, supplemented by tables and/or figures. Take care that the text does not repeat data that are presented in tables and/or figures. Only emphasize and summarize the essential features of any interventions, the main outcome measures, and the main results.

The Discussion section should be used to emphasize the new and important aspects of the study, placing the results in context with published literature, the implications of the findings, and the conclusions that follow from the study results. Typical length: no more than 5500 words, 40–80 references.

8.3. *Case Reports*

These are short discussions of a case or case series with unique features not previously described that make an important teaching point or scientific observation. They may describe novel techniques, novel use of equipment, or new information on diseases of importance. Section headings should be: Abstract, Introduction, Case Report, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

The Introduction should describe the purpose of the report, the significance of the disease and its specificity, and briefly review the relevant literature.

The Case Report should include the general data of the case, medical history, family history, chief complaint, present illness, clinical manifestation, methods of diagnosis and treatment, and outcome.

The Discussion should compare, analyze and discuss the similarities and differences between the reported case and similar previously reported cases. The importance or specificity of the case should be restated when discussing the differential diagnoses. Suggest the prognosis of the disease and possibility of prevention. Typical length: no more than 1500 words, 20–40 references.

8.4. *Short Communications*

These should be concise presentations of clinical or preliminary experimental results. Section headings should be: Abstract, Introduction, Methods, Results, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

Typical length: no more than 1000 words, 20–40 references, with no more than four figures or tables. The Editors reserve the right to decide what constitutes a Short Communication.

8.5. *Letters to the Editor*

Letters are welcome in response to previously published articles, and may also include interesting cases that do not meet the requirement of being truly exceptional, as well as other communications of general interest. Letters should have a title and include appropriate references, and include the corresponding author's mailing and e-mail addresses. Letters are edited, sometimes extensively, to sharpen their focus. They may be sent for peer review at the discretion of the Editors. Letters are selected based on clarity, significance, and space. Typical length: no more than 600 words, 5–10 references; 1 table and/or 1 figure may be included.

8.6. *Editorials*

Editorials are invited articles or comments concerning a specific paper in the Journal or a topical issue in the field. While normally invited, unsolicited editorials may be submitted. Typical length: no more than 1500 words, 15–30 references.

9. Manuscript Preparation

Text should be typed double-spaced on one side of white A4 (297 × 210 mm) paper, with outer margins of 2.5 cm. A manuscript should include a title page, abstract, text, acknowledgments (if any), conflicts of interest statement (if any), references, and figures and tables as appropriate. Each section of the manuscript should begin on a new page. Pages should be numbered consecutively, beginning with the title page.

9.1. *Title Page*

The title page should contain the following information (in order, from the top to bottom of the page):

- category of paper
- article title
- names (spelled out in full)* of all the authors, and the institutions with which they are affiliated; indicate all affiliations with a superscripted lowercase letter after the author's name and in front of the appropriate affiliation
- corresponding author details (name, e-mail, mailing address, telephone and fax numbers)

**The name of each author should be written with the family name last, e.g., Jing-Lin Chang. Authorship is restricted only to direct participants who have contributed significantly to the work.*

9.2. *Abstract and Keywords*

Abstracts should be no more than 300 words in length. Abstracts for Original Articles should be structured, with the section headings: Background/ Introduction, Purpose(s)/Aim(s), Methods, Results, Conclusion. Abstracts for Case Reports are unstructured, but should include the significance and purpose of the case presentation, the diagnostic methods of the case, the key data, and brief comments and suggestions with regard to the case. Abstracts for Review Articles and Short Communications should also be unstructured. No abstract is required for Letters to the Editor and Editorials. For the article categories that require an abstract, 3–5 relevant keywords should also be provided in alphabetical order.

9.3. *Main Text*

The text for Original Articles should be organized into the following sections: Background/ Introduction, Purpose(s)/Aim(s), Methods, Results and Discussion. Sections for Case Reports are: Introduction, Case Report, and Discussion. Each section should begin on a new page.

9.3.1. Abbreviations

Where a term/definition will be continually referred to, it must be written in full when it first appears in the text, followed by the subsequent abbreviation in parentheses. Thereafter, the abbreviation may be used. An abbreviation should not be first defined in any section heading; if an abbreviation has previously been defined in the text, then the abbreviation may be used in a subsequent section heading. Restrict the number of abbreviations to those that are absolutely necessary.

9.3.2. Units

Système International (SI) units must be used, with the exception of blood pressure values which are to be reported in mmHg. Please use the metric system for the expression of length, area, mass, and volume. Temperatures are to be given in degrees Celsius.

9.3.3. Names of drugs, devices and other products

Use the Recommended International Non-proprietary Name for medicinal substances, unless the specific trade name of a drug is directly relevant to the discussion. For devices and other products, the generic term should be used, unless the specific trade name is directly relevant to the discussion. If the trade name is given, then the manufacturer name and the city, state and country location of the manufacturer must be provided the first time it is mentioned in the text, for example, "…SPSS version 11 was used (SPSS Inc., Chicago, IL, USA)."

9.3.4. Statistical requirements

Statistical analysis is essential for all research papers except case reports. Use correct nomenclature of statistical methods (e.g., two sample *t* test, not unpaired *t* test). Descriptive statistics should follow the scales used in data description. Inferential statistics are important for interpreting results and should be described in detail.

All p values should be expressed to 2 digits to the right of the decimal point, unless $p < 0.01$, in which case the p value should be expressed to 3 digits to the right of the decimal point. The smallest p value that should be expressed is $p < 0.001$, since additional zeros do not convey useful information; the largest p value that should be expressed is $p > 0.99$.

9.3.5. Personal communications and unpublished data

These sources cannot be included in the references list but may be described in the text. The author(s) must give the full name and highest academic degree of the person, the date of the communication, and indicate whether it was in oral or written (letter, fax, e-mail) form. A signed statement of permission should be included from each person identified as a source of information in a personal communication or as a source for unpublished data.

9.4. *Acknowledgments and Conflicts of Interest Statement*

General acknowledgments for consultations, statistical analysis, etc., should be listed concisely at the end of the text, including the names of the individuals who were directly involved. Consent should be obtained from those individuals before their names are listed in this section. All financial and material support for the research and work from internal or external agencies, including commercial companies, should be clearly and completely identified. Ensure that any conflicts of interest (financial and/or non-financial) are explicitly declared.

9.5. *Abbreviation list*

A term that appears more than three times in a paper should be abbreviated. Spell out the term on first mention, followed by the abbreviated form in parentheses. Thereafter, please use the abbreviated form. Supply a list of nonstandard abbreviations used in the paper at the end of the main text, in alphabetical order, giving each abbreviation followed by its spelled-out version.

9.6. *References*

9.6.1. In the main text, tables, figure legends

- References should be indicated by numbers in square brackets in line with the text, and numbered consecutively in order of appearance in the text.
- References cited in tables or figure legends should be included in sequence at the point where the table or figure is first mentioned in the main text.
- Do not cite uncompleted work or work that has not yet been accepted for publication (i.e., "unpublished observation", "personal communication") as references. Also see Section 9.3.5. above.
- Do not cite abstracts unless they are the only available reference to an important concept.

9.6.2. In the references section

- References should be limited to those cited in the text and listed in numerical order, NOT alphabetical order.
- References should include, in order, author surnames and initials, article title, abbreviated journal name, year, volume and inclusive page numbers. The last names and initials of all the authors up to 6 should be included, but when authors number 7 or more, list the first 6 authors only followed by "et al". Abbreviations for journal names should conform to those used in MEDLINE.
- If citing a website, provide the author information, article title, website address and the date you accessed the information.

- Reference to an article that is in press must state the journal name and, if possible, the year and volume.

Authors are responsible for the accuracy and completeness of their references and for correct text citation.

Examples are given below.

Standard journal article
Chen Z, Fan M, Bian Z, Zhang Q, Zhu Q, Lu P. Immunolocalization of heat shock protein 70 during reparative dentinogenesis. Chin J Dent Res 2000;3:50–5.

Journal supplement
Kaplan NM. The endothelium as prognostic factor and therapeutic target: what criteria should we apply? J Cardiovasc Pharmacol 1998;32(Suppl 3):S78–80.

Journal article not in English but with English abstract
Nakayama H, Ishikawa T, Yamashita S, Fukui I, Mutoh T, Hikichi K, et al. CSF leakage and anosmia in aneurysm clipping of anterior communicating artery by basal interhemispheric approach. No Shinkei Geka 2011;39:263–8. [In Japanese, English abstract]

Book
Bradley EL. Medical and surgical management. Philadelphia: Saunders; 1982, p. 72–95.

Book chapter in book with editor and edition
Greaves M, Culligan DJ. Blood and bone marrow. In: Underwood JCE, editor. General and systematic pathology. 4th ed. London: Churchill Livingstone; 2004, p. 615–72.

Bulletin
World Health Organization. World health report 2002: reducing risk, promoting healthy life. Geneva, Switzerland: World Health Organization; 2002.

Company/manufacturer publication/pamphlet
Eastman Kodak Company, Eastman Organic Chemicals. Catalog no. 49. Rochester, NY: Eastman Kodak; 1977, p. 2–3.

Electronic publications
Duchin JS. Can preparedness for biological terrorism save us from pertussis? Arch Pediatr Adolesc Med 2004;158:106–7. Available from: http://archpedi.ama-assn.org/cgi/content/full/158/2/106. Accessed June 5, 2004.

Smeeth L, Iliffe S. Community screening for visual impairment in the elderly. Cochrane Database Syst Rev 2002(2):CD001054. doi:10.1002/14651858.CD1001054.

Items presented at a meeting but not yet published
Durbin D, Kallan M, Elliott M, Arbogast K, Cornejo R, Winston F. Risk of injury to restrained children from passenger air bags. Paper presented at: 46th Annual Meeting of the Association for the Advancement for Automotive Medicine; September 2002; Tempe, AZ.

Greenspan A, Eerdekens M, Mahmoud R. Is there an increased rate of cerebrovascular events among dementia patients? Poster presented at: 24th Congress of the Collegium Internationale Neuro-Psychopharmacologicum (CINP); June 20–24, 2004; Paris, France.

Khuri FR, Lee JJ, Lippman SM. Isotretinoin effects on head and neck cancer recurrence and second primary tumors. In: Proceedings from the American Society of Clinical Oncology; May 31–June 3, 2003; Chicago, IL. Abstract 359.

Item presented at a meeting and published
Cionni RJ. Color perception in patients with UV- or blue-light-filtering IOLs. In: Symposium on Cataract, IOL, and Refractive Surgery. San Diego, CA: American Society of Cataract and Refractive Surgery; 2004. Abstract 337.

Material accepted for publication but not yet published
Carrau RL, Khidr A, Crawley JA, Hillson EM, Davis JK, Pashos CL. The impact of laryngopharyngeal reflux on patient-reported quality of life. Laryngoscope. In press.

Ofri D. Incidental findings: Lessons from my patients in the art of medicine. Boston, MA: Beacon Press. In press.

Theses and dissertations
Undeman C. Fully automatic segmentation of MRI brain images using probabilistic diffusion and a watershed scale-space approach [master's thesis]. Stockholm, Sweden: NADA, Royal Institute of Technology; 2001.

Ayers AJ. Retention of resin restorations by means of enamel etching and by pins [dissertation]. Indianapolis: Indiana University; 1971.

Website
American Association of Oral and Maxillofacial Surgeons. Wisdom teeth. AAOMS Web site. http://www.aaoms.org/wisdom_teeth.php. Published January 23, 2008. Updated March 9, 2009. Accessed November 15, 2009.

9.7. Tables

Tables should supplement, not duplicate, the text. They should have a concise table heading, be self-explanatory, and numbered consecutively in the order of their citation in the text. Information requiring explanatory footnotes should be denoted using superscripted lowercase letters in alphabetical order (a, b, c, etc.). Asterisks (*, **) are

used only to indicate the probability level of tests of significance. Abbreviations used in the table must be defined and placed after the footnotes. If you include a block of data or table from another source, whether published or unpublished, you must acknowledge the original source.

9.8. *Figures*

9.8.1. General guidelines

The number of figures should be restricted to the minimum necessary to support the textual material. They should have an informative figure legend and be numbered in the order of their citation in the text. All symbols and abbreviations should be defined in the legend. Patient identification should be obscured. All lettering should be done professionally and should be in proportion to the drawing, graph or photograph. Photomicrographs must include an internal scale marker, and the legend should state the type of specimen, original magnification and stain.

Figures must be submitted as separate picture files at the correct resolution (see Section 9.7.2. below). The files should be named according to the figure number, e.g., "Article1_Fig1", "Article1_Fig2".

9.8.2. Formats

Regardless of the application used, when your electronic artwork is finalized, please "save as" or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

- EPS: Vector drawings. Embed the font or save the text as "graphics".
- TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
- TIFF: Bitmapped line drawings: use a minimum of 1000 dpi.
- TIFF: Combination of bitmapped line/half-tone (color or grayscale): a minimum of 600 dpi is required.
- DOC, XLS or PPT: If your electronic artwork is created in any of these Microsoft Office applications, please supply "as is".

Please do not:

- Supply files that are optimized for screen use (like GIF, BMP, PICT, WPG); the resolution is too low;
- Supply files that are too low in resolution;
- Submit graphics that are disproportionately large for the content.

A detailed guide on electronic artwork is available at http://www.elsevier.com/artworkinstructions.

10. The Editorial and Peer Review Process

As a general rule, the receipt of a manuscript will be acknowledged within 1 week of submission, and authors will be provided with a manuscript reference number for future correspondence. If such an acknowledgment is not received in a reasonable period of time, the author should contact the Editorial Office.

Submissions are reviewed by the Editorial Office to ensure that it contains all parts. The Editorial Office will not accept a submission if the author has not supplied all the material and documents as outlined in these author instructions.

Manuscripts are then forwarded to the Editor-in-Chief, who makes an initial assessment of it. If the manuscript does not appear to be of sufficient merit or is not appropriate for the Journal, then the manuscript will be rejected without review.

Manuscripts that appear meritorious and appropriate for the Journal are reviewed by at least two Editorial Board members or expert consultants assigned by the Editor-in-Chief. Authors will usually be notified within 6 weeks of whether the submitted article is accepted for publication, rejected, or subject to revision before acceptance. However, do note that delays are sometimes unavoidable.

11. Preparation for Publication

Once a manuscript has been accepted for publication, the authors should submit the final version of the manuscript in MS Word format, with all tables/figures as applicable, to the Editorial Office.

Accepted manuscripts are copyedited according to the Journal's style and PDF page proofs are e-mailed by the Publisher to the corresponding author for final approval. Authors are responsible for all statements made in their work, including changes made by the copy editor.

12. Publication Charges and Reprints

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☐ Authorship statement signed by all authors
☐ Signed conflicts of interest disclosure statement
☐ Signed copyright transfer agreement
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☐ References in the correct format, cited in numerical order, and all references in the List are cited in the Text/Tables/Figures, and vice versa

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☐ Acknowledgments
☐ Conflicts of interest statement
☐ Table headings and tables, each on a new page
☐ Figure legends, on a new page
☐ Electronic picture files of all figures; resolution of 300 dpi for halftone images, 600 dpi for combination art (halftone + line art), and 1000 dpi for line art

Further considerations:

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Conception and design of study: _____, _____, _____, _____;

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Acknowledgments

All persons who have made substantial contributions to the work reported in the manuscript (e.g., technical help, writing and editing assistance, general support), but who do not meet the criteria for authorship, are named in the Acknowledgments and have given us their written permission to be named. If we have not included an Acknowledgments, then that indicates that we have not received substantial contributions from non-authors.

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